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**ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA
AGRONÓMICA, ALIMENTARIA Y DE BIOSISTEMAS**

**NON-CIRCULATIVE VIRUS TRANSMISSION BY APHIDS: NEW
INSIGHTS INTO THE MECHANISMS OF TRANSMISSION AND
THE INTERFERENCE FOR RETENTION SITES IN THE
VECTOR**

TESIS DOCTORAL

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ACRONYMS AND ABBREVIATIONS

A: Ampere

AAP: Acquisition access period

AC-EPG: Alternative-current electrical penetration graph

BYDV: *Barley yellow dwarf virus* (BYDV, *Luteovirus*)

BYV: *Beet yellows virus* (BYV, *Closterovirus*)

C: *Waveform C:* extracellular aphid stylets pathway

CaMV: *Cauliflower mosaic virus* (CaMV, *Caulimovirus*)

CC: Companion cell

CLSM: Confocal laser-scanning microscopy

CMV: *Cucumber mosaic virus* (CMV, *Cucumovirus*)

CP: Capsid protein

CPm: Minor coat protein

CSIC: Consejo Superior de Investigaciones Científicas

CTV: *Citrus tristeza virus* (CTV, *Closterovirus*)

CuP: Cuticular protein

cv: Cultivar

CV: Circulative virus

Da: Dalton

DAG: Asparagine-Alanine-Glycine motif

DC-EPG: Direct-current electrical penetration graphs

DNA: Deoxyribonucleic acid

Dr.: Doctor

E1: *Waveform E1:* continuous aphid salivation into sieve elements

E2: *Waveform E2:* aphid ingestion of phloem sap from sieve elements

ELISA: Enzyme-linked immunosorbent assay

EPG: Electrical penetration graphs technique

F: *Waveform F:* Derailed stylets

g: grams

G: *Waveform G:* Aphid ingestion from xylem

GHG: Greenhouse gas

h: Hours

HC: Helper component

HC-Pro: Helper component protein

HDMS: Hexamethyldisilazane

IAP: Inoculation access period

ICA: Instituto de Ciencias Agrarias

Int: Interval (distance between two downpeaks in subphase II-2 of the pd)

Int. E1/E2: Transition phase between waveforms E1 and E2.

IVPP: Grupo de Investigación Insectos Vectores de Patógenos de Plantas

KITC: Lysine-Isoleucine-Threonine-Cysteine motif

L:D: Light:Dark

LMV: Lettuce mosaic virus (LMV, *Potyvirus*)

LIYV: *Lettuce infectious yellow virus* (LIYV, *Closterovirus*)

M: Molar

ml: Milimeter

mm: Milimeter

mM: milimolar

MCDV: *Maize chlorotic dwarf virus* (MCDV, *Waikavirus*)

Micro-CT: Micro-computed tomography

NC: Noncirculative virus

np: *Waveform np:* Non-probing of the aphid

NP: non-persistent (virus)

p: Statistical significance value

P: Persistent (virus)

PAR: Photosynthetic active radiation

pd: *Waveform 'pd' (potencial drop):* intracellular puncture of aphid stylets

PEMV: *Pea enation mosaic virus* (PEMV, *Enamovirus*)

phloem-pd: *Waveform phloem-pd:* aphid intracellular puncture into phloem cells

pi: Range value in Marascuilo test

pj: Critical value in Marascuilo test

PPC: Phloem parenchyma cell

PPUs: Pore-plasmodesmata units

Prof.: Professor

PRSV: *Papaya ringspot virus* (PRSV, *Potyvirus*)

PSEP: Phloem sieve element phase

PTK: Proline-Threonine-Lysine motif

P2: Helper protein involved in CaMV retention

P2rev5: Non-transmissible CaMV mutant

P3: Protein associated to CaMV virion

RH: Relative humidity

RNA: Ribonucleic acid

s: Seconds

SE: Sieve element

SP: Semipersistent virus

TEV: *Tobacco etch virus* (TEV, *Potyvirus*)

ToCV: *Tomato chlorosis virus* (ToCV, *Crinivirus*)

TuMV: *Turnip mosaic virus* (TuMV, *Potyvirus*)

TYLCV: *Tomato yellow leaf curl virus* (TYLCV, *Begomovirus*)

U: Mann-Whitney test value

UCR: University of California Riverside

UGR: Universidad de Granada (University of Granada)

UV: Ultraviolet radiation

V: Volt

VAP: viral attachment proteins

VLP: Virus-like protein

X: Xylem

X-waveform: Repetitive pds prior to E1 waveform

Z: Wilcoxon test value

ZYMV: *Zucchini yellow mosaic virus* (ZYMV, *Potyvirus*)

RESUMEN

En esta Tesis Doctoral se propone profundizar en el conocimiento de diversos conceptos sobre virus no-circulativos transmitidos por pulgones. El objetivo principal es generar nuevos conocimientos sobre el proceso de transmisión viral por pulgones que permita desarrollar nuevas estrategias que lleven a un mejor control de los virus vegetales. Concretamente, se han planteado los siguientes objetivos generales:

1. Estudiar el efecto del ayuno de pulgones en la transmisión de virus no-persistentes.
2. Estudiar la competencia e interferencia entre diferentes virus no circulativos por los lugares de retención en el vector.
3. Estudiar el comportamiento alimenticio de pulgones en relación a la transmisión de virus semipersistentes restringidos al floema.
4. Determinar la localización de los estiletes de pulgones en los tejidos de la planta durante la actividad clave asociada a la transmisión de virus semipersistentes restringidos al floema.

La mayor parte de la experimentación de la presente Tesis Doctoral (objetivos 1, 2 y 3) se ha llevado a cabo en el Instituto de Ciencias Agrarias del Consejo Superior de Investigaciones Científicas (ICA-CSIC) (Madrid, España). Parte de los ensayos dentro del objetivo 4 se realizaron en el marco de una Estancia Breve desarrollada en la Universidad de California Riverside (UCR) (EEUU), financiada por el Ministerio de Economía, Industria y Competitividad. Asimismo, el análisis de parte de las muestras de los experimentos dentro del objetivo 4 se llevaron a cabo en colaboración con la Universidad de Granada (UGR) (Granada, España).

En cada uno de los objetivos de la presente Tesis Doctoral se empleó la técnica de Gráficos de penetración eléctrica (Electrical penetration graphs ‘EPG’ technique). Gracias a esta técnica podemos monitorizar el comportamiento alimenticio de insectos con aparato bucal picador-chupador y asociar la posición del extremo distal del estilete

en los diferentes tejidos de la planta. De esta manera, podemos asociar las actividades de los estiletes del insecto a la inoculación y adquisición de virus vegetales.

El primer objetivo de la Tesis Doctoral (Capítulo 4) tuvo como finalidad estudiar el efecto del ayuno de pulgones previo a la adquisición de virus transmitidos de manera no persistente (NP). Para ello, se usaron individuos ayunados y no ayunados de *Myzus persicae* y *Aphis gossypii*. Los pulgones se conectaron al equipo de EPG para realizar una prueba intracelular en una planta de calabacín infectada con *Cucumber mosaic virus* (CMV, *Cucumovirus*) tras ser ayunados durante una hora o sin ayuno previo. Posteriormente, los pulgones fueron trasferidos individualmente a una planta sana de calabacín para evaluar la tasa de transmisión de CMV mediante inspección visual y detección serológica mediante test de ELISA. Los registros de EPG mostraron un aumento de la actividad del pulgón asociada a la adquisición de virus no persistentes (Collar y Fereres, 1998) en individuos ayunados tanto de *M. persicae* como *A. gossypii*. Además, se observó un incremento en la transmisión de CMV en individuos ayunados de *M. persicae* en comparación con los no ayunados. Sin embargo, la eficacia en la transmisión de CMV fue similar cuando se comparó entre individuos ayunados y no ayunados de *A. gossypii*. Por tanto, los resultados obtenidos demuestran que el ayuno de pulgones previo a la adquisición no siempre aumenta necesariamente la tasa de transmisión de todos los virus transmitidos de manera NP, como tradicionalmente había sido establecido para dicho tipo de transmisión viral (Watson, 1938; Powell, 1993). Esta diferencia en la eficiencia de transmisión entre individuos ayunados de *M. persicae* y *A. gossypii* sugirió posibles diferencias anatómicas en los estiletes de ambas especies o la presencia diferencial de compuestos en la saliva de dichos pulgones que puedan interferir o favorecer de forma específica la interacción con las partículas de CMV.

En el segundo objetivo (Capítulo 5), se estudió la competencia e interferencia por la retención en los receptores del vector entre diferentes virus no-circulativos (NC) transmitidos por pulgones. Para ello, se llevaron a cabo ensayos de transmisión secuenciales con períodos de adquisición fijos o monitorizados mediante EPG, tanto en plantas infectadas como en dieta artificial. Para el estudio de la competencia entre *Cauliflower mosaic virus* (CaMV, *Caulimovirus*) y *Turnip mosaic virus* (TuMV, *Potyvirus*), individuos de *Brevicoryne brassicae* fueron expuestos a plantas de nabo coinfectadas con TuMV y CaMV, mientras que para el estudio de la interferencia entre

ambos géneros virales, los pulgones se expusieron a dos adquisiciones/transmisiones secuenciales, primero en planta de nabo infectada con CaMV o virus purificado en dieta artificial y posteriormente en planta de nabo infectada con TuMV. En los estudios correspondientes a la interferencia entre CaMV y CMV, se usaron individuos de *M. persicae* que adquirieron CaMV en una planta infectada de nabo o la proteína helper de CaMV (P2-CaMV) en dieta artificial y posteriormente CMV en una planta de calabacín. Por último, se evaluó la interferencia entre *Zucchini yellow mosaic virus* (ZYMV, *Potyvirus*) y CMV usando *A. gossypii* como vector. Para ello, los pulgones adquirieron individualmente bien ZYMV o CMV en plantas infectadas de melón y después eran transferidos a una planta de melón infectada con CMV o ZYMV, respectivamente. Tras la realización de los experimentos los pulgones fueron transferidos individualmente a diferentes especies de plantas receptoras teniendo en cuenta las especies de virus estudiadas. La infección viral se evaluó mediante inspección visual y mediante detección serológica (test de ELISA) tres semanas después de la realización de los ensayos. Los resultados revelaron que la adquisición previa de ZYMV interfiere en la posterior adquisición CMV, reduciendo la tasa de transmisión de este último. Sin embargo, no se observó competencia o interferencia en el resto de combinaciones virus-vector estudiadas: competencia/interferencia entre CaMV y TuMV e interferencia entre CaMV y CMV. Estos resultados revelaron la interacción entre los virus CMV y ZYMV durante el proceso de adquisición por el vector, sugiriendo cierta afinidad por los mismos lugares de retención en el estilete. Los resultados obtenidos también sugirieron que los receptores usados por CaMV en los estiletes del vector pudieran ser diferentes a los empleados por potyvirus y cucumovirus.

En el tercer objetivo (Capítulo 6), se estudió el comportamiento alimenticio de *M. persicae* en relación a la inoculación y adquisición de *Beet yellows virus* (BYV, *Closterovirus*), el cual es un virus transmitido de manera semipersistente y que se encuentra restringido al floema de la planta huésped (Esau, 1968). En primer lugar, se realizaron ensayos utilizando las dos especies de vectores más eficientes en la transmisión de BYV, *M. persicae* y *Aphis fabae* (Sylvester, 1956; Bennett, 1960; Limburg *et al.*, 1997), así como tres especies de plantas huésped de BYV: *Chenopodium album*, *Claytonia perfoliata* y *Beta vulgaris* (Sylvester, 1956; Bennett, 1960) para determinar el sistema vector-planta huésped más eficiente. Los resultados mostraron que *M. persicae* fue el vector más eficaz, y remolacha azucarera la especie de planta

más susceptible a la infección por BYV, por lo que se escogió el modelo *M. persicae*-BYV-remolacha azucarera para la realización de los posteriores estudios monitorizando el comportamiento alimenticio del pulgón mediante EPG. Para ello, se conectaron individuos no virulíferos al equipo de EPG en remolacha azucarera infectada con BYV y se interrumpió la alimentación de *M. persicae* tras observar unos patrones específicos de onda. Así, las diferentes actividades del pulgón producidas en la planta fuente de virus eran posteriormente asociadas a la adquisición de BYV. Una vez terminado artificialmente el proceso de alimentación, los pulgones eran transferidos individualmente a una planta sana de remolacha para evaluar la eficacia de transmisión de BYV. Por otra parte, se conectaron pulgones virulíferos al equipo de EPG y se colocaron sobre plantas sanas de remolacha azucarera para estudiar las actividades de los estiletes del pulgón relacionadas con la inoculación de BYV. Las plantas receptoras fueron analizadas visualmente y mediante detección por ELISA para evaluar la infección viral, cuatro semanas después de la realización de los experimentos. El análisis de los registros de EPG reveló la producción de una prueba intracelular que difería significativamente, tanto en la duración total como en la caída de potencial, del resto de pruebas intracelulares, realizadas fuera de los tejidos floemáticos y asociadas a la transmisión de virus no persistentes (Martín *et al.*, 1997). Dicha nueva prueba intracelular de características diferentes a las pds estándar se denominó "phloem-pd" o pd de floema, debido a que siempre ocurría precediendo a la onda asociada como primer contacto de los estiletes del pulgón con los vasos cribosos del floema (onda E1), lo que sugería su posible localización en células del floema. Además, la caída de potencial de la "phloem-pd" era similar a la caída de potencial observada en la subsiguiente E1. Además, la "phloem-pd" resultó ser la onda clave en la inoculación de BYV, obteniéndose el óptimo de inoculación tras la su realización. Todo ello sugería que los estiletes de *M. persicae* se podrían encontrar en células de floema durante la realización de las "phloem-pd".

En el cuarto objetivo (Capítulo 7), se llevó a cabo la localización exacta de la posición del extremo distal del estilete de *M. persicae* durante la realización de la nueva inserción intracelular, "phloem-pd", en plantas de remolacha azucarera. Para ello, se llevó a cabo la criofijación del estilete durante la observación de dicha prueba intracelular, y el posterior estudio de la posición del extremo distal usando tanto la microscopía confocal (CLSM) como la tomografía microcomputerizada (Micro-CT). Asimismo, se llevaron a

cabo dos tratamientos control, crio fijando los estiletes durante la observación de i) una prueba intracelular estándar ("standard-pd": prueba asociada a penetraciones de los estiletes en tejidos no floemáticos) y ii) onda E1 (inserción de los estiletes en tubos cribosos de floema). El análisis de las imágenes producidas por microscopía confocal mostró la localización del extremo distal del estilete penetrando tejidos floemáticos, concretamente células acompañantes y también tubos cribosos del floema. Las imágenes producidas por Micro-CT confirmaron la intrusión de los estiletes en tubos cribosos de floema. Los tratamientos control reflejaron la inserción de los estiletes en células del mesófilo o vaina del haz vascular durante la realización de la "standard-pd", y penetraciones en tubos cribosos del floema durante la visualización de la onda E1, tal y como se había descrito previamente para ambas ondas (Tjallingii, 1990; Prado y Tjallingii, 1994).

Los resultados obtenidos en la presente Tesis Doctoral serán clave en el desarrollo de futuras líneas de investigación relacionados con las interacciones virus-vector así como en la creación de nuevas estrategias que permitan interferir con el proceso de transmisión y consiguiente dispersión de los virus vegetales en los cultivos.

SUMMARY

The main objective of this Thesis was to study several aspects concerning non-circulative plant virus transmission by aphids and generate basic knowledge that could be applied to develop new strategies to interfere with their transmission and spread. More precisely, the following specific objectives were covered in the present PhD Thesis:

1. To evaluate the effect of the pre-acquisition fasting period in the transmission of viruses transmitted in a non-persistent manner by aphids.
2. To study the competition and interference of different non-circulative viruses for the retention sites in their aphid's vector.
3. To study the feeding behaviour of aphids associated with the transmission of semipersistently transmitted, phloem-limited viruses.
4. To localize the stylet tip position of aphids in plant tissues during the occurrence of key feeding behavioural pattern associated with the transmission of phloem-limited viruses transmitted in a semipersistent manner.

The experiments concerning objectives 1, 2 and 3 of this Thesis were developed at Institute of Agricultural Sciences (ICA) facilities of the Spanish National Research Council (CSIC) (Madrid, Spain). Experiments within objective 4 were conducted in a short-term research placement at University of California Riverside (UCR) (Riverside, CA., USA) in collaboration with Prof. James Ng at the Department of Plant Pathology and Prof. Gregory P. Walker at the Department of Entomology. Results of experiments of objective 4 were confirmed by the analysis of leaf and insect samples at the University of Granada (Spain) in collaboration with Prof. Javier Alba-Tercedor at the Department of Zoology.

The Electrical penetration graphs (EPG) technique was used in all the objectives of this PhD Thesis. Using this technique, we can monitor the feeding process of pierce-sucking insects in the plant and determine the position of the stylets tip in the different tissues of the plant. Therefore, we can determine the different aphid activities in the plant involved in the acquisition and inoculation of plant viruses.

The first study aimed to evaluate the pre-acquisition fasting effect of *Myzus persicae* and *Aphis gossypii* on the transmission of *Cucumber mosaic virus* (CMV, *Cucumovirus*) (Chapter 4). Fasted and non-fasted aphids were wired and connected to the EPG device and allowed to produce a single intracellular puncture (waveform ‘pd’: potential drop) as acquisition access period (AAP) in a CMV-infected zucchini plant. To determine if the aphids acquired CMV during the AAP, they were individually transferred to a non-infected test zucchini plant to assess CMV infection by visual inspection and ELISA test three weeks after the experiments. EPG recordings showed an increase of probing activities in fasted aphids favoring the acquisition of non-persistent viruses (Collar and Fereres, 1998). In fact, fasted *M. persicae* individuals showed an increase in CMV transmission. However, fasted *A. gossypii* individuals did not increase the transmission rate of CMV compared to non-fasted. Therefore, here we showed that a pre-acquisition fasting period does not always enhance the transmission of all viruses transmitted in a NP manner, as previously postulated for this type of virus-vector relationship (Watson, 1938; Powell, 1993). Results suggested possible anatomical differences in the stylets of *M. persicae* and *A. gossypii* individuals or presence of different compounds in the saliva that could favor or interfere with the interaction with the CMV particles.

The competition or interference for the retention sites in their aphid vectors was also studied for several non-circulative (NC) virus species (Chapter 5). The transmission of different combinations of virus species by their most efficient vectors were studied: i) competition and interference between *Cauliflower mosaic virus* (CaMV, *Caulimovirus*) and *Turnip mosaic virus* (TuMV, *Potyvirus*) in *Brevicoryne brassicae*, ii) interference between CaMV and CMV in *M. persicae*, and iii) interference between *Zucchini yellow mosaic virus* (ZYMV, *Potyvirus*) and CMV in *A. gossypii*. After virus acquisition on different sources, aphids were individually transferred to different non-infected receptor test plants depending on the virus-vector combination studied. Virus infection was assessed by visual inspection and ELISA test three weeks after the experiments. Results revealed that CaMV did not interfere with the subsequent transmission of either TuMV or CMV. However, the acquisition of ZYMV interfered in subsequent acquisition and transmission of CMV, with no impact in the rate of transmission of ZYMV after first acquisition of CMV. Therefore, we found that potyviruses and cucumoviruses interfered with each other during the acquisition process by their main vector, suggesting that they

bind to the same receptors in the stylets. Conversely, CaMV probably binds to receptors different from those used by potyviruses and cucumoviruses.

Furthermore, we aimed to investigate the aphid activities involved in the transmission of the semipersistently transmitted, phloem-limited virus, as it is *Beet yellows virus* (BYV, *Closterovirus*) (Esau, 1968), using *M. persicae* as vector. First, we performed preliminary tests using the two most efficient vectors of BYV: *M. persicae* and *Aphis fabae* (Sylvester, 1956; Bennett, 1960; Limburg *et al.*, 1997), and three BYV-host plants: *Chenopodium album*, *Claytonia perfoliata* and *Beta vulgaris* (Sylvester, 1956; Bennett, 1960). Our goal was to determine the most efficient vector-virus-plant combination to perform the EPG experiments. Results showed that *M. persicae*-*B. vulgaris* was the most efficient combination. Therefore, we monitored the feeding behaviour of *M. persicae* on *B. vulgaris* by EPG and terminated the feeding process after the observation of specific EPG patterns to determine which stylet activities were involved in the acquisition and transmission of BYV. In order to determine the aphid stylet activities involved in BYV acquisition, we monitored the feeding process of non-viruliferous *M. persicae* individuals during an AAP in BYV-infected sugar beets and then individually transferred the aphids to non-infected receptor sugar beets which were assessed for BYV transmission efficiency by visual inspection and ELISA test four weeks later. Development of BYV infection in the receptor plants indicated that the aphids had acquired BYV during the AAP. Additionally, we monitored the feeding process of viruliferous *M. persicae* in non-infected sugar beet plants to associate the aphid stylet activities involved in BYV inoculation. The analysis of the EPG recordings revealed a new brief intracellular puncture (hereafter called as phloem-pd) that was associated with inoculation of BYV. The highest BYV inoculation rate was observed when feeding was terminated after the observation of the phloem-pd waveform. Phloem-pds significantly differed from the standard intracellular punctures (standard-pds) previously associated with the transmission of non persistent viruses by aphids that occurred in non-vascular plant tissues (Martín *et al.*, 1997). This new distinct pattern had a shorter duration and lower potential drop magnitude than standard-pds. Also, this particular EPG pattern always preceded the E1 phase (continuous salivation into phloem sieve elements). Therefore, we proposed the name phloem-pd due to the likely possibility that this newly distinctive potential drop occurred in phloem cells (phloem parenchyma, companion or sieve element cells).

Therefore, we performed studies to localize the exact position of the stylet tip in the phloem tissues during the occurrence of the phloem-pd (Chapter 7). We cryofixed both the aphid and leaf tissue surrounding the aphid stylets during a phloem-pd and then identified the type of cell punctured by the stylets using confocal laser-scanning microscopy (CLSM) and micro-computed tomography (micro-CT). Two control treatments were conducted by cryofixing the stylets during a standard-pd and also during the first E1 phase of the aphid. Images from CLSM showed the stylets punctured companion and sieve element cells during phloem-pds. Images from samples analyzed by Micro-CT confirmed that aphid stylets penetrated sieve elements during a phloem-pd. Therefore, we demonstrated by using two different microscopic techniques that aphids can briefly penetrate sieve elements and also companion cells during phloem-pds that occur prior to a penetration that results in continuous salivation phase of the aphid in sieve elements (E1 waveform). During the production of a standard-pd, the stylets penetrated mesophyll or bundle sheath cells, whereas during E1, the stylets penetrated sieve element cells, as previously shown for these two reported EPG patterns (Tjallingii, 1990; Prado and Tjallingii, 1994).

The present Thesis provides new insights on several aspects concerning the transmission of NC viruses by aphids. In addition, results from the present Thesis will help to develop new strategies to interfere or block the retention and transmission of NC by their insect vectors.

CHAPTER 1. INTRODUCTION

1.1 CHALLENGES OF THE FUTURE AGRICULTURE. THE INCREASE OF WORLD POPULATION AND GLOBAL FOOD SECURITY

The quick and high increase of world population represents a big challenge for the near future of the agriculture, with strong efforts needed in order to save the future crop production as well as to improve the yield. The U.N. forecast an 0.96% annual increase in global population between now and 2030 and thereafter a yearly increase of 0.63% through 2050, resulting in an overall increase of global population from its current 7.3 billion to 9 billion in 2050 (Popp *et al.*, 2013; McCarthy *et al.*, 2018). Also, some current trends point to an increase up to 10.9 billion (Prosekov and Ivanova, 2018) (for each country rate, see Fig. 1.1). Such rates of population growth will require an increase in food supply at least by 50% (Ray *et al.*, 2013) and up to 70-75% according to more recent forecasts (McCarthy *et al.*, 2018; Prosekov and Ivanova, 2018).

Figure 1.1. Expected areas of population growth and decline within the range 2000-2080. (From Prosekov and Ivanova, 2018).

Crop production worldwide is threatened by a series of biotic and abiotic factors that have a negative influence in the growth and development, thus reducing the yield. However, quantifying yields losses is such a time-consuming task and works focused on identifying their causes are still limited (Oerke, 2006). Among biotic factors, the losses concerning global food production caused by plant diseases are estimated in 10%,

with viruses as the second factor, just after phytopathogenic fungi (Strange and Scott, 2005; Oerke, 2006). Crop protection practices have reduced the potential losses in agriculture from 50% to about 29% (Oerke, 2006). However, in case of two major source crops for population worldwide, as they are wheat and maize, losses caused by diseases have increased from 9.1% and 9.4%, to 12.6% and 11.2%, respectively, from 1964 to 2003 (Cramer 1967; Oerke *et al.*, 1994; Oerke and Dehne, 2004; Oerke, 2006).

Global food demand is growing quickly, however much of the world's current cropland has yields below their potential, and the current global trajectory with regard to agricultural land expansion has serious long-term implications for the environment (Tilman *et al.*, 2011). For instance, in case of grain production, whereas the total production has more than doubled, the amount of land devoted for arable agriculture has increased by only 9% (Pretty, 2008). Agricultural intensification through technology transfer and enhancement of soil fertility in underyielding nations would greatly reduce the yield gap, decreasing the GHG (greenhouse gas) emissions and species extinctions that otherwise would have resulted from land clearing (Lobell *et al.*, 2009; Burney *et al.*, 2010). Moreover, several authors have pointed out that increasing the crop yield is the most sustainable path for food security rather than clearing more land for food production (Godfray *et al.*, 2010; Tilman *et al.*, 2011; Ray *et al.*, 2013). However, if the forecasted yield change rates do not increase, land clearing could be needed if global food security is to increase or even maintained (Tilman *et al.*, 2011; Ray *et al.*, 2013).

Pests have reduced the productivity of crops since the dawn of agriculture, and farmers have been looking for ways of protecting their crops from these organisms (Oerke, 2006). Weeds represent the most important pest in many crops; however, the efficacy of their control reaches almost the 75%, compared to 39% for animal pests, 32% for pathogens, and just 5% for plant viruses (Oerke, 2006). The use of synthetic insecticides has been a long tradition to control arthropod pest and therefore interfering with virus transmission. However, the high frequency of their application has often resulted in the emergence of resistance of arthropods to the active ingredient. Also, the increased use of pesticides since 1960 obviously has not resulted in a significant decrease of crop losses (Oerke, 2006). Moreover, chemical methods applied for disease control can result in environmental contamination and presence of chemicals in the food, in addition to social and economic problems (Song *et al.*, 2017). Plant viruses, as strict intracellular

pathogens, cannot be controlled chemically, therefore new strategies interfering virus transmission by insect vectors need to be developed. Even though many pesticide applications focus on limiting the population of insect virus-vectors, this is not a totally effective method in the case of interfering with transmission of viruses as vectors can evolve resistance (Foster *et al.*, 2014). New strategies against plant viruses will need to focus mainly on the avoidance of sources of infection, control of the vectors, study of resistance of new plant varieties, the use of proteins that could disrupt virus retention in the vector as well as creation of new transgenic plant varieties (Hull, 2014; van Hemden, 2018).

In the present Thesis, we aimed to provide new information concerning the transmission mechanism of viruses that produce significant crop losses, such as *Beet yellows virus* (BYV, *Closterovirus*) and *Cucumber mosaic virus* (CMV, *Cucumovirus*). The viruses and aphid species used to conduct the experiments have an important impact in the current agriculture, presenting a wide range of host plant species included within three key botanical families cultivated worldwide (*Cucurbitaceae*, *Brassicaceae* and *Amaranthaceae*).

1.2 APHIDS AS PESTS AND VIRUS VECTORS

Arthropods are likely the most successful animal phylum, with Insecta as the largest class of this phylum. Within Insecta, the order Hemiptera comprises small sap-sucking insects such as aphids (Hemiptera: Aphididae) and whiteflies (Hemiptera: Aleyrodidae) with a needle-like stylet bundle consisting of two mandibular and two maxillary stylets. Concretely, 300 Hemipteran species are responsible for the transmission of more than 70% of all known insect-borne viruses (Feres and Raccach, 2015). Particularly, the family Aphididae includes the greatest proportion of insect vectors, transmitting about one third of all plant viruses studied (Hogenhout *et al.*, 2008).

There are two major types of aphid life cycle based on how the insect utilizes host plants: host alternation (heterocious) and non-host alternating (monoecious or autoecious). Host-alternating aphids live on one plant species in winter (primary host – usually woody plants), migrate to an unrelated plant species (secondary host – usually herbaceous plants) in spring/summer and migrate back to the primary host in autumn. After reproducing parthenogenetically during the summer, sexual forms appear in the

fall. Sexual reproduction gives rise to eggs that are laid on the primary host after males and sexual females (oviparae) have mated. Aphids that interrupt parthenogenesis with sexual reproduction in this way are termed holocyclic, whether heterocious or monoecious (Hardie, 2018). Non-host-alternating aphids remain either on the same host species or utilize closely-related host species throughout the years; that is, they can sexually produce eggs on the same group of host plants that is fed on by all the parthenogenetic generations. Some aphid species reproduce continuously by parthenogenesis and never produce an egg stage (anholocyclic life cycle) (Hardie, 2018). Mostly important, they are such a potential harmful factor in plants since aphid populations increase in a very short period of time by means of telescopic generations (Blackman and Eastop, 2000). Also, aphids exhibit dimorphism with apterae and winged morphs, responsible for long distance dispersal contributing to virus spread (Loxdale *et al.*, 2018).

Aphids are considered one of the most important pests worldwide not only because of the direct damage they cause, but also their alimentary habits involve indirect damage. They excrete honeydew, fostering the development of sooty molds that eventually reduces the amount and quality of production. However, the most important indirect damage caused by aphids is the transmission of plant viruses (Field *et al.*, 2018). Most plant viruses require insect vectors in order to move to a new healthy host plant. Hemipteran vector mouthparts are designed for the efficient extraction of plant sap, described as ‘piercing-sucking’, but they also are the sites involved in acquisition and inoculation of plant viruses. Within the proboscis, the mandibular and maxillary stylets form a fine elongated needle-like structure capable of penetrating plant cells walls without causing major damage to plant tissues.

Aphid vectors make their initial foraging decisions by integrating visual and odor cues, which convey information about plant presence, identity and quality (Visser *et al.*, 1996). Flying aphids are attracted to plant-reflected wavelengths (in the green-yellow part of the visual spectrum) during landing and by UV light when taking off from a plant and looking for a new host (Kennedy *et al.*, 1961). After contacting the new host plant, vectors assess additional cues from leaf or stem surfaces, parenchyma, and vascular tissues through olfactory and gustatory sensory systems (Powell *et al.*, 2006). The insect probing, feeding and dispersal behaviours in response to plant cues directly determine

the probability that virions will be acquired, retained and transported (Ferreles, 2016; Mauck *et al.*, 2018). Also, it has been proposed that viruses evolve traits that induce host phenotypes and effects on vectors that encourage virus spread. There is an increasing evidence of plant viruses manipulating vector behaviour to favor their transmission to new host plants by interacting with the vector tissues following acquisition from infected hosts (Carmo-Sousa *et al.*, 2016; Mauck *et al.*, 2018, 2019).

The four main aphid vector species studied in the present Thesis transmit numerous virus species infecting important commercial plants within several important family plants:

- **The cotton aphid (*Aphis gossypii* Glover):** *A. gossypii* is a polyphagous cosmopolitan pest that colonizes more than 100 crop plants, mainly within *Cucurbitaceae*, *Rutaceae* and *Malvaceae* family plants. *A. gossypii* is able to transmit more than 50 plant viruses (Blackman and Eastop, 2018) (Fig. 1.2).
- **The green peach aphid (*Myzus persicae* Sulzer):** *M. persicae* is also a cosmopolitan and extremely polyphagous pest, including more than 40 different plant families within its secondary hosts. The great economic importance of *M. persicae* is due its efficiency as a virus vector, as it has been shown to be able to transmit more than 100 plant viruses (Blackman and Eastop, 2018) (Fig. 1.2).
- **The cabbage aphid (*Brevicoryne brassicae* L.):** *B. brassicae* is an aphid distributed worldwide that mainly colonizes plants in the *Brassicaceae* family. A waxy layer with a grayish-white to blue powdery appearance covers apterae morphs (Fig. 1.2). *B. brassicae* is able to transmit around 20 plant viruses (Olivares, 2017).
- **The black bean aphid (*Aphis fabae* Scopoli):** *A. fabae* occurs nearly world-wide, feeding on about 200 host plants in different botanical families, causing important yield losses on broad bean and sugar beet (Blackman and Eastop, 2018). It has been implicated in the transmission of over 40 plant viruses (Blackman and Eastop, 2018) (Fig 1.2).

Figure 1.2. Four aphid species used in the experiments. Top left: the green peach aphid *Myzus persicae*; top right: the cabbage aphid *Brevicoryne brassicae*. Below left: the cotton aphid, *Aphis gossypii*; below right: the black bean aphid *Aphis fabae*.

1.3 PLANT VIRUSES: MODES OF TRANSMISSION AND RETENTION IN THE VECTOR

Vector-borne plant viruses are obligate intracellular parasites that depend on the host machinery to multiply and invade plant tissues, changing the physiology of their host plants. Plant viruses have been classified in two major categories depending on ability to persist in the vector: circulative viruses (CV), also referred to as persistent viruses, and non-circulative (NC) (Fereres and Raccach, 2015; Blanc and Michalakakis, 2016) (Table 1.1). Within CV category, virus particles cross gut barriers of the vector and enter the circulatory system and ultimately reach the salivary glands, with few virus genera also able to propagate within the vector (Table 1.1; Figure 1.3). On the the other hand, NC virus particles are attached to the aphid cuticle for a short period of time without any circulation within the vector body (Raccach and Fereres, 2009; Blanc *et al.*, 2014).

Table 1.1. Principal characteristics of the modes of virus transmission by insects.

Feature	Noncirculative (NC)		Circulative (CV)	
	Nonpersistent (NP)	Semipersistent (SP)	Persistent Propagative	Persistent Nonpropagative
<i>Acquisition time</i>	Seconds to minutes	Seconds to hours	Minutes to hours	
<i>Inoculation time</i>	Seconds to minutes	Seconds to hours	Minutes to hours	
<i>Retention time</i>	Seconds to minutes/hours	Minutes to hours/days	Days to months	
<i>Latent period</i>	Not required	Not required	Required	
<i>Replication in the vector</i>	No	No	Yes	No
<i>Passage through moult</i>	Negative	Negative	Positive	
<i>Insect species specificity</i>	Low	Intermediate	High	
<i>Association with plants</i>	Not restricted to phloem	Some restricted to the phloem	Restricted to the phloem	

NC viruses are also divided in two different categories depending on the duration of virus retention in the vector: nonpersistent (NP) and semipersistent (SP) viruses (Ferreles and Raccach, 2015; Mauck *et al.*, 2018) (Table 1.1). NP viruses persist briefly in the vector (few hours) and are acquired and inoculated during brief intracellular stylet punctures (seconds), without a latent period in the vector. However, SP viruses persist for longer time in the vector (few hours to days) and need longer periods of time (hours) for acquisition and inoculation, with also no latent period. In both cases, virus transmissibility is lost when the vector molts. CV viruses are also commonly referred to as persistent viruses (P) since they persist for days/months in the vector (Ferreles and Raccach, 2015). These viruses also need longer periods of time for their transmission (hours to days), and require a latent period in the vector (Table 1.1).

Whereas NP viruses are transmitted during short intracellular punctures by aphids in epidermal cells, SP viruses comprise a heterogeneous group that includes viruses that differ in their mode of transmission, which depends on the virus distribution in the tissues of the host plants (Table 1.1; Fig. 1.3). For instance, some SP viruses, such as *Cauliflower mosaic virus* (CaMV, *Caulimovirus*) are located in all plant tissues in their host plants. Therefore, these viruses can be inoculated within seconds to minutes during short aphid probes, similar to NP viruses (Moreno *et al.*, 2012). However, CaMV

acquisition is mainly associated to phloem activities and the transmission rate increases significantly after phloem feeding (Palacios *et al.*, 2002). On the other hand, some other SP viruses such as *Beet yellows virus* (BYV, *Closterovirus*) and *Citrus tristeza virus* (CTV, *Closterovirus*) are restricted to the phloem tissues of the host plant (Esau, 1968; Price, 1966). Therefore, successful transmission of these viruses is achieved only when the aphid stylets reach phloem cells, thus taking longer time for their transmission, similar to CV viruses (Sylvester, 1956; Bennett, 1960; Campolo *et al.*, 2014).

During virus transmission by insect vectors, four steps have been described:

- Acquisition phase: process in which a non-viruliferous vector feeds on an infected plant in order to acquire the virus. In virus transmission experiments, the term acquisition access period (AAP) is defined as the period of time needed for the vector to acquire the virus.
- Latent period: period between virus acquisition and the ability of the vector to transmit the virus.
- Retention period: period subsequent to acquisition in which the vector is competent for virus transmission.
- Inoculation phase: process in which the virus is inoculated by a viruliferous vector to the plant. In virus transmission experiments, the term inoculation access period (IAP) is defined as the period of time needed for a viruliferous vector to inoculate the virus.

The mode of transmission of NC viral particles has been classified according to the way they attach or bind to the vector in two different modes: the ‘CP’ and ‘helper’ strategies. Certain NC particles bind to the aphid cuticle by simply interaction of proteins of the capsid protein (CP) and denominated as ‘CP strategy’ (Normand and Pirone, 1968; Gera *et al.*, 1979; Chen and Francki, 1990). *Cucumber mosaic virus* (CMV, *Cucumovirus*) is the most studied NC virus binding to the vector by the CP strategy (Perry *et al.*, 1994; Perry *et al.*, 1998; Ng *et al.*, 2000, Fereres and Perry, 2019). A highly conserved negatively charged loop structure (the β H- β I loop) was observed on the surfaces of virions and hypothesized to mediate virus binding (Liu *et al.*, 2002).

Figure 1.3. Distribution of circulative (CV) and non-circulative (NC) virus particles in plant tissues and their localization in the vector. CV particles are limited to phloem tissues with only phloem activities of vector stylets playing a role in their acquisition and inoculation. NC particles could be either restricted to the phloem tissues [semipersistently transmitted (SP) phloem-limited viruses] or located in all plant cells [non-persistent (NP) transmitted viruses]. CV particles are retained in the salivary glands, whereas NC particles could be retained either in the foregut or the stylet common duct (From Mauck *et al.*, 2018).

However, the β H- β I loop is not the only part of the CP that is involved in the binding process, since mutations of conserved amino acids in two adjoining loop structures also disrupt transmission (Fereres and Perry, 2019). Also, additional properties beyond physical stability and a surface ligand could be playing a role in aphid transmissibility. Studies of the structure of CMV revealed a cluster of amino acids with acid-base pair interactions to form stabilizing salt bridges between subunits of the asymmetric unit at the quasi-threefold axis of symmetry (Smith *et al.*, 2000). Since this cluster of amino acids is not exposed on the surfaces of the virion, therefore it was hypothesized that dynamic properties may be essential for the binding or release of virions during aphid transmission process (Bricault and Perry, 2013).

On the other hand, some other NC viruses need the presence of an additional virus-encoded nonstructural protein (commonly named as ‘helper component’) that mediates the virus-vector interaction (Pirone and Blanc, 1996; Uzest and Blanc 2016). For instance, potyviruses such as *Turnip mosaic virus* (TuMV, *Potyvirus*) and *Zucchini*

yellow mosaic virus (ZYMV, *Potyvirus*) need the presence of the commonly named “helper component” for this virus genus (HC-Pro), in order to get bound to the cuticle of the aphid stylets. Therefore, successful retention of potyviruses requires first binding of HC-Pro or at the same time as virus particles (Pirone and Blanc, 1996). The HC-Pro is a 50-kDa protein composed of two helix-rich domains, located near the N- and C-terminal ends of the protein and linked by a hinge. Two conserved motifs in HC-Pro are involved in the binding to the virion and to the aphid cuticle. The conserved motif Pro-Thr-Lys (PTK) present in the C-terminal of the HC assists the binding of virus particles (Huet *et al.*, 1994; Peng *et al.*, 1998) through the amino-acid triplet Asp-Ala-Gly (DAG) present N-terminal of the capsid protein (Atreya *et al.*, 1990; Gal-On *et al.*, 1992). On the other hand, the KITC domain located in the N-terminal is the responsible of the binding of the HC-Pro to the aphid’s mouthparts (Blanc *et al.*, 1998; Wang *et al.*, 1996).

Retention sites of SP viruses in the vector have been found for a couple of viruses. For instance, the semipersistent CaMV is retained following the HC strategy, however in this case, the retention to the aphid cuticle is mediated by two helper proteins (denominated P2 and P3) (Drucker *et al.*, 2002; Palacios *et al.*, 2002; Moreno *et al.*, 2012). The P2 protein binds to a specific aphid mouthpart structure named acrostyle that is located in the stylet common duct where both food and salivary canals join together (Uzest *et al.*, 2007). The P2 motif directly involved in specific vector recognition was identified at position 6 of the N-terminus of P2 (Moreno *et al.*, 2005). On the other hand, α -helices at the C-terminus engage in coiled-coil interactions with α -helices of the N-terminal end of the virion-associated protein P3 (Leh *et al.*, 2001). P2 is acquired primarily from epidermal and mesophyll cells during brief intracellular punctures where the complex P3-CaMV is mainly acquired once phloem phase is occurring (Palacios *et al.*, 2002; Drucker *et al.*, 2002). However, the semipersistent and phloem-limited CTV has been found to be retained in the foregut of its main vector *Toxoptera citricida* (Killiny *et al.*, 2016). A summary of virus retention sites and distribution of viral particles in plant tissues is indicated in Fig. 1.3.

In the present PhD Thesis, we focused on some of the main and most studied NC viruses transmitted by four major aphid species worldwide. We applied different virus-vector combinations on the basis of their transmissibility by their most efficient vectors: i)

CMV-*M. persicae*/*Aphis gossypii*, ii) ZYMV-*A. gossypii*, iii) TuMV-*B. brassicae*, iv) CaMV-*M. persicae*/*B. brassicae*, v) BYV-*M. persicae*/*A. fabae*.

- **CMV:** this virus is transmitted by more than 80 aphid species in 33 genera, with *M. persicae* and *A. gossypii* as main aphid vector species (Palukaitis and García-Arenal, 2003). The most efficient vector has been shown to be *A. gossypii* in cucurbits in the field (Labonne *et al.*, 1982) as well as in laboratory tests (Garzo *et al.*, 2004). However, the number of potential vectors could be much higher if more aphid species are tested (Ferreles and Perry, 2019). The only aphid species tested so far that failed to transmit CMV in replicated tests is *Sitobion avenae* (Gildow *et al.*, 2008). CMV has a very broad host plant range, infecting more than 1200 plant species in 100 families including important commercial, such as cucurbits, pepper, lettuce and bean (Scholthof *et al.*, 2011). CMV virion particles are isomeric with a tripartite genome of messenger-sense, single stranded RNA (Ferreles and Perry, 2019). Symptoms of CMV infection include yellowish patches and green and yellow mottling on leaves, and reduced growth in infected plants (Fig. 1.4).
- **ZYMV:** a single-stranded positive-sense RNA virus, also causing severe symptoms in cucurbits such as leaf mosaic, yellowing, fruit distortions and mottling (Simmons *et al.*, 2013) (Fig. 1.4). A large number of aphid species (26) have been shown to transmit the virus (Katis *et al.*, 2006), although two species, *M. persicae* and *A. gossypii*, have the highest reported transmission efficiencies (Castle *et al.*, 1992; Garzo *et al.*, 2004).
- **TuMV:** one of the most important plant viruses transmitted in a NP manner worldwide infecting plants mainly within *Brassicaceae* family and transmitted by at least 89 aphid species (Shattuck, 1992). TuMV has a linear and monopartite genome, with a positive-sense single stranded RNA. The main symptoms caused by TuMV are chlorotic local lesions, mosaics, mottling and puckering (Yasaka *et al.*, 2017) (Fig. 1.4).

Figure 1.4. Symptoms produced by different plant viruses in their host plants. On top, NP viruses: left, *Cucumber mosaic virus* (CMV) infection in zucchini; center, *Turnip mosaic virus* (TuMV) infection in turnip; right, *Zucchini yellow mosaic virus* (ZYMV) infection in zucchini. Below, SP viruses: left, *Beet yellows virus* (BYV) infection in sugar beet; right, *Cauliflower mosaic virus* (CaMV) infection in turnip.

- **CaMV**: the main SP virus infecting plant within the *Brassicaceae* family. CaMV has a circular double-stranded DNA genome (Hull, 2002). Although at least 27 aphid species are listed as known vectors of CaMV (Kennedy *et al.*, 1962), *B. brassicae* and *M. persicae* have been reported as the two main aphid species transmitting CaMV (Broadbent, 1957). Early symptoms of CaMV show vein clearing, with a later chlorosis of old leaves ending in a leaf necrosis (Fig. 1.4).
- **BYV**: it has been the most studied SP phloem-limited virus (Sylvester, 1956; Bennett, 1960; Limburg *et al.*, 1997). BYV is composed of a single stranded flexuous RNA (Agranovsky *et al.*, 1994), and causes vein clearing in basal leaves, with later yellowing and final necrosis and leaf death (Figure 1.4). The main vectors transmitting BYV are *M. persicae* and *A. fabae* (Sylvester 1956;

Limburg *et al.*, 1997). Early BYV infection decreases storage root growth around 20-30% (Clover *et al.*, 1999).

1.4 ELECTRICAL MONITORING OF APHID FEEDING BEHAVIOUR

The electrical penetration graph (EPG) technique allows us to study the different aphid stylet activities produced by aphids in plant tissues (McLean and Kinsey, 1964; Tjallingii, 1978). Aphids are attached to a gold wire of an insect electrode and inserted into the input connector of an amplifier (EPG probe). EPG recording is performed inside a Faraday cage to prevent electrical noise. EPG data acquisition and analysis are conducted by specific software. The different EPG waveforms produced by aphids (Fig. 1.5) have been previously described and associated to a specific stylet tip position in the plant tissues. The patterns so far described for aphids are:

- **Non-probing ‘np’**: flat line associated to the non-probing activity of the aphid prior to stylet penetration into the leaf cuticle.
- **Waveform C**: waveform with extracellular potential. During this waveform, aphid stylets are located in the extracellular pathway (apoplast) before reaching phloem tissues. Two other waveforms are usually included in waveform C: i) waveform A, representing the first intercellular stylet penetration of the aphid stylets in the epidermis and ii) waveform B, associated with the production of gelling saliva and the salivary sheath.
- **Waveform pd (‘potential drop’)**: this waveform represents an intracellular stylet penetration. Three phases have been described within the pd: phase I, with a potential drop produced by aphid stylets insertion through the plasmalemma; phase II, representing the stylet tip presence inside the cell, and phase III, with stylet withdrawal from the cell. Phase II is also divided in three subphases (subphase II-1, II-2 and II-3). Subphase II-1 represent saliva injection into the cytoplasm and is responsible for the inoculation of NP viruses (Martín *et al.*, 1997, Powell, 2005). Subphase II-3 represents ingestion of cell contents and acquisition of NP viruses (Powell, 1995; Collar *et al.*, 1997). Specific aphid activities during subphase II-2 are not well understood. It has been postulated that either aphid salivation or egestion of plant sap previously ingested by the aphid is occurring during the subphase II-2 of the pd (Moreno *et al.*, 2012).

Figure 1.5. Main EPG waveforms described for aphids (Tjallingii, 1978; 1990).

- **Waveform E:** this waveform represents an intracellular potential with aphid stylet tip presence in the sieve elements. Two different patterns are observed within that waveform: E1 and E2. Waveform E1 represents ejection of watery saliva into sieve elements, and it has been associated with the inoculation of persistently transmitted, phloem-limited viruses (Prado and Tjallingii, 1994). Waveform E2 represents phloem sap ingestion with periodic ejection of watery saliva and it has been associated with the acquisition of phloem-limited persistently transmitted viruses (Prado and Tjallingii, 1994).

- **Waveform F:** this waveform is observed when the aphid encounters mechanical difficulties during penetration of plant tissues, likely representing derailed stylet activities.
- **Waveform G:** waveform with extracellular potential representing active ingestion from xylem vessels. Even though this waveform does not play any role in virus transmission by aphids, active xylem ingestion is key in the transmission of xylem-limited bacteria such as *Xylella fastidiosa* by Cicadellidae (Almeida and Backus, 2004) or by Aphrophoridae (Cornara *et al.*, 2018).

1.5 STUDY OF THE PROBING BEHAVIOUR OF APHIDS IN ASSOCIATION WITH VIRUS TRANSMISSION

Monitoring of the feeding behaviour of aphids by the EPG technique to determine specific aphid stylet activities involved in successful inoculation of acquisition of viruses has been paramount in studies concerning aphid-vectored viruses (Scheller and Shukle, 1986; Prado and Tjallingii, 1994; Martín *et al.*, 1997; Moreno *et al.*, 2012; Fereres, 2016). These studies have provided novel information about the key aphid stylet activities involved in transmission of NP, SP and P viruses by aphids, as well as elucidating the main aphid activities (salivation, ingestion, egestion) underlying the different EPG waveforms.

The different EPG waveforms involved NP virus transmission have been well studied. Inoculation of NP viruses occurs during the pd subphase II-1, with acquisition process taking place during the subphase II-3 (Powell *et al.*, 1995; Martín *et al.*, 1997). Inoculation of some SP viruses, such as CaMV is also associated with brief superficial potential drops (pds), similarly to NP viruses. However, only the subphase II-2 plays a role in the inoculation of CaMV (Moreno *et al.*, 2012), but the mechanisms involved in dislodging CaMV particles during subphase II-2 remain unknown. Subphase II-2 could represent an additional salivation phase with possible egestion of cell content previously ingested (Moreno *et al.*, 2012).

Several gaps still exist concerning the mechanism of SP virus transmission, specifically within the phloem-limited virus group (Ng and Zhou, 2015; Fereres, 2016). Although several studies have elucidated the IAPs and AAPs of semipersistently transmitted,

phloem-limited viruses (for BYV: Sylvester, 1956, Bennett, 1960; for CTV: Herron *et al.*, 2006), the specific aphid stylet activities that play a role in their transmission remain unknown. In contrast, the processes for transmission of persistently transmitted, phloem-limited viruses, such as *Barley yellow dwarf virus* (BYDV, *Luteovirus*) have been well studied. Several studies have identified aphid activities associated with inoculation and acquisition of BYDV by artificially ending EPG recordings after specific waveform patterns were observed (Scheller and Shukle, 1986; Prado and Tjallingii, 1994). Studies performed using the English grain aphid *Sitobium avenae* (Hemiptera: Aphididae) as the aphid vector, showed successful inoculation when aphids performed the X-waveform, which is associated to brief and repetitive intracellular punctures prior to ingestion from the phloem (Ip waveform) (Scheller and Shukle, 1986). When using the Bird cherry oat aphid *Rhopalosiphum padi* (Hemiptera: Aphididae) as vector, inoculation and acquisition was associated mainly to waveform E1 and E2, respectively (Prado and Tjallingii, 1994). Nevertheless, a few cases of virus acquisition and inoculation were observed to occur prior to waveforms E1 and E2, suggesting that transmission of persistently transmitted, phloem-limited viruses also occurs during brief intracellular punctures into phloem cells. However, authors could not associate any specific EPG pattern to those intracellular punctures involved in BYDV transmission and presumably occurring into phloem cells. Therefore, the transmission of SP, phloem-limited viruses also may occur prior to E1/E2 phases, on the basis of the knowledge available concerning the transmission of BYDV. Thus, more studies concerning the transmission of SP, phloem-limited viruses are needed in order to understand better the mechanisms of transmission of NC virus by aphids.

Here, in the present Thesis, I specifically focused on providing new insights related to the transmission of NC viruses by aphids. Numerous studies have been conducted in the present PhD Thesis towards fulfilling several information gaps still existing in the field of NP and SP transmission. Results reveal novel and important information about the mechanisms of transmission of NC viruses by aphids. The experiments performed were conducted by using some of the most important viruses and aphid species negatively influencing vegetables, so the data reported will be useful in developing new strategies for interfering with the transmission and spread of viruses among the crops cultivated

worldwide. Also, this new knowledge will lead into future lines of research that will help to further our understanding of the specific mechanisms involved in the transmission of NC viruses by hemipteran insects.

CHAPTER 2. OBJECTIVES

The general objective of this PhD Thesis was to investigate and generate new information regarding NC virus transmission by aphids. More precisely, I first challenged the universal hypothesis about the increased transmission rate after a pre-acquisition fasting period. Later I studied the competition between different NC viruses for retention sites in the vector by performing serial sequential transmission tests. To investigate further the mechanisms of transmission of phloem-limited SP viruses, I studied the specific stylet activities produced by aphids associated with transmission of closteroviruses. Finally, I studied the localization of the maxillary stylet tips within the plant tissues during the inoculation of phloem-limited viruses using different microscopy techniques.

Therefore, the following specific objectives were covered in the present PhD Thesis:

1. To study the pre-acquisition fasting effect on the transmission of the NP *Cucumber mosaic virus* (CMV, *Cucumovirus*) and on the probing behaviour of *Myzus persicae* and *Aphis gossypii*.
2. To test the interference between different genera of NC viruses (CaMV and TuMV; CaMV and CMV; ZYMV and CMV) for retention sites in their main aphid vectors (*Brevicoryne brassicae*, *Myzus persicae* and *Aphis gossypii*).
3. To study the feeding behaviour of *Myzus persicae* associated to the transmission of the semipersistent and phloem-limited *Beet yellows virus* (BYV, *Closterovirus*).
4. To localize by confocal laser-scanning microscopy and microcomputed tomography the stylet tips of *Myzus persicae* in the plant tissues during the occurrence of specific activities associated with the inoculation of the semipersistently transmitted, phloem-limited BYV.

CHAPTER 3. MATERIAL AND METHODS

3.1 EXPERIMENTAL SITES

The experiments concerning objectives 1, 2 and 3 of this Thesis were conducted at the Institute of Agricultural Sciences (ICA) facilities of the Spanish National Research Council (CSIC) (Madrid, Spain). Some experiments within objective 4 were conducted in a short-term (4-month) research placement at University of California Riverside (UCR, Riverside, CA., USA) in collaboration with Prof. James Ng at the Department of Plant Pathology and Prof. Gregory P. Walker at the Department of Entomology. Experiments of objective 4 were completed by the analysis of leaf and insect samples at the University of Granada (Spain) in collaboration with Prof. Javier Alba-Tercedor at the Department of Zoology.

3.2 BIOLOGICAL MATERIAL

3.2.1 PLANTS

The following plant species were used for the experiments at ICA-CSIC and UCR facilities. Seeds of zucchini (*Cucurbita pepo* cv. 'Précoce mâraichère': Vilmorin), melon (*Cucumis melo* cv. 'Siglo', cv 'Margot' and cv 'Primal'; 'Siglo' and 'Primal': Syngenta; 'Margot': kindly provided by Dr. María Luisa Gómez-Guillamón), turnip (*Brassica rapa* cv. 'Just Right': Takii & Co., LTD, Kyoto, Japan), sugar beets (*Beta vulgaris* cv. 'Julietta': KWS Ibérica) and a variety from breeding line resistant to powdery mildew (UCR), chenopodium (*Chenopodium album*: Semillas Cantueso), montia (*Claytonia perfoliata*: B&T World Seeds). All plants were germinated in 12cm-diameter pots filled with 1:1 mixture of vermiculite (Asfaltex S.A., Barcelona, Spain) and soil substrate (Jiffy Products International BV, Moerdijk, The Netherlands).

Plants were watered three times a week containing water-soluble 20-20-20 (N-P-K) Nutrichem 60 fertilizer (Miller Chemical & Fertilizer Corp., Pennsylvania, USA) at a 1g·L⁻¹ dosage. Plants used as test plants or for aphid rearing were grown and maintained inside a 'walk-in' chamber (Ibercex S.A., Arganda del Rey, Spain) at 23:20 °C temperature (day:night), a photoperiod of 16:8 (day:night), 60-80% RH, 120 µEinstein

$\text{m}^{-2} \text{ s}^{-1}$ PAR (30 cm from light source) (Fig. 3.1). At UCR, plants were kept in greenhouse at a temperature of $25 \pm 4^\circ\text{C}$ and ambient light conditions.

Figure 3.1. Insect-free climatic chamber for plant development at ICA-CSIC facilities.

Virus source plants were kept in another walk-in chamber at a temperature of $24:20^\circ\text{C}$ (day:night), a photoperiod of 16:8 (day:night) and a PAR of $120 \mu\text{Einsteins m}^{-2} \text{ seg}^{-1}$ (30 cm from light source). After the transmission tests were conducted, the receptor plants were grown in a greenhouse under natural light conditions and temperature control (temperature of $23 \pm 4^\circ\text{C}$). Both virus source and test plants were watered and fertilized similarly as the test plants.

3.2.2 APHID SPECIES

Four different aphid species were used throughout the experiments: *Myzus persicae* Sulzer (Hemiptera: Aphididae), *Aphis fabae* Scopolii (Hemiptera: Aphididae), *Aphis gossypii* Glover (Hemiptera: Aphididae) and *Brevicoryne brassicae* L. (Hemiptera: Aphididae). Information about the different aphid species is indicated in Table 3.1. All colonies were started from a single viviparous apterae female collected in the field. Aphids were reared at medium-high density by placing 2-3 individuals per rearing plant.

Table 3.1. Different aphid species used in the experiments. Collection sites and year, aphid collection plant, rearing host plant used and rearing climatic conditions are indicated.

Aphid species	Place	Year	Plant species origin	Plant species rearing	Rearing conditions
<i>Myzus persicae</i> Sulzer.	‘El Encín’, Alcalá de Henares, Madrid (Spain)	1989	Pepper	Sugar beet cv. ‘Julietta’	23:18 °C 60-80% RH 16:8 L:D
<i>Myzus persicae</i> Sulzer.	Imperial County, California (USA)	*	Broccoli	Sugar beet	22:20 °C 60-80% RH 16:8 L:D
<i>Aphis gossypii</i> Glover	Almería (Spain)	1998	Melon	Melon cv. ‘Siglo’	23:18 °C 60-80% RH 16:8 L:D
<i>Aphis fabae</i> Scopoli	‘La Poveda’, Madrid (Spain)	2016	Chenopodium	Sugar beet ‘Detroit Dark Red’	23:18 °C 60-80% RH 16:8 L:D
<i>Brevicoryne brassicae</i> L.	San Martín de la Vega, Madrid (Spain)	1999	Cabbage	Turnip cv. ‘Just Right’	23:18 °C 60-80% RH 16:8 L:D

*No date available about aphid collection from the field.

Figure 3.2. Insect-rearing ‘walk-in’ climatic chamber at ICA-CSIC facilities.

For EPG and transmission experiments, newly emerged adult aphids were used (10-12 days of age). After 14 days, the colony was discarded and a new colony was re-started by repeating the same protocol. To obtain winged morphs of *M. persicae* (alatae), aphids were allowed to complete three generations (21 days) in the same plant to overcrowd the colony and induce the appearance of alatae morphs. Aphid colonies in the 'walk-in' chamber were maintained inside plastic cages covered with a fine net to allow ventilation (Fig. 3.2).

3.3 VIRUS - INFECTED SOURCE PLANTS

To produce virus-infected source plants for acquisition and transmission experiments, two different inoculation methodologies were used: mechanical inoculation and inoculation by aphids.

3.3.1 MECHANICAL VIRUS INOCULATION

Virus source plants infected by *Turnip mosaic virus* (TuMV), *Zucchini mosaic virus* (ZuMV), *Cucumber mosaic virus* (CMV) and *Cauliflower mosaic virus* (CaMV) were generated by mechanical inoculation. For inoculation of TuMV and CaMV, turnip plants were inoculated at growth stage 11 (BBCH-scale). For inoculation of ZYMV and CMV, melon and zucchini plants were inoculated at stage 11. Plants were mechanically inoculated by using infected either fresh or dehydrated vegetal material maintained at 4°C or -80°C. Infected material was homogenized in an inoculation buffer (disodic phosphate 0.03M, containing 0.2% by weight of DIECA in a 1:10 (wt/vol) proportion, pH=7.0). Before inoculation to healthy plants, leaves were dusted with silicon carbide (carborundum) (ASTM N° 600) to produce small wounds to break cells walls, allowing the entry of virus particles and survival of the wounded cells. Later, the buffer containing infected vegetal material was gently spread with the forefinger on the adaxial side of the dusted leaf.

3.3.2 VIRUS INOCULATION BY APHIDS

Aphid inoculation was used to generate new virus-infected source plants infected with the phloem-limited BYV. Thus, non-viruliferous aphids collected from the colony were allowed to feed on a BYV-infected plant for 24h as an acquisition access period (AAP). BYV-infected plants were inoculated 4-5 weeks before placing the aphids. Groups of

10 aphids were then transferred to a non-infected sugar beet plant (stage 10) for an inoculation access period (IAP) of 24h. After the IAP, aphids were carefully removed from the new source plants using a fine brush and plants were weekly checked to remove any living nymphs.

3.4 VIRUS DETECTION: ELISA TEST

Viral infection assessment of test plants was conducted by first visualization of symptoms produced by the different viruses studied, followed by a serological confirmation by *Enzyme-Linked Immunosorbent Assay* (ELISA). ELISA tests were conducted 3-4 weeks after virus transmission experiments. For assessment of infection by potyviruses (TuMV and ZYMV) and caulimoviruses (CaMV) an indirect ELISA test was performed. For detection of CMV and BYV a *Double Antibody Sandwich* ELISA (DAS-ELISA) was performed (Clark and Adams, 1977) (Table 3.2).

Table 3.2. Characteristics of the different antibodies used for the viral infection assessment by ELISA test.

Virus Species	Provider	Dilution	Conjugate enzyme	Type of antibody
TuMV	BIOREBA AG, Switzerland	1:200	Alkaline Phosphatase	Polyclonal
ZYMV	BIOREBA AG, Switzerland	1:200	Alkaline Phosphatase	Polyclonal
CaMV	AGDIA Inc., USA	1:250	Peroxidase	Monoclonal
CMV	BIOREBA AG, Switzerland	1:200	Alkaline Phosphatase	Monoclonal
BYV	SEDIAG, France	1:1000	Alkaline Phosphatase	Monoclonal

Buffers were prepared as indicated by the provider and the ELISA protocol was conducted following instructions included in the different Elisa Kits. Different buffers were used depending on the virus detected, type of antibody and type of ELISA test. Absorbance was measured at a 405 nm (or 650 nm in case of CaMV) by a BioTek spectrophotometer (BioTek Instruments Inc., Winooski, USA). A sample was

considered as positive infected when its value was three times higher than the average values of the non-infected samples used as negative controls.

3.5 ELECTRICAL PENETRATION GRAPHS (EPG) TECHNIQUE

Aphid probing and feeding was monitored by the use of either a GIGA-8 or GIGA-4 direct current-EPG (DC-EPG) device (EPG Systems, The Netherlands). Adult apterae aphids were attached to the gold wire of an insect electrode using water-based silver glue (EPG Systems, The Netherlands; Inframat Advanced Materials, Manchester, CT, USA). For wiring, aphids were immobilized at the tip of a pipette tip connected to a vacuum pump. Silver conductive paint (Pelco® Colloidal Silver no. 16034, Ted Pella Inc., Redding, CA, USA) was used to attach the opposite end of the gold wire (18.5µm diameter, 2-2.5cm long) to a 3-cm-long copper wire (0.2mm diameter) that was soldered to a brass nail (1.2mm diameter) using flux enhancement. This electrode nail was inserted into the input connector of the first-stage amplifier (EPG probe). The plant electrode was a copper post (10 cm long, 2 mm diameter), which was inserted into the potting soil of a plant. EPG recording was performed inside a Faraday cage to prevent electrical noise (Fig. 3.3). Then, after the paint was dry (aprox. 15 min) aphids were placed on the abaxial side of the leaf after securing the leaf to a plastic bar with the help of a hair clip to prevent movement (Fig. 3.4).

Figure 3.3. Electrical penetration graph (EPG) set-up. (Adapted from Tjallingii, 1999)

The signal was digitized at 100 samples per second using a Dataq 710 or 720 (DATAQ Instruments) analog to digital card converter and recorded on Windaq Pro software (hardware and software from Dataq Instruments, Akron, OH, USA) or using Stylet+ Software for Windows (EPG Systems, The Netherlands). Substrate voltage was adjusted following the calibration instruction of the DC-EPG equipment so that EPG output signals fit into the +5V to -5V provided by the software (Fig. 3.3).

Figure 3.4. Left: Monitoring the feeding process of *Myzus persicae* apterae adults in *Beta vulgaris* by Electrical penetration graph (EPG). Right: wired *M. persicae* individual feeding on *Beta vulgaris* (leaves were often secured with a hair clip to avoid leaf movement).

3.6 STATISTICAL PROCEDURES

Statistical analyses were conducted using SPSS software package (version 22 and 25) (IBM SPSS Statistics, 2013; 2017), StatView 4.0 (Abacus concepts, 1992) and R (v.3.3.3) (R Development Core Team, 2016). The different statistical tests conducted to study significant differences between the variables analyzed in each experiment are described in the specific material and methods section. Virus transmission rates were compared performing a Chi-square test or following the Marascuilo procedure. A Fisher's Exact test was performed when expected values were lower than 5. The different aphid behavioural variables as well as potential drop magnitude were analyzed by conducting parametric tests (Gaussian variables) or non-parametric tests (non-Gaussian variables) ($p < 0.05$). If needed, behavioural variables were transformed by either $\ln(x+1)$ or $\sqrt{x+1}$ prior to analysis and later checked for normality using Shapiro Wilk W test.

CHAPTER 4. STUDY OF THE PRE-ACQUISITION FASTING EFFECT ON THE TRANSMISSION OF *Cucumber mosaic virus* (CMV, *Cucumovirus*) AND PROBING BEHAVIOUR OF APHIDS¹

4.1 INTRODUCTION

Fasting aphids prior to an acquisition access period (AAP) has been known for many years to enhance non-persistent virus transmission (Watson, 1938; Watson and Roberts, 1939). Hereafter referred to as the pre-acquisition fasting effect, the phenomenon was first reported to occur when *Myzus persicae* Glover (Hemiptera: Aphididae) was prevented from feeding for as little as five minutes prior to an AAP for cucumber virus 1 (synonym of *Cucumber mosaic virus*, CMV), *Hyoscyamus virus* 3 and potato virus 3 (synonyms of the potyviruses: *Henbane mosaic virus*, HMV, and *Potato virus Y*, PVY). Subsequently the pre-acquisition fasting effect has been evaluated and confirmed for several potyviruses [e.g., *Tobacco etch virus* (TEV, *Potyvirus*), *Turnip mosaic virus* (TuMV, *Potyvirus*)] using the same vector *M. persicae* (Cockbain *et al.*, 1963; Day, 1954; Sylvester, 1950, 1955; Watson, 1946; Hamlyn, 1953; Powell, 1993). This effect, however, has not been observed for all potyviruses. For example, a pre-acquisition fasting period did not significantly increase the transmission of *Zucchini yellow mosaic virus* (ZYMV, *Potyvirus*) by *M. persicae*, but did so when *Aphis gossypii* was used as vector (Fereres *et al.*, 1992). Similarly, a pre-acquisition fasting period was reported to have no impact on *Cauliflower mosaic virus* (CaMV, *Caulimovirus*) transmission by *M. persicae* and *Brevicoryne brassicae* (Hamlyn, 1955; Martini, 1956).

Two hypotheses have been proposed to explain the pre-acquisition fasting effect on virus transmission. First, compounds from either the plant or aphid saliva could accumulate in the mouthparts of vectors during feeding, and subsequently prevent virus acquisition and retention.

¹Published in: Jiménez J., Webster C.G., Moreno A., Almeida R.P.P., Blanc S., Fereres A., Uzeš M. 2017. Fasting alters aphid probing behaviour but does not universally increase the transmission rate of non-circulative viruses. *Journal of General Virology*, 98: 3111-3121.

Accordingly, such inhibitory compounds would be removed upon fasting (Watson, 1938). Wang and Pirone (1996) reported that fasted *M. persicae* retained virions in their mouthparts more efficiently than non-fasted aphids. This observation was positively correlated with an increase in virus transmission efficiency after a pre-acquisition fasting period. Wang and Pirone also reported that the fasting effect disappeared when *M. persicae* were allowed to feed for an extended period of time on sucrose solutions. Under these conditions, both fasted and non-fasted aphids displayed similar transmission efficiency to fasted aphids reared on healthy plants (Wang and Pirone, 1996). Therefore, these authors suggested that during the fasting period, aphids could release plant inhibitory compounds previously ingested and retained in their stylets. An alternative hypothesis proposes that fasting alters aphid probing behaviour (Bradley, 1952; 1956; Collar and Fereres, 1998). Instead of having stereotypical phloem-searching behaviour, fasted aphids were observed to make repetitive, superficial probes that favour the transmission of non-circulative viruses (Bradley, 1952; 1956). Detailed monitoring of the probing behaviour of fasted and non-fasted aphids by the electrical penetration graph (EPG) technique (Tjallingii, 1978) on PVY-infected plants during the AAP also revealed differences in the intracellular punctures (commonly named as potential drops: pds) (Collar and Fereres, 1998). Fasted aphids produced pds with a significantly longer II-3 sub-phase, a sub-phase linked to the acquisition of non-circulative viruses (Martin *et al.*, 1997; Powell *et al.*, 1995; Palacios *et al.*, 2002; Symmes *et al.*, 2008). Similarly, a higher number of pulses in fasted aphids were also observed during II-3 sub-phase, a variable showed to increase PVY transmission (Collar *et al.*, 1997). For ZYMV, the length of sub-phase II-3 was correlated with virus acquisition by *M. persicae*, but not with the number of archlets occurring during this sub-phase (Symmes *et al.*, 2008).

The paradigm that pre-acquisition fasting positively impacts on non-circulative virus transmission efficiency only stands on studies of a few virus-vector combinations (Watson, 1938; Watson and Roberts, 1939; Cockbain *et al.*, 1963; Sylvester, 1956; Powell, 1993; Fereres *et al.*, 1992; Hamlyn, 1955; Martini, 1956; Wang and Pirone, 1996). Nevertheless, aphid fasting prior to transmission experiments is a widely used experimental approach. However, available data do not clearly justify the use of a fasting period prior to virus acquisition. More importantly, the biological mechanism(s) driving the observations associated with aphid fasting and virus transmission are not

understood. Furthermore, since Watson's initial report in 1938, little work has been done on Cucumoviruses and other non-circulative viruses, and this effect, that may be dependent on virus/vector combination, remains to be thoroughly investigated. In summary, a pre-acquisition fasting period is included in the design of most experiments involving non-circulative virus transmission [nonpersistent (NP) and semipersistent (SP) viruses] and aphid probing behaviour (Swenson, 1962; Shuckle *et al.*, 1987; Collar *et al.*, 1998; Symmes and Perring, 2007; Symmes, 2008; Krenz *et al.*, 2015), despite the fact that the biological role of this artificial experiment detail is unknown, is poorly characterized and that the outcome of this application is not always the same. Because epidemiological models tend to use experimental data on virus transmission rate to simulate pathogen spread, understanding the impact of a fasting period on vector transmission of viruses is of applied relevance.

4.2 OBJECTIVE

The present study aimed to evaluate if a pre-acquisition fasting period universally increases the transmission rate of all NP-transmitted viruses by studying the effect of fasting on CMV transmission, using *M. persicae* and *A. gossypii* as aphid vectors. Transmission tests were conducted with fasted and non-fasted aphids, to assess the impact of the aphid species and the plant species used as a virus-infected source on virus transmission efficiency. Additionally, the feeding behaviour of both fasted and non-fasted aphid vectors during the AAP was evaluated by using the EPG technique, to determine the specific aphid probing activities linked to the transmission of non-persistent viruses.

4.3 MATERIAL AND METHODS

4.3.1 VIRUS AND APHID MAINTENANCE

M. persicae were reared on *Solanum melongena* cv. "Barbentane" and *A. gossypii* were reared on *Cucumis melo* cv. "Siglo", following the same procedures indicated in Chapter 3 (section 3.2.2). Isolates of CMV (Fny, M6, V698, Ls, Val 24 and B20) were mechanically inoculated on *Cucurbita pepo* cv. 'Précocé Maraîchère' following the procedures indicated in Chapter 3 (section 3.3.1).

4.3.2 TRANSMISSION TESTS TO STUDY THE PRE-ACQUISITION EFFECT IN THE CMV ACQUISITION BY *Aphis gossypii* AND *Myzus persicae*

To assess whether a fasting effect was observed for different viral strains in a single virus species, transmission tests were performed on six CMV isolates (Fny, M6, V698, Ls, Val 24 and B20) using *A. gossypii* as vector. In these series of experiments, 5 aphids per test plant were allowed a 5 minutes AAP on CMV-infected plants, followed by a 2 hours IAP. To study the pre-acquisition fasting effect using *M. persicae* as vector, transmission tests were performed with AAP visualized through a binocular microscope. Both non-viruliferous fasted and non-fasted aphids were moved by paintbrush to upper, fully expanded CMV-Fny-infected leaves. Apterous *A. gossypii* and *M. persicae* adults were collected from the colony and either isolated for one hour in small plastic cages (fasted aphids) or transferred directly from the rearing plant on a CMV-infected plant (non-fasted aphids). In case of *M. persicae*, individuals were considered to have successfully completed an AAP when they were observed to have sustained contact between the rostrum of the aphid and the infected source leaf for 20 to 30 seconds as an AAP. Aphids that did not show sustained contact of labium-leaf surface within the first two minutes were discarded. Two *M. persicae* individuals were then transferred to each zucchini test plant (cv. ‘Précoce Maraîchère’) and allowed to feed for an IAP of 24 hours, before the aphids were killed by an insecticide treatment. Symptoms of virus infection were recorded 21 days later by visual inspection (Perry *et al.*, 1998; Desbiez *et al.*, 2003; van Munster *et al.*, 2017) and ELISA test, following the procedures indicated in Chapter 3 (section 3.4). The virus transmission efficiency was calculated by the number of plants developing infection divided by the total number of test plants.

4.3.3 STUDY OF THE PROBING BEHAVIOUR OF FASTED AND NON-FASTED APHIDS

To investigate the probing behaviour of fasted and non-fasted *A. gossypii* and *M. persicae*, an EPG device (Giga-8; EPG-Systems) (EPG Systems, Wageningen, The Netherlands; Tjallingii, 1978) was used to study the aphid stylet activities during the AAP in the CMV-infected source plant [for a detailed EPG set-up description, see Chapter 3 (section 3.5)]. Newly emerged non-viruliferous *M. persicae* and *A. gossypii* were used for the experiments. After wiring, some aphids were suspended in the air by the wire and not allowed to feed (fasted), or allowed to feed on a healthy melon leaf (*A. gossypii*) or on a healthy eggplant leaf (*M. persicae*) (non-fasted). After one hour, both

fasted and non-fasted aphids were connected to the EPG device and placed on a CMV-Fny infected source zucchini plant until a single intracellular puncture (pd) was observed. Aphids that did not start to probe within 5 minutes were discarded. Once the pd was observed, recordings were manually ended by softly touching the aphids with a brush. Duration of some EPG waveforms, previously described for aphids (Tjallingii, 1988), and relevant for non-circulative virus transmission (Powell, 1991) were considered as variables to compare the probing behaviour of fasted and non-fasted aphids: (i) time spent from start of the recording until first probe (Start – C; C waveform reflecting the apoplastic stylets pathway), (ii) time from first probe until intracellular puncture (C – pd), (iii) duration of intracellular puncture (pd duration), and (iv) time spent from the start of the recording until the intracellular puncture (Start – pd). Finally, among the three distinct subphases occurring during an intracellular puncture (II-1, II-2 and II-3), the duration of II-3 sub-phase of the pd as well as the number of archlets within this sub-phase were quantified. Longer duration of this sub-phase and thus larger number of archlets, have been positively correlated with acquisition efficiency of non-circulative viruses (Martín *et al.*, 1997; Powell *et al.*, 1995).

4.3.4 STATISTICAL ANALYSIS

Virus transmission rates obtained by either fasted or non-fasted aphids were compared performing a Chi-square test or a Fisher's Exact test when expected values were lower than 5, using Stat View 4.0 statistical package ($p < 0.05$) (Abacus Concepts, 1992). The analysis of the duration of the different behavioural variables studied by either EPG recording or binocular microscope was conducted using SPSS v.22 software package (IBM SPSS Statistics, 2013). Behavioural variables were transformed by either $\ln(x+1)$ or $\sqrt{x+1}$ prior to analysis and later checked for normality using Shapiro-Wilk test. Mean comparison between groups of fasted and non-fasted aphids that followed a Gaussian distribution was analyzed by Student's *t*-test to detect any significant difference ($p < 0.05$). When behavioural variables did not follow a Gaussian distribution, non-parametric Mann-Whitney *U*-test test was then applied.

4.4 RESULTS

4.4.1 PRE-ACQUISITION FASTING EFFECT ON CMV TRANSMISSION EFFICIENCY BY *Aphis gossypii* AND *Myzus persicae*

Experiments testing the pre-acquisition fasting effect of *A. gossypii* in the transmission of six CMV isolates showed that fasting only increased the transmission of strain V698 (Fig. 4.1; $p=0.013$). No significant impact of fasting on the transmission rate was observed for the other five CMV strains tested: CMV-Fny ($p=0.710$), M6 ($p=0.138$), Ls ($p=0.999$), Val24 ($p=0.999$) and B20 ($p=0.391$) (Fig. 4.1). The lack of fasting effect in the combination CMV-*Aphis gossypii* was also observed in transmission tests performed in Chapter 5 (section 5.4.3; Fig. 5.3 B), concerning studies to test the interference between NP-transmitted viruses for retention sites in the vector. Concerning the experiments to test the pre-acquisition fasting period prior to CMV-Fny acquisition using *M. persicae* as vector, fasting clearly favored acquisition and subsequent transmission of CMV. Whereas transmission efficiency obtained for non-fasted aphids was only 1.75% (1/57), CMV transmission efficiency was significantly increased for fasted aphids (21.05%; 12/57) ($\chi^2=10.51$; $p<0.001$).

Figure 4.1. Transmission of various CMV strains (Fny, M6, LS, Val24, B20 and V698) by fasted and non fasted *Aphis gossypii*. Box plots show the percentage of virus transmission using 5 aphids per test plant in a single replicate of 26 to 28 test plants. A single source plant was used for each virus strain.

*Statistical differences for each fasted/non-fasted pair determined by a Chi-square test ($p < 0.05$).

4.4.2 EFFECT OF FASTING ON APHID PROBING BEHAVIOUR ASSESSED BY THE EPG TECHNIQUE

To better evaluate the behaviour of *A. gossypii* and *M. persicae*, EPG was used to record the type and duration of feeding events of aphids fed on CMV-Fny-infected zucchini plants (Table 4.1). The time spent by fasted aphids from the start of the EPG recording until the first stylet insertion into the leaf tissue (C waveform) was significantly reduced relatively to non-fasted aphids, from 92.35 to 47.75 s for *A. gossypii* and 38.84 to 19.64 s for *M. persicae* (Table 4.1). The duration of the C waveform, was not significantly different between the treatments for either aphid. The duration of the first pd increased from 7.29 to 9.81 s for *A. gossypii* and from 5.43 to 6.21 s for *M. persicae* for non-fasted and fasted aphids, respectively. Additionally, the duration of the II-3 sub-phase observed in fasted aphids was significantly longer than for non-fasted individuals, from an average of 4.79 and 3.27 s to an average of 7.22 and 4.04 s per aphid for *A. gossypii* and *M. persicae*, respectively (Table 4.1). The number of archlets during the II-3 sub-phase in fasted aphids was significantly higher than in non-fasted aphids: from an average of 5.35 and 4.34 to an average of 8.78 and 6.15 per aphid for *A. gossypii* and *M. persicae*, respectively (Table 4.1).

Duration of II-3 sub-phase and number of archlets during II-3 sub-phase differed significantly between fasted *M. persicae* and *A. gossypii* ($p < 0.001$ and $p < 0.001$, respectively; Table 4.2). However, when non-fasted, the two aphid species did not show such significant differences ($p = 0.224$ for II-3 duration; $p = 0.896$ for number of archlets) (Table 4.2).

Table 4.1: Effect of fasting on the duration of probing behaviour variables (measured in seconds, with exception of No. II-3 pulses) analysed by electrical penetration graph (EPG) of *Myzus persicae* and *Aphis gossypii* on *Cucumber mosaic virus* CMV-Fny infected *Cucurbita pepo*.

Behavioural Variables	Treatment Test value	N	Mean $\pm$ SE	Min.	Max.	P value
<i>Myzus persicae</i>						
Start EPG – C	Fasted	99	19.64 $\pm$ 1.70	1.17	74.90	0.001*
	Non-fasted	99	38.84 $\pm$ 4.13	0.61	203.38	
C – pd	Fasted	99	9.60 $\pm$ 0.90	2.15	58.20	0.225
	Non-fasted	99	14.65 $\pm$ 2.16	1.65	127.66	
pd duration	Fasted	99	6.21 $\pm$ 0.15	3.17	10.01	0.001*
	Non-fasted	99	5.43 $\pm$ 0.18	2.55	11.60	
II-3 Duration	Fasted	99	4.04 $\pm$ 0.13	1.23	7.27	0.000*
	Non-fasted	99	3.27 $\pm$ 0.17	0.50	8.93	
No. II-3 pulses	Fasted	99	6.15 $\pm$ 0.28	0	12	0.000*
	Non-fasted	99	4.34 $\pm$ 0.36	0	15	
<i>Aphis gossypii</i>						
Start EPG – C	Fasted	109	47.75 $\pm$ 5.15	2.01	297.60	0.000*
	Non-fasted	104	92.35 $\pm$ 8.11	5.39	299.96	
C – pd	Fasted	109	19.43 $\pm$ 4.04	2.88	350.90	0.128
	Non-fasted	104	32.80 $\pm$ 4.41	2.93	232.81	
pd duration	Fasted	109	9.81 $\pm$ 0.36	2.65	20.67	0.000*
	Non-fasted	104	7.29 $\pm$ 0.41	2.65	23.12	
II-3 Duration	Fasted	109	7.22 $\pm$ 0.35	0.80	17.15	0.000*
	Non-fasted	104	4.79 $\pm$ 0.40	0.42	19.98	
No. II-3 pulses	Fasted	109	8.78 $\pm$ 0.50	0	21	0.000*
	Non-fasted	104	5.35 $\pm$ 0.61	0	32	

*Significant differences ($P < 0.05$) according to a ¹t-student test (Gaussian variables) and to a ²Mann-Whitney U test (Non-Gaussian variables).

Table 4.2: Comparison of sub-phase II-3 duration (seconds) and number of II-3 pulses between fasted and non-fasted *Aphis gossypii* and *Myzus persicae* probing on *Cucumber mosaic virus* CMV-Fny infected *Cucurbita pepo*.

Variable	Treatment	N	Mean $\pm$ SE	Min.	Max.	P value
U-value						
II-3 Duration						
Fasted	<i>M. persicae</i>	99	4.04 $\pm$ 0.13	1.23	7.27	0.000*
	<i>A. gossypii</i>	109	7.22 $\pm$ 0.35	0.80	17.25	
Non-Fasted	<i>M. persicae</i>	99	3.27 $\pm$ 0.17	0.50	8.93	0.224
	<i>A. gossypii</i>	104	4.79 $\pm$ 0.40	0.42	19.98	
II-3 Pulses						
Fasted	<i>M. persicae</i>	99	6.15 $\pm$ 0.28	0	12	0.000*
	<i>A. gossypii</i>	109	8.78 $\pm$ 0.50	0	21	
Non-Fasted	<i>M. persicae</i>	99	4.34 $\pm$ 0.36	0	15	0.896
	<i>A. gossypii</i>	104	5.35 $\pm$ 0.61	0	32	

* Significant differences ($P < 0.05$) according to a Mann-Whitney U test.

4.5 DISCUSSION

Many variables have been shown to impact the acquisition, retention and inoculation of non-circulative viruses, including virus strain, aphid clone, plant host and previous feeding activities of the aphid vector (Symmes and Perring, 2007; Fereres and Moreno, 2009; Fereres and Raccach, 2015). The fasting of aphids prior to an AAP on virus-infected plants or solutions containing purified virus is generally assumed to significantly increase non-circulative virus transmission (Watson, 1938). However, the underlying mechanisms explaining this phenomenon are largely unknown. Furthermore, fasting does not appear to increase transmission rate for all virus/aphid combinations (e.g. Symmes and Perring, 2007). We confirmed this observation, that fasting does not increase transmission efficiency in all virus/aphid combinations, as observed for CMV transmission by *A. gossypii*. We also showed that, at least for CMV, the occurrence of a fasting effect is also affected by virus isolate. These results challenge the broadly accepted notion that fasting (or the starvation effect) universally increases non-circulative virus transmission rate.

As previously reported by Wang and Pirone (1996), the ‘fasting effect’ is suppressed when aphids are transitorily fed on a sucrose solution prior to an AAP on virus-infected plants. We observed this same effect in the experiments performed and included in the published manuscript where the results of this chapter were reported (Jiménez *et al.*, 2017). These results support the hypothesis that compounds of plant origin present in the stylets of non-fasted aphids probably interfere with virus uptake and/or virus retention. Aphids feeding on a sucrose solution also seemed to negatively impact the transmission efficiency of *M. persicae* and *A. gossypii* for the viruses tested when compared to aphids reared on plants, which generally transmitted the three viruses more efficiently. Therefore, the presence of a plant competitor molecule impairing efficient virus uptake and retention at the binding sites in aphid mouthparts alone cannot account for the ‘fasting effect’ observed.

Fasting period of aphids prior to an AAP affected the probing behaviour. While not significant in all comparisons, these variations among treatments support the hypothesis that changes in aphid probing behaviour may explain the fasting effect on non-circulative virus transmission rate (Bradley, 1952; Bradley, 1956; Collar and Fereres, 1998). We attempted to identify the underlying causes of the fasting effect on transmission by comparing the probing behaviour of *M. persicae* and *A. gossypii* monitored by EPG during acquisition of CMV-Fny. Similar to a previous report (Collar and Fereres, 1998), fasting affected both aphid species in a similar manner, causing insects to initiate probing faster and make longer first pds, have longer II-3 sub-phase, as well as a higher number of archlets on sub-phase II-3. During that specific pd sub-phase, it has been suggested that aphids ingest cytosol contents from punctured cells by active uptake using the cibarium as a pump. Therefore, aphids showing this behaviour are likely to acquire non-circulative viruses more efficiently (Powell *et al.*, 1995; Martín *et al.*, 1997). The EPG recording indicated that fasted *M. persicae* spent only 9.6 s from the start of C waveforms until the first potential drop was observed. Therefore, during the given 30 s AAP, an aphid would be able to do more than one pd and acquire the virus in several cells (average time to produce one pd is 6.21 s). Such a change in behaviour could increase the chances of CMV acquisition or retention and therefore transmission during fixed duration AAPs. However, these EPG variables (longer first pd, longer II-3 subphase) of fasted aphids did not always correlate with increased

transmission rate. Fasted *A. gossypii* transmitted CMV with less efficiency than fasted *M. persicae*, despite a higher number of archlets in II-3 sub-phase when monitoring the aphid probing behaviour (Jiménez *et al.*, 2017). The overall longer duration of the C waveform meant that fasted and non-fasted *A. gossypii* likely produced only a single pd during the AAP of 30 s, potentially explaining why fasting did not significantly increase CMV transmission.

Wang *et al.* (1998) suggested possible differences between aphid vector species when comparing the transmission of TEV and TuMV by *M. persicae* and *Myzus ascalonicus* van der Goot (Hemiptera: Aphididae). In Wang's study the probing behaviour of fasted individuals of both species used in that study was similar (same occurrence of potential drops and duration of pd subphases). However, differences in transmission efficiency were observed. Other variables may explain the contrasting effect of fasting observed for different virus/vector combinations. For example, virus infection modifies the composition and/or the concentration of volatiles emitted by plants, some of which act as aphid attractants (Mauck *et al.*, 2016). At this stage, we cannot rule out the possibility that non-fasted and fasted aphids evaluate the attractiveness for infected and non-infected plants differently. Regarding the insect partner, differences in the chemical composition of the inner surface of aphid stylets (Uzest *et al.*, 2010) and/or other physiological aspects, such as flushing out of virions by egested saliva and the composition of the saliva itself, might differ between *M. persicae* and *A. gossypii*. Interestingly, differences in the composition of aphid saliva are supported by the observation of similar probing behaviours by *A. gossypii* and *M. persicae* on a melon accession carrying the Vat resistance gene (Chen *et al.*, 1997; Villada *et al.*, 2009); only *A. gossypii* triggered a hypersensitive response after superficial intracellular stylet punctures. Additional studies will be required to better understand how fasting impacts the transmission of non-circulative viruses, and to determine which factors in addition to aphid probing behaviour are involved in the transmission success. Such knowledge would undoubtedly bring novel information on plant–virus–insect interactions.

CHAPTER 5. COMPETITION AND INTERFERENCE BETWEEN NON-CIRCULATIVE VIRUSES FOR THE RETENTION SITES IN THEIR APHID VECTORS

5.1 INTRODUCTION

Most plant viruses require vectors in order to infect new host plants, contributing to spread. Therefore, vectors are key in the survival of plant viruses, with the most economically important vectors located in the class Insecta (Uzest and Blanc, 2016). Aphids and whiteflies transmit more than 500 plant virus species, representing the major groups of vectors of plant viruses (Fereres and Raccach, 2015). Aphids alone transmit about one third of all plant viruses studied (Hogenhout *et al.*, 2008; Whitfield *et al.*, 2015).

Two major virus categories have been established depending on the ability of viruses to persist within the vector: circulative viruses (CV), also referred to as persistent viruses, and non-circulative (NC) (Fereres and Raccach, 2015; Blanc and Michalakakis, 2016). Within CV category, viruses cross gut barriers and enter the circulatory system of the insect and ultimately reach the salivary glands, from where the virions are transmitted through salivation to inoculate the plant (Brault *et al.*, 2010). In contrast, NC virus particles are attached to the aphid cuticle lining for a short period of time without any circulation or replication within the vector body (Blanc *et al.*, 2014). The attachment of NC virus particles to the specific retention sites in the aphid cuticle is exclusively mediated by protein interactions. The mechanisms undergoing NC virus-vector interactions as well as retention sites have been widely studied (Dombrovsky *et al.*, 2005, 2007; Moreno *et al.*, 2005; Uzest *et al.*, 2007; Uzest and Blanc, 2016; Webster *et al.*, 2017, 2018; Deshoux *et al.*, 2018)

There are two molecular mechanisms involved in the retention of NC virus particles to specific attachment sites within their aphid vectors. Virus transmissibility and specificity depends on the coat protein (CP strategy) or other unique proteins that act as a bridge between the virion and aphid cuticle (helper strategy) (Raccach and Fereres,

2015; Uzest and Blanc, 2016). The sole involvement of CP is the responsible of retention of cucumoviruses, such as *Cucumber mosaic virus* (CMV, *Cucumovirus*) (Chen and Francki, 1990; Perry and Fereres, 2019). However, for some other plant virus species, an additional virus-encoded nonstructural protein (commonly named as ‘helper’) mediates virus-vector interaction (Uzest and Blanc, 2016). For instance, potyviruses such as *Turnip mosaic virus* (TuMV) and *Zucchini yellow mosaic virus* (ZYMV) need the presence of that helper component (HC-Pro) to bind to the cuticle of the aphid stylets (Pirone and Blanc, 1996). First binding of HC or at the same time as virus particles is essential for virion retention (Racchah *et al.*, 2001). HC-Pro is a 50-kDa protein composed of two helix-rich domains, located near the N- and C-terminal ends of the protein and linked by a hinge. The conserved motif Pro-Thr-Lys (PTK) present in the C-terminal of the HC assists the binding of virus particles (Huet *et al.*, 1994; Peng *et al.*, 1998) through the amino-acid triplet Asp-Ala-Gly (DAG) present N-terminal of the capsid protein (Atreya *et al.*, 1990; Gal-On *et al.*, 1992). On the other hand, the KITC domain located in the N-terminal is the responsible of the binding of the HC-Pro to aphid mouthparts (Blanc *et al.*, 1998; Wang *et al.*, 1996).

Cauliflower mosaic virus (CaMV, *Caulimovirus*) is also a NC virus whose strategy of transmission has been accurately studied and described (Chalfant and Chapman, 1962; Pirone and Blanc, 1996; Palacios *et al.*, 2002; Moreno *et al.*, 2005, 2012). CaMV also uses the ‘helper strategy’ with an 18-kDa non-structural viral protein (P2) binding viral particles to the aphid mouthparts (Drucker *et al.*, 2002) by interacting with the virion-associated protein P3 linked to the virion (Leh *et al.*, 2001). The P2 protein binds to a specific aphid mouthpart structure named acrostyle that is located in the stylet common duct where both food and salivary canals join together (Uzest *et al.*, 2007). The P2-P3 assembling occurs in the aphid cuticle rather than in the infected plants as both complexes are spatially separated in infected plants. Whereas P2 is preferentially acquired from epidermal and a mesophyll cell during brief intracellular punctures, the complex P3/CaMV is mainly acquired once phloem phase is reached (Palacios *et al.*, 2002; Drucker *et al.*, 2002). Moreover, a novel study suggested that CaMV can perceive the presence of the aphid vector in the host plant by triggering a redistribution of the specialized transmission bodies (TB) within cells surrounding the aphid salivary sheath to enhance its transmission (Martienière *et al.*, 2013). However, the specific

mechanisms involved in the triggering of this process, such as the breakage of the cell wall by the aphid stylets or injection of elicitors from the watery saliva as still unknown.

The assumption that other NC viruses besides CaMV are retained in the acrostyle is tempting (Pelletier *et al.*, 2012; Boquel *et al.*, 2013), however receptors on any other NC viruses rather than CaMV remain poorly studied. The cuticular protein *stylin-01* has been identified as putative binding receptor of the helper-protein P2 of CaMV (Webster *et al.*, 2018). However, the role of *stylin-01* in the binding of several other NP virions is uncertain. Also, little knowledge is available about remarkable factors playing a role before and during the binding process, such as cell plant components, gelling and watery saliva as well as other factors influencing virus-vector interactions (Drucker and Then, 2015).

5.2 OBJECTIVE

The focus of our work was to study if there is any interference/competition for retention sites within the aphid mouthparts between different types of NC viruses. Here, we hypothesized that NC viruses with different transmission strategies (capsid *versus* helper protein) may share the same retention sites. We tested our hypothesis by performing a series of sequential and EPG-assisted transmission tests using different combinations of aphid-transmitted NC viruses (interference experiments) or exposing aphids to either plants showing mixed-infections or artificial diets containing different combinations of virions/helper proteins (competition experiments). On the basis of reported information of CaMV retention sites and putative receptors we aimed to test if there was any interference or competition between first acquisition and retention of P2-CaMV protein in the acrostyle with subsequent acquisition and transmission of CMV (CP strategy) or TuMV (helper component: HC-Pro). Also, a series of sequential transmission tests between CMV and ZYMV were conducted to find out if there was any interference for retention sites between cucumoviruses and potyviruses. Results showed no interference or competition between CaMV and TuMV or CMV. However, CMV transmission efficiency decreased when ZYMV was acquired first but no interference in ZYMV transmission occurred when CMV was acquired in advance. The results obtained in relation with the two different binding strategies and putative receptors for the different NC virus species are discussed.

5.3 MATERIAL AND METHODS

5.3.1 APHID CLONES, VIRUS STRAINS AND HOST PLANTS

Colonies of virginiparous *Brevicoryne brassicae*, *Myzus persicae* and *Aphis gossypii* were used as aphid vectors in the adult stage. *B. brassicae* and *M. persicae* were maintained in *Brassica rapa* var. ‘Just Right’. *A. gossypii* was reared in *Cucumis melo* var. ‘Siglo’. All aphid colonies were reared in environmental growth chambers at ICA-CSIC following the procedures indicated in Chapter 3 (section 3.2.2).

Virus strains were propagated by mechanical inoculations following the procedures indicated in Chapter 3 (section 3.3.1). For the virus species-plant combinations tested, the same host plant was used to avoid a possible plant effect (turnip plants were used for both CaMV and TuMV; melon plants were used for both ZYMV and CMV). In case of viruses infecting different host plant species (CaMV and CMV), a combination of aphids feeding in artificial diet (CaMV) and on a virus-infected source plant (CMV) was applied. Virus-infected source plants were used three weeks after virus inoculation. Both TuMV (isolate UK-1 kindly provided by Dr. F. Ponz, CBGP-UPM-INIA, Madrid, Spain) and CaMV (isolate Cabb-S, kindly provided by Dr. S. Blanc, INRA CIRAD SupAgro, Montpellier, France) were maintained in *B. rapa* var. ‘Just right’. CMV (isolate FNY, kindly provided by Prof. Keith Perry, University of Cornell, USA) was maintained in *C. pepo* var. ‘Prècoce maraîchère’. CMV isolate M6 and ZYMV isolate C-71 (both kindly provided by Dr. Enrique Moriones, IHSM-LaMayora-CSIC, Málaga, Spain) was maintained in *C. melo* var ‘Margot’.

B. rapa var. ‘Just right’, *Cucurbita pepo* var. ‘Prècoce Maraîchère’ and *C. melo* var. ‘Primal’ were used as test (receptor) plants. Receptor plants were used at the 2-leaf stage (turnip) and at the 1-leaf stage (zucchini and melon). After transmission tests, adult aphids and nymphs were removed from the test plants with a paint brush and plants were then sprayed with an insecticidal treatment Confidor® 20 LS (Bayer CropScience) (100 ppm ai). All receptor plants were then transferred to the greenhouse at natural light conditions and a temperature of $23 \pm 4^{\circ}\text{C}$ until symptom development. Virus infection was checked by visual inspection and by ELISA, following the procedures indicated in Chapter 3 (section 3.4).

5.3.2 P2, P2Rev5, P3 AND CaMV PURIFICATION

All purified helper proteins and virions used in the transmission tests described in this chapter were kindly provided by Dr. M. Uzest and Dr. S. Blanc (INRA CIRAD SupAgro, Montpellier, France). The CaMV viral protein P2 and the non-transmissible mutant P2Rev5 were produced in the baculovirus insect cell system. Sf9 cells were infected with recombinant baculoviruses at 28°C as described in Hébrard *et al.* (2001) and Moreno *et al.* (2005). Insect cells were harvested 72 h post infection. After a freeze-thaw cycle at -20°C, pellets were resuspended in SES buffer (Blanc *et al.*, 1993) and kept at -20°C until use. P2 and P2rev5 relative concentrations were evaluated by western blot analyses using specific P2 antiserum (Hébrard *et al.*, 2001).

The CaMV viral protein P3 was produced from the bacterial clone pETCa3 (Jacquot *et al.*, 1998) according to Plisson (Plisson *et al.*, 2003). Induced *E. coli* cells were resuspended in P3 buffer [20 mM Tris-HCl (pH 8.0), 200 mM NaCl] supplemented with Complete Antiprotease Cocktail (Roche, Penzberg, Germany). Cells were ultrasonicated. P3 being heat-stable, the soluble fraction was subjected to heating at 65°C for 10 min. Denaturated proteins were discarded by centrifugation (20,000 g for 10 min). P3 was then purified by differential ammonium sulfate precipitation. Finally, the pellet containing P3 was resuspended in P3 buffer and dialyzed to remove ammonium sulfate. P3 was kept at -20°C until use.

CaMV particles (Cabb-BJI isolate) were purified from 3 weeks-infected turnips according to Hull procedure (1976). Infected leaves were ground in phosphate buffer (KH₂PO₄ 0.5M pH 7.2) supplemented with 1M urea and 2.5% Triton X-100. After clarification, virus particles were pelleted by ultracentrifugation (145,000 g for 2 h), and purified on a 10-40% sucrose gradient. The viral fraction was ultracentrifuged (75,000 g for 3 h) and virus particles were finally resuspended in Hepes 10 mM pH 7.0.

5.3.3 ELECTRICAL PENETRATION GRAPHS (EPG) SETUP

The EPG technique was used to monitor specific sequential transmission tests to assess if there is interference between caulimoviruses and potyviruses or caulimoviruses and cucumoviruses [for a detailed EPG set-up description, see Chapter 3 (section 3.5)]. Non-viruliferous *B. brassicae* and *M. persicae* were collected from the colony and immobilized at the edge of a pipette tip using a vacuum pump. A gold wire (20 µm

diameter, 2-2.5 cm long) was attached to the dorsum by applying a small droplet of water-based silver paint (EPG Systems, Wageningen, The Netherlands). The opposite end of the gold wire was also attached to a 3 cm long copper wire that was welded to a copper nail, serving as the input electrode.

5.3.4 ‘COMPETITION’ AND ‘INTERFERENCE’ BETWEEN VIRUS PARTICLES IN THE VECTOR DURING THE ACQUISITION PROCESS

Two different sets of transmission tests were performed within the different virus-vector combinations studied. First, we tested the competition for retention sites by exposing aphids either to plants showing mixed-virus infection or to artificial diets containing purified virions and their helper components. Also, virus acquisition interference experiments were conducted by exposing aphids sequentially to different infective sources either from i) two different virus-infected source plants or ii) first acquisition of purified virions/helper components from a membrane (artificial diet) and subsequent acquisition from a source plant.

5.3.5 TRANSMISSION TESTS TO STUDY IF COMPETITION OR INTERFERENCE EXISTS BETWEEN CaMV (CAULIMOVIRUSES) AND TuMV (POTYVIRUSES)

B. brassicae was specifically chosen as vector in these experiments. This aphid species is a competent vector of both CaMV and TuMV, and it was shown to retain CaMV longer than *M. persicae* (Moreno *et al.*, 2005). Therefore, if TuMV needs to bind to the acrostyle to ensure its transmission from plant to plant, then the chances to observe an impact of CaMV binding on TuMV transmission should be here optimized.

To test if competition exists between CaMV and TuMV particles for retention sites in the vector during the acquisition process, *B. brassicae* individuals were exposed to a turnip plant doubly infected with CaMV and TuMV. Non-viruliferous aphids were fasted for 1h and later subjected to an AAP of 5 min on the abaxial side of a full-expanded young leaf of the mixed infected plant. As control treatments, aphids were exposed for an AAP of 5min on either a TuMV-infected or a CaMV-infected turnip. After the AAP, aphids were individually transferred to a turnip receptor plant for an IAP

of 24h (Table 5.1; I, A). Then, the receptor plants were transferred to the greenhouse and 3 weeks later screened for virus infection.

To test if interference exists between CaMV and TuMV, sequential transmission tests were performed. Non-viruliferous aphids were fasted for 1h and then subjected to two different AAP on CaMV-infected source plants, either 5 min or 8 h (Table 5.1; I, B) since two optimal acquisition access periods were reported for CaMV when *B. brassicae* acts as vector (Chalfant and Chapman, 1962). Afterwards, aphids were individually placed on the abaxial side of a TuMV-infected plant for an AAP of 5 min followed by a 24h inoculation access period (IAP) on a non-infected turnip test plant. (Table 5.1; I, B). Control tests were conducted by placing single aphids on a non-infected turnip plant for either 5 min or 8 h before having access to a TuMV-infected source plant for a 5 min AAP followed by a 24h as an IAP on a non-infected turnip test plant. Also, single aphids were used to individually transmit either TuMV (5 min as an AAP) or CaMV (8 h as an AAP) directly from an infected source plant to a turnip receptor test plant with no intermediate passage.

Another set of interference tests were performed by monitoring the feeding behaviour of *B. brassicae* during virus acquisition from a virus-infected source plant. Adult aphids were attached to a gold wire, connected to a copper electrode and then released on a parafilm membrane with a sucrose solution (10% sucrose) containing the purified P2-CaMV helper protein for 2 hours prior to subsequent EPG-assisted transmission tests using TuMV-infected as a source plant (Table 5.1; I, B). A total 160 µl of solution [72.8 µl distilled water, 40 µL P2 protein, 32 µl sucrose 50%, 12 µl Tris 20mM (pH=8), 3.2 µl Sodium Citrate 500 mM (pH=7.0)] was placed at the flat top of a 3 cm long/1cm diameter plastic tube. A stretched parafilm membrane was then placed over the solution, covering all upper plastic tube surface. Copper electrodes with aphids attached were pinned on modeling clay and the aphids then placed smoothly on the parafilm membrane for artificial feeding. After an AAP of 2h on the membrane, aphids were connected to an EPG probe and allowed to produce a single intracellular puncture (pd) on a TuMV-infected turnip plant. As a control treatment, the same protocol was followed but aphids were exposed to the same artificial diet but without P2.

Table 5.1. Different treatments applied to study the interference between NC viruses in three vector species. I) Competition and interference tests with CaMV and TuMV using *Brevicoryne brassicae* as vector. II) Competition and interference tests with CaMV and CMV using *Myzus persicae* as vector. III) Interference tests between ZYMV and CMV using *Aphis gossypii* as vector.

I) Caulimoviruses (CaMV) and Potyviruses (TuMV) – <i>B. brassicae</i>	
A) Competition tests (CaMV&TuMV)	
Mixed CaMV/TuMV-infected turnip (5min AAP) → test turnip plant (24h IAP)	
TuMV-infected turnip (5min AAP) → test turnip plant (24h IAP)	
CaMV-infected turnip (5min AAP) → test turnip plant (24h IAP)	
B) Interference tests	
5min AAP on CaMV- infected turnip	
CaMV-infected turnip (5min AAP) → TuMV-infected turnip (5min AAP) → test turnip plant (24h IAP)	
Non-infected turnip (5min AAP) → TuMV-infected turnip (5min AAP) → test turnip plant (24h IAP)	
8h AAP on CaMV-infected turnip	
CaMV-infected turnip (8h AAP) → TuMV-infected turnip (5min AAP) → test turnip plant (24h IAP)	
Non-infected turnip (8h AAP) → TuMV-infected turnip (5min AAP) → test turnip plant (24h IAP)	
CaMV-infected turnip (8h AAP) → test turnip plant (24h IAP)	
TuMV-infected turnip (5min AAP) → test turnip plant (24h IAP)	
2h AAP on artificial diet + EPG-assisted transmission tests	
P2-CaMV on parafilm membrane (2h AAP) → TuMV-infected turnip (1pd AAP) → test turnip plant (24h IAP)	
Control diet (2h AAP) → TuMV-infected turnip (1pd AAP) → test turnip plant (24h IAP)	
II) Caulimoviruses (CaMV) and cucumoviruses (CMV) – <i>M. persicae</i>	
Interference tests (CaMV → CMV) – <i>M. persicae</i>	
8h AAP on CaMV-infected turnip	
CaMV-infected turnip (8h AAP) → CMV-infected zucchini (5min AAP) → test zucchini plant (24h IAP)	
Non-infected turnip (8h AAP) → CMV-infected zucchini (5min AAP) → test zucchini plant (24h IAP)	
CaMV-infected turnip (8h AAP) → test turnip plant (24h IAP)	
2h AAP on artificial diet + EPG-assisted transmission tests	
P2/P3-CaMV on parafilm membrane (2h AAP) → CMV-infected zucchini (1pd AAP) → test zucchini plant (24h IAP)	
Control diet (2h) → CMV-infected zucchini (1pd AAP) → test zucchini plant (24h IAP)	
III) Interference between cucumoviruses (CMV) and potyviruses (TuMV) – <i>A. gossypii</i>	
A) Interference tests (ZYMV → CMV)	
ZYMV-infected melon (5min AAP) → CMV-infected melon (5min AAP) → test melon plant (24h IAP)	
Non-infected melon (5min AAP) → ZYMV-infected melon (5min AAP) → test melon plant (24h IAP)	
ZYMV-infected melon (5min AAP) → test melon plant (24h IAP)	
B) Interference tests (CMV → ZYMV)	
CMV-infected melon (5min AAP) → ZYMV-infected melon (5min AAP) → test melon plant (24h IAP)	
Non-infected melon (5min AAP) → CMV-infected melon (5min AAP) → test melon plant (24h IAP)	
CMV-infected melon (5min AAP) → test melon plant (24h IAP)	

A pre-acquisition fasting period of 1h was applied in all treatments; 1 aphid/test plant (*2 aphids/test plant); AAP=acquisition access period; IAP=inoculation access period

5.3.6 TRANSMISSION TESTS TO STUDY IF INTERFERENCE EXISTS BETWEEN CaMV (CAULIMOVIRUSES) AND CMV (CUCUMOVIRUSES)

Interference between *Caulimoviruses* and *Cucumoviruses* was studied by exposing first *M. persicae* individuals to a CaMV-infected source plant and then to a CMV-infected source plant to test subsequent acquisition/retention and transmission of CMV. Non-viruliferous apterous adults were confined in plastic cages for a 1h-preacquisition fasting period. Aphids were then subjected to an 8h AAP on a CaMV-infected turnip. Afterwards, aphids were individually transferred to the abaxial side of a CMV-infected zucchini leaf for an AAP of 5 min, followed by a 24h IAP on a non-infected zucchini receptor test plant (Table 5.1, II). Control tests were conducted by placing single aphids on a non-infected turnip plant for 8h before having access to CMV-infected plants. Single aphids were used to transmit CaMV directly from an infected source (AAP of 8h) to a turnip test plant with no intermediate passage.

Also, interference tests were performed by monitoring the feeding behaviour of *M. persicae* during the AAP in the second test plant. Adult aphids were attached to a gold wire and connected to a copper electrode and then placed on an artificial membrane with a sucrose solution (10%) containing purified P2/P3/CaMV complex for 2 hours prior to subsequent EPG-assisted transmission tests using CMV-infected zucchini as a source plant. Wired aphids were released on the artificial membrane containing the P2/P3-CaMV complex plus the feeding solution (Table 5.1; II). A total of 160 µl of solution [72.8 µl distilled water, 40 µl P2 protein, 6 µL P3 protein, 6 µl CaMV (2.25 mg/ml), 32 µl sucrose 50%, 3.2 µl Sodium Citrate 500 mM (pH=7.0)] was placed at the top of a 3cm long/1cm diameter plastic tube. Electrodes with attached aphids were pinned on modeling clay and the aphids placed smoothly on the parafilm membrane for artificial feeding. After an AAP of 2h on the membrane, aphids were connected to an EPG probe and allowed to produce a single intracellular puncture (pd) on a CMV-infected zucchini plant. As control treatment, the same protocol was followed but aphids were exposed to a control diet without the P2-CaMV helper protein (Table 5.1, II).

5.3.7 TRANSMISSION TESTS TO STUDY IF INTERFERENCE EXISTS BETWEEN ZYMV (POTYVIRUSES) AND CMV (CUCUMOVIRUSES)

A. gossypii was used as a vector to conduct the interference experiments as it has been reported to be an efficient vector of both CMV and ZYMV (Labonne *et al.*, 1982; Castle *et al.*, 1992; Garzo *et al.*, 2004).

Sequential transmission tests were performed to test if there is any interference between ZYMV and CMV for retention sites, using *A. gossypii* as a vector. Aphids were fasted for 1h and then subjected to an AAP of 5 min on either a ZYMV or CMV-infected melon source plant and later transferred to either an infected CMV or ZYMV-infected melon, respectively, for another AAP of 5min (Table 5.1; III: A, B). Finally, aphids were individually transferred to a non-infected melon test plant for an IAP of 24h. Controls were conducted by placing single aphids on a non-infected melon plant for 5 min before exposure to a 5 min AAP on either a CMV or a ZYMV-infected melon. Then, aphids were individually transferred to a non-infected melon test plant for an IAP of 24 h. As additional controls single aphids were used to transmit either CMV or ZYMV directly from an infected source to a melon test plant immediately after fasting.

5.3.8 STATISTICAL ANALYSIS

All virus transmission efficiencies were compared by Chi-square goodness of fit test ($p < 0.05$) and by Fisher's exact test when expected values were lower than 5 ($p < 0.05$). All pairwise comparisons were analyzed using Statview 4.0 package for Macintosh (Abacus Concepts, 1992).

5.4 RESULTS

5.4.1 COMPETITION AND INTERFERENCE BETWEEN CaMV (CAULIMOVIRUSES) AND TuMV (POTYVIRUSES) FOR RETENTION SITES

Competition experiments between TuMV and CaMV resulted in a similar transmission rate between all treatments. Virus transmission efficiencies from experiments using a coinfecting CaMV/TuMV-turnip as source virus plant were 11.1% and 14.8% for TuMV and CaMV, respectively. No differences were observed between the co-infection tests the single transmission tests with aphids acquiring either CaMV or TuMV for 5 min

(control treatments) [14.1% for TuMV ($\chi^2=0.47$, $p=0.486$); 12.5% for CaMV ($\chi^2=0.28$; $p=0.595$)] (Fig. 5.1 A).

Interference studies showed that TuMV transmission efficiencies were similar when *B. brassicae* individuals fed either on a CaMV-infected (9.3%) or on a non-infected turnip (9.2%) ($\chi^2<0.001$; $p=0.97$) for 5 min prior to TuMV acquisition (Fig. 5.1 B1). When aphids were subjected to an AAP of 8h on CaMV prior to TuMV acquisition, TuMV transmission efficiency was only 5.7%, similar to that obtained when aphids fed directly on a non-infected turnip for 8h prior to TuMV acquisition (7.1%) ($\chi^2=0.14$; $p=0.704$). The transmission rate of CaMV of the sequential transmission test was 28.16%, with no significant differences ($p<0.05$) to the rate obtained when aphids acquired CaMV for 8h and then transferred directly to a receptor plant with no intermediate passage (37.5%) ($\chi^2=2.37$; $p=0.124$). TuMV transmission efficiency was 15.5% when aphids acquired the virus straight after fasting (Fig. 5.1 B2).

B. brassicae individuals feeding on an artificial membrane containing P2-CaMV helper protein and then making a single intracellular puncture on a TuMV-infected turnip plant were able to transmit TuMV at a rate of 6.4 % (3/47). No significant differences were observed with the control diet treatment (1/11; 9.1 %) ($\chi^2=0.1$; $p=0.750$) (Fig. 5.1 C).

No experiments to test the interference of first acquisition of TuMV prior to subsequent acquisition and transmission of CaMV were performed. CaMV can doubly perform as a non-persistent and a truly semipersistent virus when *B. brassicae* acts as a vector (Palacios *et al.*, 2002; Moreno *et al.*, 2005). CaMV is acquired and retained for long periods (8h) by *B. brassicae* after several series of post-acquisition feeding or fasting periods (Chalfant and Chapman, 1962) and also after a series of consecutive intracellular stylet punctures (Moreno *et al.*, 2005). Consequently, first CaMV acquisition would be more likely to interfere with subsequent retention of TuMV than in the other way around.

Figure 5.1. *Cauliflower mosaic virus* (CaMV) and *Turnip mosaic virus* (TuMV) transmission efficiencies obtained in the different treatments to test the competition and interference between *Caulimovirus* and *Potyvirus* using *Brevicoryne brassicae* as vector. A) Competition experiments with aphids fed for 5min on a mixed CaMV/TuMV-infected turnip. B) Interference tests applying either 5min (B.1) or 8h (B.2) as an AAP for CaMV prior to AAP of 5 minutes on a TuMV-infected turnip. C) Interference tests combining feeding for 2h on artificial diet for P2-acquisition and subsequent EPG-assisted AAP on TuMV-infected turnip.

Different letters show significant differences ($p < 0.05$) according to a Chi-square test or a Fisher exact test when expected values were lower than 5. Upper case and lower case are used to compare same virus species within the same competition or interference set studied.

5.4.2 INTERFERENCE BETWEEN CaMV (CAULIMOVIRUSES) AND CMV (CUCUMOVIRUSES) FOR APHID TRANSMISSION

The transmission rate of CMV by *M. persicae* was 8.6% (12/140) when aphids first fed on a CaMV-infected turnip prior to CMV acquisition. No significant differences were observed in the transmission rate for the control, when aphids fed on a non-infected turnip prior to CMV acquisition (8/140; 5.7%) ($\chi^2=0.81$; $p=0.353$) (Fig. 5.2 A). Therefore, our results suggest that there is no interference between CaMV and CMV for retention sites (Fig. 5.2 A).

Figure 5.2. *Cauliflower mosaic virus* (CaMV) and *Cucumber mosaic virus* (CMV) transmission efficiencies obtained in the different treatments to test the competition and interference between *Caulimovirus* and *Cucumovirus* using *Myzus persicae* as vector. A) Interference of first acquisition for 8h on CaMV-infected turnip with subsequent acquisition on a CMV-infected zucchini. B) Interference tests with individuals acquiring P2-P3-CaMV from artificial diet and subsequent AAP of 5min on a CMV-infected zucchini.

Different letters show significant differences ($p < 0.05$) according to a Chi-square test or a Fisher exact test when expected values were lower than 5. (CMV transmission rates are compared within A and B).

Aphids acquiring the P2/P3/CaMV complex from the artificial feeding membrane prior to making a single intracellular puncture in a CMV-infected source plant were able to transmit CMV at a rate of 25.6% (10/39). No significant differences were observed in the transmission rate of CMV (16.9%; 10/59) under the control treatment ($\chi^2=1.10$; $p=0.296$) when aphids were exposed to the non-transmissible P2rev5 instead of the P2-CaMV helper protein (Fig. 5.2 B). Therefore, there was no interference between P2/P3/CaMV on CMV transmission as there was no reduction in the transmission rate of CMV when aphids were first exposed to the P2/P3/CaMV complex.

5.4.3 INTERFERENCE BETWEEN CMV (CUCUMOVIRUSES) AND ZYMV (POTYVIRUSES) FOR APHID TRANSMISSION

Transmission rate of ZYMV was 10.5 % when *A. gossypii* fed on a CMV-infected melon plant prior to ZYMV acquisition. A similar transmission rate of ZYMV was obtained when aphids fed on a non-infected melon plant prior to ZYMV acquisition (11.4 %) ($\chi^2=0.06$; $p=0.805$) (Fig. 5.3 A). No differences were observed when aphids were exposed to a ZYMV-infected melon directly after fasting (19.7 %) ($\chi^2=3.43$; $p=0.064$). Therefore, our results suggest no interference for retention sites between CMV and ZYMV when aphids acquired CMV first.

Figure 5.3. *Zucchini yellow mosaic virus* (ZYMV) and *Cucumber mosaic virus* (CMV) transmission efficiencies obtained for the different treatments to test the interference between *Potyvirus* and *Cucumovirus* using *Aphis gossypii* as vector. An AAP of 5 min was applied to acquire either ZYMV or CMV. A) Interference of first acquisition and retention of CMV in the later acquisition of ZYMV. B) Interference of first acquisition and retention of ZYMV on the subsequent acquisition of CMV.

Different letters show significant differences ($p<0.05$) according to a Chi-square test or a Fisher exact test when expected values were lower than 5. In A), ZYMV transmission efficiencies are compared. In B), CMV transmission efficiencies are compared.

However, when aphids were exposed first to a ZYMV-infected plant before transmission of CMV there was a significant reduction in the transmission rate of the latter (Fig. 5.3 B). In control treatments, CMV transmission rate was 45.3 % when *A. gossypii* acquired CMV directly after fasting. A similar transmission rate of CMV (40.8 %) was obtained when aphids fed on a non-infected melon plant prior to CMV acquisition ($\chi^2=0.33$; $p=0.564$). However, CMV transmission efficiency was significantly reduced to 21.6 %

when *A. gossypii* individuals were previously exposed for an AAP of 5min on a ZYMV-infected melon plant prior to CMV acquisition ($\chi^2=8.81$; $p=0.003$) (Fig. 5.3 B). Therefore, our results suggest that ZYMV/or its HC can interfere with the subsequent retention of CMV in the aphid stylets.

5.5 DISCUSSION

Whereas the binding domains of NC viruses particles in the aphid mouthparts involved in transmission are really well known, the specific receptors in the aphid cuticle as well as cuticle proteins involved in their binding have been poorly studied. So far retention sites within the aphid mouthparts have been accurately described only for the semipersistently transmitted CaMV. The specific comma-like area along the common duct of the maxillary stylets (the acrostyle) is where the CaMV helper component P2 binds, serving as the bridge to the P3-CaMV complex which subsequently binds to P2 (Uzest *et al.*, 2007; Uzest *et al.*, 2010). That unique structure was specifically identified in 9 different aphid species, including two of the aphid species used in our work (*B. brassicae* and *M. persicae*) and presumably present in most, if not all, aphid species (Uzest *et al.*, 2010).

Furthermore, in very recent work, the protein *stylin-01* from the RR-1 subfamily (Rebers and Riddiford, 1988) was postulated as a candidate for receptor for the transmission of NC viruses (Webster *et al.*, 2017). The *in vivo* silencing of *stylin-01* gene through RNA interference was shown to have a negative effect in the transmission efficiency of CaMV by *M. persicae*. Moreover, the previously named Mpcp4 cuticular protein (Dombrovsky *et al.*, 2007) appeared to interact with the coat protein of CMV using a yeast-two-hybrid (YTH) system (Liang and Gao, 2017). The Mpcp4 protein was in fact the same as the *stylin-01* described later by Webster *et al.* (2018). However, whether or not this interaction in yeast reflects a true binding of CMV to Mpcp4 in the aphid stylets needs further investigation since attempts to successfully attach CMV particles to Mpcp4 have failed so far (Webster *et al.*, 2018).

Our experimental procedure showed no interference in sequential transmission tests when *M. persicae* acquired CaMV or P2 from plants or artificial diet respectively prior to CMV acquisition. This result could initially suggest that CaMV and CMV do not share the same

receptors. However, the lack of interference between CaMV and subsequent retention of CMV could also be explained by the dislodging of CaMV particles during subsequent acquisition of CMV. While aphids carrying CaMV probe on a CMV-infected source they salivate from the onset of stylet penetration on the plant (Moreno *et al.*, 2011) and therefore can dislodge CaMV particles during the process of CMV acquisition. In fact, persistence of CaMV in *M. persicae* has been reported to be much shorter than in other aphid vector species (Moreno *et al.*, 2005). Therefore, we cannot conclude that CaMV and CMV do not share the same retention sites. Competition experiments, however, support the hypothesis that *stylin-01* may act as a receptor for both the P2-CaMV complex and CMV (Rodrigo Almeida, unpublished data). Results of competition experiments between CaMV and CMV showed a decrease in CMV transmission when aphids fed on an artificial diet containing CMV virions together with the P2-P3-CaMV complex, suggesting that both viruses could compete for the same retention sites and therefore may share the same receptors. These results of competition experiments do not contradict our results on the lack of interference between CaMV and CMV. When CMV is acquired together with CaMV by aphids from the same source there could be a higher affinity of CaMV for the same retention sites that could make CMV retention more difficult. Such results would suggest that CaMV and CMV share the same retention sites but CaMV outcompetes CMV because of its higher affinity for the receptors present in the acrostyle. However, we cannot discard a blockage of CMV accessibility to receptors in the aphid cuticle by the masking of the CMV capsid proteins by the P3-P2-CaMV complex. If such is the case, the reduction in the transmission ability of CMV when aphids acquire simultaneously both viruses would suggest some kind of competition between both viruses but receptors of CMV in the aphid stylets could still be different to those of CaMV.

In case of ZYMV, putative receptors were suggested within the RR-2 subfamily in *M. persicae* stylets (Dombrovsky *et al.*, 2007). Therefore, ZYMV receptors would be different to those of the RR-1 suggested for CMV in *M. persicae* (Liang and Gao, 2017; Webster *et al.*, 2018). However, virus transmission efficiencies presented in Fig. 5.3 B show evidences that the acquisition of ZYMV by *A. gossypii* interfered with subsequent acquisition and transmission of CMV, suggesting that both viruses are using the same receptors in *A. gossypii* stylets. Whereas peptide arrays within the RR-1 domain were suggested to interact with CMV in *M. persicae* (Liang and Gao, 2017; Webster *et al.*,

2018), peptide arrays of the RR-2 protein subfamily were showed to interact with CMV in case of *Acyrtosiphon pisum* (Webster *et al.*, 2017). Whether peptides of RR-1 or RR-2 subfamily proteins or even both subfamilies are involved in the retention of both viruses when using *A. gossypii* as vector needs additional biochemical and functional validation.

However, our studies show that ZYMV transmission was not influenced when the reverse sequential transmission test was conducted (acquisition of CMV first followed by subsequent ZYMV acquisition and transmission), challenging our initial hypothesis of same receptors for ZYMV and CMV in *A. gossypii* mouthparts. However, differences in the mechanisms of retention/release between both virus species could explain the results obtained. ZYMV binding is thought to be more stable than that of CMV, since the binding occurs through an intermediate bridge molecule, the helper component protein (HC-Pro) (Pirone and Blanc, 1996). The interaction between the *Potyvirus* particles and the HC is more specific than previously thought. Whereas the HC of ZYMV assisted transmission of both ZYMV and TuMV virions, the HC of TuMV did not assist transmission of ZYMV (Dombrovsky *et al.*, 2005). However, CMV binding to the aphid mouthparts is mediated by the CP strategy, suggesting that CMV particles could be released when aphids were allowed to probe, salivate and ingest on a ZYMV-infected source. This result suggests that ZYMV-HC Pro or HC Pro alone remains attached to the stylet receptors when aphids make a second acquisition probe in a CMV-infected source, while CMV is unable to remain in the stylets when aphids make a second acquisition probe in a ZYMV-infected source. Acquisition and retention of the complex ZYMV/HC-Pro first interfered with subsequent CMV retention, probably by reducing the number of receptors available for retention of CMV virions. Here we postulate that HC-Pro remains attached to the stylets even after release of the virions of ZYMV to the plant and that the binding of a potyvirus (or its HC-Pro) to its receptor sites is more stable than for CMV.

The hypothesis that potyviruses and cucumoviruses are selectively transmitted was suggested when *M. persicae* and *A. gossypii* were allowed to feed on a doubly TuMV/CMV-infected *Brassica campestris* and then transferred to a series of test plants (Fujisawa, 1985). Wingless *M. persicae* subjected to a 10 min AAP and later sequentially transferred to five individual receptor plants transmitted mostly TuMV, with low number of double infections and no single CMV transmissions at all. Similarly, both TuMV and CMV were transmitted together to the first and second turnip test plant when using

wingless *A. gossypii* as a vector, with only a single case of TuMV infection and CMV transmission occurring in the third plant. One could conclude that TuMV particles are more likely to be retained than CMV in the aphid stylets with the help of its HC-Pro suggesting also a competition between viruses during the acquisition and retention process.

Conversely, our results indicated that caulimoviruses and potyviruses did not compete for the same binding sites, nor interfered during the transmission process. Therefore, the *stylin-01* positively associated with CaMV retention is not likely mediating TuMV binding to aphid stylets. Our results globally suggest that both cucumoviruses and potyviruses are sharing the same or very similar receptors in the aphid's stylets but different to those used by CaMV.

The aphid cuticle lining is a very tangled surface, with a massive network of heavily cross-linked proteins and chitin fibers with different molecular processes playing a role in virus particles retention (Webster *et al.*, 2017). This fact makes non-persistent and semipersistent virus particles quite difficult to study in order to decipher their retention sites in the vector. Moreover, the acrostyle is a surface continuously in contact with aphid saliva, phloem sap and plant cell contents with many transient factors likely mediating virus retention. In fact, both RR-1 and RR-2 proteins have been detected at the tip of maxillary stylets, with domains in direct contact with contaminated phloem sap. Molecular studies have so far clarified the interaction of one protein of the RR-1 domain proteins directly involved in CaMV binding and also presumably to CMV (Webster *et al.*, 2018; Deshoux *et al.*, 2018). Further studies are needed to elucidate the specific cuticular proteins involved in the binding to the different families of aphid-transmitted non-circulative viruses.

CHAPTER 6. STUDY OF THE FEEDING BEHAVIOUR OF APHIDS ASSOCIATED WITH THE TRANSMISSION OF THE SEMIPERSISTENT AND PHLOEM-LIMITED *Beet yellows virus* (BYV, *Closterovirus*)²

6.1 INTRODUCTION

There are numerous studies on aphids as virus vectors and the transmission mechanisms of plant viruses transmitted in a nonpersistent and persistent manner. However, there is still a lack of information regarding semipersistently aphid-transmitted plant viruses such as closteroviruses (eg. *Beet yellows virus*; BYV, *Closterovirus*) (Sylvester, 1956; Nault, 1997). Plant species inside *Amaranthaceae* family represent over half of the host plants of BYV. Within this family, sugar beet (*Beta vulgaris* L.) is the most important commercial crop affected by BYV (Godfrey and Mauk, 1993). BYV has an important effect on the physiology of infected plants by inducing leaf chlorosis, decreasing photosynthesis efficiency and thus reducing root yield (Clover *et al.*, 1999). Characteristics of BYV have been studied for several decades including molecular properties and its transmission by its main aphid vectors. BYV is a phloem-limited virus as most *Closterovirus* species (Esau, 1968) and composed of a flexuous filamentous virus particle containing single-stranded RNA (Agranovsky *et al.*, 1994). In few cases it can be translocated out of phloem tissues and reach mesophyll cells in some host plants such as *Nicotiana benthamiana* (Medina *et al.*, 1999) or even *B. vulgaris* and *Tetragonia tetragonoides* (Esau, 1960). However, BYV infection of non-phloem tissues seems only incidental in *B. vulgaris* as it occurs sporadically only in mesophyll cells adjacent to phloem tissues and during late stages of the virus infection (when yellowing symptoms occur) (Esau, 1960).

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BYV has a wide range of aphid vectors as it is transmitted by more than 20 aphid species. The main aphid vectors are *Aphis fabae* and *Myzus persicae*, with *M. persicae* being the most efficient vector (Watson, 1951; Heathcote and Cockbain, 1964; Kirk *et al.*, 1991; Limburg *et al.*, 1997). BYV was first included in the persistent viruses category (Watson and Roberts, 1940) due to virus acquisition and inoculation increasing with longer feeding periods on infected and healthy plants, respectively. However, years later BYV was described as a semipersistent aphid-transmitted virus due to its discrepancy with either nonpersistent or persistent viruses. Indeed, BYV showed a retention period ranging from hours to day, in contrast with few minutes/hours retention of non-persistent viruses, and to life-span retention of persistent viruses (Sylvester, 1956; Ng and Zhou, 2015). There have been many attempts in order to determine both the optimum acquisition access period (AAP) and the inoculation access period (IAP) of BYV. Most previous works have been performed with both *A. fabae* and *M. persicae*. BYV acquisition has been shown to be a much longer process than inoculation in the different studies conducted with *M. persicae* (Sylvester, 1956; Bennett, 1960). Whereas Sylvester's work showed 12 h as the optimum time to reach maximum BYV acquisition (25-30%), Bennett's work indicated 6 hours as providing the highest acquisition rate (58%). However, the maximum inoculation efficiency was detected after 6-10 h (11%) and after 1h (57%) of feeding in Sylvester and Bennett works, respectively. The higher BYV transmission efficiencies obtained by Bennett are explained by the larger number of insects used per test plant (3 aphids/plant). However, the specific *M. persicae* stylets activities associated with successful inoculation or acquisition of BYV that could explain these differences between the IAP and the AAP remains unclear. Furthermore, the main BYV characteristics such as retention sites in the vector, transmission strategy and the feeding behaviour of their vectors associated with transmission has not been investigated before (Ng and Zhou, 2015).

The EPG technique has been used to identify specific stylet penetration (probing) activities in aphids related to virus transmission (Martín *et al.*, 1997; Moreno *et al.*, 2012) as well as to other insect transmitted plant pathogens such as, bacteria by psyllids (Sandanayaka *et al.*, 2014; Antolinez *et al.*, 2017). Monitoring aphid feeding behaviour by EPGs allows us to determine in real time the stylet tip position in the different plant tissues and the specific stylet activities by feeding aphids (Tjallingii, 1985, 1988; Collar *et al.*, 1997). EPG studies showed that nonpersistent potyviruses and cucumoviruses are

inoculated by their aphid vectors during superficial brief intracellular punctures (potential drop waveform; ‘pd’) in the early steps of host plant recognition (Martín *et al.*, 1997; Collar *et al.*, 1997; Powell, 2005). However, persistently transmitted plant viruses such as luteoviruses are dependent on aphid stylet activities in a phloem sieve element (SE) for their transmission. Early work by Prado and Tjallingii (1994) showed that the acquisition of *Barley yellow dwarf virus* (BYDV, *Luteovirus*) occurs during the phloem sap ingestion phase (E2) whereas inoculation occurs during aphid salivation into sieve elements (E1). Similarly, the feeding behaviour of several other vector species related to semipersistent phloem restricted viruses has been also studied with some virus species such as criniviruses and waikaviruses. *Lettuce chlorosis virus* (LCV, *Closterovirus*) inoculation by the whitefly *Bemisia tabaci* (formerly *Bemisia argentifolii*) was showed to occur primarily during the phloem salivation (E1) (Johnson *et al.*, 2002) the same as for *Maize chlorotic dwarf virus* (MCDV, *Waikavirus*) transmitted by the black-faced leafhopper *Graminella nigrifrons* (Wayadande and Nault, 1993).

In preliminary observations of EPG signals from *M. persicae* on sugar beet plants in our laboratory a new type of pd (named as phloem-pd; based on the findings derived from this work) was observed that deviated from the regular or standard pd: the intracellular stylet puncture occurring during stylet path from the very beginning of a probe (Tjallingii, 1978). A typical aphid probe encompasses intercellular stylet insertion (C waveforms) with intermittent intracellular punctures (potential drops: ‘pd’), reflecting insertion of the aphid stylets in the cell walls, prior to reaching phloem tissues (E1 waveform). We observed that the phloem salivation phase (E1) was always preceded by a single or several of the new type of pds. The standard-pd can be divided in three different phases (I, II & III) representing some activities during the stylets penetration in the cell membrane, during the intracellular stylet tip presence, and stylet withdrawal from the cell, respectively (Tjallingii, 1985). Within phase II, three different sub-phases have been distinguished. Sub-phase II-1 represents saliva injection into the cell and is responsible for the inoculation of nonpersistent viruses (Martín *et al.*, 1997; Powell, 2005). Sub-phase II-3 represents ingestion of cell contents and the acquisition of nonpersistent viruses (Powell *et al.*, 1995; Martín *et al.*, 1997; Collar *et al.*, 1998). Sub-phase II-2 aphid activities are not well understood. However, II-2 has been related to the inoculation of *Cauliflower mosaic virus* (CaMV, *Caulimovirus*), that is transmitted in a semipersistent manner by *M. persicae* and *Brevicoryne brassicae* (Moreno *et al.*, 2012). These authors

suggested that the inoculation of CaMV during subphase II-2 occurs when either opening of the pharyngeal valve and fluid egestion dislodges CaMV particles retained in the food canal of the stylets, or when injection of a different type of saliva releases the virions into the punctured cell. The virion releasing mechanism of semipersistently aphid-transmitted viruses from their retention sites in aphid mouthparts remains unclear.

6.2 OBJECTIVE

Our aim was to understand the specific feeding behaviour activities associated with inoculation and acquisition of the phloem-limited BYV by *M. persicae*. We artificially terminated the probing behaviour of viruliferous *M. persicae* individuals after the observation of specific EPG patterns while the aphids fed on healthy plants and then determined whether or not the plants became infected. Most importantly, we described a new type of brief intracellular puncture (named phloem-pd) that occurs in phloem tissues and is involved in the inoculation of BYV.

6.3 MATERIAL AND METHODS

6.3.1 APHIDS AND VIRUS MAINTENANCE

For running the transmission tests on the efficacy of BYV transmission by both *Aphis fabae* and *Myzus persicae*, aphid colonies were maintained on sugar beet (*Beta vulgaris* L. cv “Detroit Dark Red”) which is a suitable host for both vector species. For experiments to compare BYV transmission by apterae and alatae *M. persicae* and EPG experiments, aphids were reared in *B. vulgaris* var. “Julietta”. The methodology of generation of alatae *M. persicae* and apterous *M. persicae* and *A. fabae* individuals is indicated in Chapter 3 (section 3.2.2).

BYV isolate PV-0981 (Leibniz-Institut, Plant Virus Collection, Germany) was isolated in *Chenopodium foliosum* and later transmitted by serial passes to *B. vulgaris*, *Claytonia perfoliata* and *Chenopodium album* by using *M. persicae* and *A. fabae* individuals, following procedures indicated in Chapter 3 (section 3.3.2).

6.3.2 TRANSMISSION TESTS

A series of preliminary transmission test of BYV were conducted to test two different aphid vector species (*M. persicae* and *A. fabae*) and three host plant species (*B. vulgaris*, *C. perfoliata* and *C. album*) to determine the most efficient vector and the most susceptible host plant species to BYV. For these experiments, *B. vulgaris*, *C. perfoliata* and *C. album* plants were used as test and source plants. A second transmission test was performed to compare the transmission efficiency of BYV by alatae and apterae adults of *M. persicae*, using *B. vulgaris* as test and source plants. The acquisition access period (AAP) and inoculation access period (IAP) was 24h and groups of 5 aphids per test plant were used. Infection was assessed by DAS-ELISA (Stevens *et al.*, 1997) 3-4 weeks after experiments following the procedure indicated in Chapter 3 (section 3.4). Experiments consisted of three replicates of 28 plants for each aphid species and plant species combination.

6.3.3 EPG-ASSISTED TRANSMISSION TESTS

Aphid probing was monitored by a Giga-8, DC-EPG device (EPG Systems, The Netherlands). Newly emerged apterous adults of *M. persicae* were attached to the gold wire of an insect electrode using water-based silver glue (EPG Systems, The Netherlands), as detailed in the EPG methodology in Chapter 3 (section 3.5). Amplifier-connected aphids were allowed to start probing either on a BYV-infected or a non-infected sugar beet test plant for acquisition and inoculation experiments, respectively.

6.3.4 WAVEFORMS FEATURES OF INTRACELULAR PUNCTURES: STANDARD AND PHLOEM-PDS

Phloem-pds differed from standard-pds by having fewer intervals and a lower frequency (repetition rate) of the downward peaks in subphase II-2, as well having a smaller magnitude of the potential drop. Intervals were defined as the time between two peaks in subphase II-2 (Fig. 6.1 A, B). To study the main differences between both types of pd, the last pd observed before E1 (phloem-pd) was compared to the last standard-pd detected within the same probe. We compared the output voltage of both types of pd and the potential drop magnitude expressed as the percentage of the potential drop of the preceding standard-pd as well as the total duration of the pd and the duration of the three sub-phases (II-1, II-2 and II-3) (Fig. 6.1 B). We also measured the drop in potential

observed at the start of the E1 period subsequent to the last phloem-pd. A total of 57 EPG recordings were used to compare the distinctive features of the two types of potential drops (standard and phloem-pds).

6.3.5 ACTIVITIES ASSOCIATED WITH THE ACQUISITION AND INOCULATION OF BYV

The EPG signals were visually observed on a PC monitor and interrupted using a paintbrush to remove the aphid from the source or test plant after observing one of the following six EPG waveforms or patterns of waveforms: 1) Short stylet pathway phases before any intracellular puncture, representing activities in the intercellular apoplastic space (waveform C), 2) Stylet pathway phase followed by a single, five, or more intracellular standard potential drops (standard-pds), 3) Stylet pathway phase followed by one or more phloem potential drops (phloem-pds), 4) Stylet pathway phase followed by a single E1 phase (20-25s), representing salivation into sieve elements, 5) Stylet pathway followed by E1 and an E1/E2 transition phase (10-15s), in which waveforms E1 and E2 overlap, and 6) Stylet pathway phase followed by E1, E1/E2 transition. And one or more E2 episodes, correlated with passive phloem sap ingestion from sieve elements (Fig. 6.1 A, B, C).

For the BYV acquisition experiments (Table 6.1), individually wired non-viruliferous aphids were allowed to produce the different waveform patterns previously described on a 4 week-old BYV-infected sugar beet. After interruption, each aphid was immediately transferred to a healthy test plant for a 24 h IAP to check for BYV transmission. For the inoculation experiments (Table 6.2), single viruliferous aphids previously fed for 24 hours on BYV-infected sugar beets were allowed to probe into a two-leaf stage healthy sugar beet test plant (Plant A) until the six previously described waveform patterns were observed. Then, aphids were lifted and removed from plant A and transferred to a second healthy sugar beet test plant (Plant B) in order to assess its initial virus acquisition. Aphids that were unable to infect either of the two test plants (A or B) were considered as non-viruliferous and discarded from the analysis. After 24 h of IAP on Plant B, aphids were removed and plants were sprayed with Confidor® 20 LS (Bayer CropScience) (100 ppm ai) and maintained in greenhouse for 4 weeks after which virus infection was determined by DAS-ELISA (Stevens *et al.*, 1997). Each replicate was conducted using a different plant and aphid per EPG recording. The minimum number of replicates per treatment was

17 and 14 for the acquisition and inoculation EPG-assisted transmission tests, respectively.

Figure 6.1. A) Overview of electrical penetration graphs produced by *Myzus persicae* on sugar beet. Different Roman numbers indicate where aphid feeding was manually ended: 1) C waveforms: extracellular stylets pathway without any intracellular puncture; 2) C waveforms including standard-pds: intracellular punctures produced in non-vascular tissues; 3) C waveforms including standard pds and at least a single phloem-pd; 4) E1 phase: saliva secretion into phloem tissues; 5) Transition E1/E2: salivation/ingestion in sieve elements; 6) E2 phase: ingestion from sieve elements. Y axis, output voltage after 100x gain. B) Expanded view of the standard pd and the novel phloem potential drop (phloem-pd): potential drop characteristics studied and compared between both potential drop waveforms: ΔV (potential drop magnitude; mV), number of intervals/sec during sub-phase II-2 (Int./s), duration of II-1, II-2 and II-3 sub-phases and total pd duration (s). C) Expanded view of rest of waveforms studied: 1) C waveform; 4) assumed end of E1 waveform (indicated with the arrow) and the beginning of the transition between E1/E2; 5) transition between E1/E2 and 6) assumed end of the transition E1/E2 (indicated with the arrow) and the beginning of E2 waveform.

6.3.6 STATISTICAL ANALYSIS

Pairs of transmission rates were compared using a Pearson's Chi-squared test with Yates' continuity correction. Differences in transmission rates obtained in the EPG experiments were simultaneously tested using the Marascuilo procedure, with statistical differences observed when the value $(p_j - p_i)$ exceeded the critical range value. Potential drop magnitude, pd subphase durations, total duration of the pd, numbers of intervals per second within subphase II-2 of the pd and duration of different EPG waveform aspects were compared between standard and phloem-pds by a *t*-student test (Gaussian variables) or by a Mann-Whitney *U*-test (pairs) or (Non-Gaussian variables) ($p < 0.05$). All analyses were conducted using the SPSS 22.0 (IBM SPSS Statistics, 2013) and R 3.3.3 (R Development Core Team, 2016) packages.

6.4 RESULTS

6.4.1 DETERMINING THE MOST EFFICIENT APHID VECTOR AND SUSCEPTIBLE PLANT SPECIES TO BYV

Transmission efficiency of BYV by viruliferous *M. persicae* was significantly higher in *B. vulgaris* (65/84; 77.38%), compared to *C. perfoliata* (44/84; 52.38%) ($\chi^2=10.45$; $p=0.001$) and *C. album* (35/84; 41.67%) ($\chi^2=20.78$; $p<0.0001$). Regarding *A. fabae*, the maximum percentage of transmission was obtained with *C. album* (21/84; 25%), followed by *C. perfoliata* (7/84; 8.33%) ($\chi^2=7.24$; $p=0.007$) and *B. vulgaris* (3/84; 3.57%) ($\chi^2=14.05$; $p<0.0001$). Among the two aphid vector species tested, *Myzus persicae* was significantly more efficient than *A. fabae* transmitting BYV to the three host plants species studied: *B. vulgaris* ($\chi^2=91.93$; $p<0.0001$), *C. perfoliata* ($\chi^2=36.49$; $p<0.0001$) and *C. album* ($\chi^2=4.53$; $p=0.033$). Apterae *M. persicae* were more efficient transmitting BYV (66/84; 78.57%) than alatae (48/84; 57.15%) ($\chi^2=5.36$; $p=0.021$). Thus, the most successful combination (*M. persicae* apterae on sugarbeet) was used in all further experiments.

6.4.2 DIFFERENCES BETWEEN STANDARD AND PHLOEM-PDS

The most obvious feature of phloem-pds was its significantly lower potential drop magnitude (output voltage mean of 2530.18 ± 76.24 mV) than the potential drop observed on standard-pds (output voltage mean 3002.54 ± 81.93 mV) ($t=3.431$; $p=0.001$) (Fig. 6.1 and 6.2A). The magnitude of the potential drop of the phloem-pd was always lower than

the one observed for the standard-pd, representing 84.24 ± 1.02 % of the mean value of the potential drop magnitude observed in the preceding standard pd ($U=57.0$; $p<0.0001$) (Fig. 6.2 A). Interestingly, the potential drop magnitude of E1 phase (output voltage 2328.23 ± 76.12 mV) was not significantly different from the magnitude of the potential drop of phloem-pds ($t=1.545$; $p=0.125$) but was significantly different from the potential drop of standard pds ($t=4.935$; $p<0.0001$) (Fig. 6.2 A). In addition, the frequency of intervals between downward peaks in sub-phase II-2 in phloem-pds (2.65 ± 0.06 int./s) was less than half that in standard pds (5.51 ± 0.11 int./s) ($U'=3,249.0$; $p<0.0001$) (Fig. 6.1 and 6.2 B). The maximum number of intervals per second in a phloem-pd was 3.45, whereas the minimum number of intervals per second in a standard-pd was 4.40. In summary, phloem-pds were mainly distinguished from the standard-pds on the basis of the magnitude of the potential drop and the number and rate of intervals between peaks within sub-phase II-2.

In 57 recordings that recorded an E1 waveform, E1 was always preceded by a single or several phloem-pd(s) (Fig. 6.1). The mean number of phloem-pds per recording was 1.56 ± 0.11 ; in 35 recordings a single phloem-pd preceded E1, in 13 recordings, there were two, in 8 recordings, three, and in one recording, 4 phloem-pds preceded E1. Every single E1 phase was preceded by a phloem-pd with a mean elapsed time of 24.28 ± 0.48 s between the end of the last phloem-pd and the beginning of E1 phase. The mean time spent by *M. persicae* from the end of the first phloem-pd until the beginning of E1 was 46.76 ± 4.43 s. Comparing both types of pds, the mean total duration of phloem-pd was shorter (3.84 ± 0.10 s) than standard pd (4.33 ± 0.11 s) ($t=3.51$; $p=0.001$) (Fig. 6.2 C). The main cause of this difference was shorter sub-phases II-1 and II-3 in phloem-pd than in standard pd (sub-phase II-1: 1.63 ± 0.04 s vs 1.91 ± 0.05 s, ($U=815.50$; $p<0.000$); sub-phase II-3: 0.79 ± 0.04 s vs 0.92 ± 0.03 s ($U=1,039.0$; $p=0.001$). No difference was observed in sub-phase II-2 duration (1.01 ± 0.04 s for phloem-pd vs 1.05 ± 0.05 s for standard pd; $U=1,524.5$; $p=0.571$) (Fig. 6.2 C).

Figure 6.2. Comparison of features of standard pds, phloem-pds E1 during 57 EPG recordings that were manually ended after detection of a single E1 phase (n=57). A) Comparison of the potential drop magnitude observed in the standard and phloem pds and E1 phase. Left vertical axis indicates the potential drop magnitude of the three patterns whereas right vertical axis indicates the percentage of potential drop of both the phloem-pd and E1 taking the drop of the standard-pd as 100% of the drop. Box-plots show the median, first and third quartiles of the potential drop. B) Comparison of number of intervals per second in the sub-phase II-2. C) Comparison of total duration of both standard and phloem pds as well as the three pd sub-phases (II-1, II-2 and II-3).

*Asterisks and different letters show significant differences according to a t-student test (Gaussian variables) or U-Mann-Whitney test (non-Gaussian variables) ($p < 0.05$).

6.4.3 *Myzus persicae* ACTIVITIES ASSOCIATED WITH BYV ACQUISITION

Table 6.1 shows the transmission rates obtained after manually interrupting probing after aphids produced different EPG waveforms. *M. persicae* did not acquire BYV from the infected-source plant before reaching the phloem salivation phase. No transmission was obtained when aphid probing was interrupted during waveform C with or without standard or phloem-pds. BYV acquisition only occurred after the phloem phase activities (after the E1 phase). A very low percentage of transmission was obtained when aphids were removed from the source plant during waveform E1 (1.42 %) and no transmission

was observed during the E1/E2 transient waveforms (0/57). However, the transmission rate constantly rose when the duration of sieve ingestion E2 increased as follows: 0-1h (2.94 %), 1-2h (8.70 %), 2-4h (11.36 %). Maximum of BYV acquisition was obtained after 4-6 h of phloem sap ingestion (23.52 %) with no significant differences in transmission rates observed for longer E2 ingestion periods: 6-8 h (27.78 %), 8-10 h (27.58 %) (Table 6.1).

Table 6.1. Relationship between *Myzus persicae* activities and the acquisition of *Beet yellows virus* (BYV). Acquisition rate was scored after subsequent 24h IAP on a test plant (1 aphid/test plant).

Aphid stylets activities (EPG waveforms observed) *	¹ N	Infected plants	Total	² Transmission efficiency
Stylet pathway (waveform C)	63	0	0/63	0 % a
Stylet pathway + a single standard-pd	62	0	0/62	0 % a
Stylet pathway + five standard-pds	62	0	0/62	0 % a
Stylet pathway + standard-pds + one or more phloem-pds	17	0	0/17	0 % a
Stylet pathway + standard-pds + one or more phloem-pds + a single E1 episode	70	1	1/70	1.42 % a
Stylet pathway + standard-pds + one or more phloem-pds + a single E1/E2 transition phase	56	0	0/56	0 % a
Stylet pathway + standard-pds + one or more phloem-pds + one or several E1+E2 episodes (0 – 1h E2)	68	2	2/68	2.94 % ab
(1 – 2h E2)	23	2	2/23	8.70 % bc
(2 – 4h E2)	44	5	5/44	11.36 % c
(4 – 6h E2)	34	8	8/34	23.52 % d
(6 – 8h E2)	36	10	10/36	27.78 % d
(8 – 10h E2)	29	8	8/29	27.58 % d

Different letters show significant differences according Marascuilo procedure, with statistical differences observed when the value (pj-pi) exceeded the critical range value (pj-pi)

*Aphids were removed from test plants after specific EPG waveform patterns were observed

¹Total number of test plants studied

²Virus transmission efficiency was calculated by dividing the number of total infected plants by the total number of test plants studied

6.4.4 *Myzus persicae* ACTIVITIES ASSOCIATED WITH BYV INOCULATION

Table 6.2 shows the transmission rates obtained after manually interrupting probing after aphids produced different EPG waveforms. No BYV inoculation (0/40) was obtained during the extracellular pathway phase (C waveform) or when a single or more standard-pds occurred (0/34). Interestingly, a sudden significant increase in the percentage of BYV inoculation (20/25: 80%) was observed when EPGs were interrupted after one or more phloem-pds occurred and before any E1 or E2 phloem phase activity was observed.

Table 6.2. Relationship between *Myzus persicae* activities and the inoculation of *Beet yellows virus* (BYV). Aphids were subjected to a 24h AAP on BYV-infected plants prior to transmission tests.

Aphid stylets activities (EPG waveforms observed) *	¹ Discarded data (A-B-)	Infected Plants		Total	³ Transmission efficiency
		² Plant A+ (A+B+ or A+B-)	Only B+ (A-B+)		
Stylet pathway (waveform C)	63	0	40	0/40	0 % a
Stylet pathway + one or more standard-pds	92	0	34	0/34	0 % a
Stylet pathway + standard- pds + one or more phloem- pds	31	20	5	20/25	80.0 % c
Stylet pathway + standard- pds + one or more phloem- pds + a single E1 episode	43	10	4	10/14	71.43 % bc
Stylet pathways + standard- pds + one or more phloem- pds + Int. + a single E1/E2 transition phase	34	18	10	18/28	64.29 % b
Stylet pathway + standard- pds + one or more phloem- pds + a single E2 episode (>20 min)	63	15	8	15/23	65.22 % b

Different letters show significant differences according to Pearson's Chi-squared test with Yates' continuity correction ($p > 0.05$).

*Viruliferous aphids were removed from test plants after specific EPG waveform patterns were observed (1 aphid/test plant).

¹Number of aphids that were unable to infect either of the two test plants (A or B) in each treatment, then considered as non viruliferous, and discarded from the analysis.

² Plant A was that where the aphid feeding was monitored until the production of the six waveform patterns previously described. Plant B was that where aphid was transferred from the Plant A in order to assess whether or not it acquired virus from plant A.

³Virus transmission efficiency was calculated by dividing the number of recordings where plant A became infected by the total number of recordings where either A, B or both test plants became infected. See main text for a complete explanation of the calculation procedure.

Lower inoculation rate was obtained when aphids were allowed to perform E1 salivation in a sieve element cell for 20-30 sec (71.43%). Similar transmission efficiency was obtained when aphids were allowed to perform an E1-E2 transient waveform period (Interphase E1/E2: Int E1/E2) (64.29%) or an E2 phase for more than 20 min (65.22%).

6.4.5 OPTIMUM AAP AND IAP OF BYV

To estimate the BYV inoculation and acquisition thresholds, we calculated the optimum time for both processes, expressed as the time spent by aphid to reach specific stylets activities key in the transmission of the virus from the first C waveform produced (1st probe). We calculated it as the time spent to reach the aphid activity resulting in the highest BYV transmission rate and significantly different to the previous treatment (phloem-pd for BYV inoculation; 4h of phloem sap ingestion for BYV acquisition). To estimate the optimum AAP, we studied the 8 recordings interrupted after 4-6 hours of phloem sap ingestion (E2), with successful BYV acquisition, and the 20 recordings artificially ended after detecting at least one phloem-pd with successful inoculation of BYV to estimate the optimum IAP (Table 6.3).

Table 6.3. Mean duration (h) of EPG variables involved in optimum BYV acquisition and inoculation by *Myzus persicae*. Recordings interrupted after 4-6 hours of sap ingestion with successful BYV acquisition (n=8) were analyzed in case of acquisition (Acq.). Recordings interrupted after a phloem-pd, and before a puncture in a sieve element cell, with successful BYV inoculation (n=20) in case of inoculation (Ino.). (1st probe: first C waveform produced by the aphid in the recording).

EPG patterns	Process	Times			Test Value	P
		Minimum	Maximum	Mean $\pm$ SE		
1 st probe – 1 st phloem-pd	Acq.	0.63	8.11	2.90 $\pm$ 0.80	1.011	0.321
	Ino.	0.59	3.99	2.02 $\pm$ 0.20		
1 st probe – End Recording	Acq.	6.27	16.54	9.51 $\pm$ 1.45	6.343	0.000*
	Ino.	0.60	6.46	2.60 $\pm$ 0.38		

*Significant differences according to a t-student test ($p < 0.05$)

Mean probing time spent by aphids to reach the maximum acquisition success was 9.51 $\pm$ 1.45 h, measured as the duration from the first probe (first C waveform produced by the aphid) until the end of the recording. However, the inoculation process was observed to be much shorter (2.60 $\pm$ 0.38 h) comparing to acquisition ($t = 6.343$; $p < 0.001$). No

differences were observed in the mean time spent by aphids to produce the first phloem-pd in either acquisition or inoculation process (2.9 ± 0.80 h and 2.02 ± 0.20 h, respectively; $t=1.011$; 0.321). We found out that the minimum time to have a successful inoculation of BYV was 0.59 h (time from first probe to 1st phloem-pd) while the minimum time needed for the successful acquisition of BYV was 1.65 h (time from the first probe to E2), detected in one of the two recordings interrupted after 0-1h of E2 phloem sap ingestion. According to the obtained results, the AAP and IAP with maximum BYV acquisition and inoculation success were found to be 9.5 and 3 hours, respectively.

6.5 DISCUSSION

The main finding of our study is the discovery of a new type of potential drop (phloem-pd) that differs from the previously described standard-pd (Tjallingii, 1985) and is linked to inoculation of BYV. Standard-pds play a key role in non-persistent virus transmission by aphids (Martín *et al.*, 1997; López-Abella *et al.*, 1988) as well as in the transmission of the semipersistent non-phloem restricted *Cauliflower mosaic virus* (CaMV, *Caulimovirus*) (Moreno *et al.*, 2005). The phloem-pds clearly differed from the standard-pd in their waveform features and in their number and timing of occurrence within the aphid probes. Phloem-pds always occurred shortly before E1 phloem salivation (24.28 s in average), whereas standard-pds occur earlier during stylet pathway phase. The potential drop of phloem-pds was similar in magnitude to that observed at the start of the subsequent E1 phase and was smaller than the drop in electrical potential observed for the standard-pds (Fig. 6.2). Also, within the phloem-pd, pd sub-phases II-1 and II-3 were shorter than in standard pds but subphase II-2 did not differ. Within pd subphase II-2, the number of intervals per second was lower than in standard pds. The lower magnitude of the potential drop in the phloem-pds appeared to reflect that this feature is caused by a higher electrical resistance between the stylet tips when located in phloem cells as compared to non-phloem cells. So, testing the possible role of these new phloem-pds on the transmission of the phloem-limited BYV became a key question in our study.

Non-phloem activities by *M. persicae* did not play any role in the BYV acquisition. Once aphids ingested sap from a sieve element cell, acquisition increased with the E2 duration and reached a maximum of 23.52% after 4-6 h, consistent with previous studies (Sylvester, 1956; Bennett, 1960). Interestingly, a single case of transmission (1/70; 1.42%) was detected in a recording manually ended during the salivation phase in phloem

tissues (E1). As known, E1 represents secretion of watery saliva into sieve element cells (Prado and Tjallingii, 1994), so no phloem sap ingestion is presumed to occur. Interestingly, this aphid (EPG recording) showed two pds with the phloem-pd feature with a complete II-3 sub-phase (associated with cell content uptake) in which some viral particles could be acquired when puncturing BYV-infected phloem cells.

Also, we showed that BYV inoculation occurs much faster than the acquisition process as observed in previous studies using *M. persicae* as a vector of BYV (Sylvester, 1956; Bennett, 1960). The mean time to the first phloem-pd from the beginning of the first probe was 2.02 h, with a mean time of 2.6 h from the first probe until the interruption in these recordings. This is similar to the 3 hours optimum inoculation access time previously reported for BYV (Sylvester, 1956). Interestingly, one of our aphids was able to produce a phloem-pd in 0.59 h, resulting in successful BYV inoculation. This could explain Bennett's (Bennett, 1960) result showing that BYV inoculation occurs within an hour of inoculation access period (IAP) and using 3 aphids per test plant. Our maximum BYV acquisition efficiency showed a mean duration of 9.51h, matching well with optimum times previously reported in the range of 6-12 hours (Sylvester, 1956; Bennett, 1960).

The results provided in the present work could be underlying and supporting previous studies on the feeding behaviour of aphids associated with the transmission of phloem-limited viruses where virus transmission was detected to occur prior to the phloem phase (waveforms E1 and E2) (Prado and Tjallingii, 1994; Scheller and Shukle, 1986). The phloem-pds could be the EPG pattern underlying the inoculation of *Barley yellow dwarf virus* (BYDV, *Luteovirus*) by *Rhopalosiphum padi* in *Triticum aestivum* prior to the E1 waveform (Prado and Tjallingii, 1994). Moreover, transmission of BYDV was also reported to occur during the X-waveform produced by *Sitobion avenae* in *Avena sativa* when using AC-EPG (Scheller and Shukle, 1986). This specific EPG waveform represents repetitive stylet punctures in phloem tissues prior to the continuous phloem ingestion phase (Ip waveform). Our phloem-pd waveform reported here could represent the same biological feature as the X-waveform reported for aphids when using AC-amplifiers (Scheller and Shukle, 1986). Furthermore, the inoculation of BYV when phloem-pd is produced by aphids is also consistent with transmission data obtained from phloem-limited viruses transmitted by whiteflies. Such studies show that transmission of phloem-limited criniviruses occurs at a low rate during stylet activities previous to the E1

phase. The inoculation of the phloem-limited *Lettuce chlorosis virus* (LCV, *Closterovirus*) by whiteflies was reported to occur when no phloem phase was detected (Johnson *et al.*, 2002). Also, *Tomato chlorosis virus* (ToCV, *Crinivirus*) was transmitted at a low efficiency (3.45%) before *B. tabaci* showed the phloem salivation phase E1 (Maluta *et al.*, 2017). However, unlike BYV, ToCV transmission rate increased when a single E1 episode occurred (52.2%). Similarly, Jiang *et al.* (2000) studied the feeding behaviour of *B. tabaci* associated with the transmission of the *Tomato yellow leaf curl virus* (TYLCV, *Begomovirus*) and some whiteflies that showed no E1 episode (only stylet pathway phase) were able to inoculate the virus with low efficiency (2.45%).

Previous studies, therefore, suggest that the inoculation of these phloem-limited plant viruses may occur during brief stylet punctures in phloem tissues. However, establishing a relation between phloem punctures (parenchyma cells, and in particular the specialized form companion cells, and sieve element cells) with virus transmission and to associate them to a specific EPG pattern was not the aim of previous studies. Also, the pds in whiteflies are very different from those produced by aphids (Jiang *et al.*, 1999). They occur much less frequently than in aphids and have no subphases during phase II. They generally occur close in time to phloem phase before E1 and therefore might in fact be short phloem punctures. So, the observed low efficiency in virus transmission occurring during early probing may possibly reflect phloem cell punctures before the long E1 salivation phase. The specific plant tissues in which a phloem-pd is occurring cannot be derived from the EPG prior to the study described in Chapter 7 of this PhD dissertation. Although Tjallingii and Hogen Esch (1993) demonstrated by combined EPG and histological studies that *A. fabae* was able to puncture phloem parenchyma (including companion cells) and sieve element cells several times before initiating the E1 salivation into a sieve element cell, they could not relate the cell types to any specific EPG waveform features.

The lower potential drop magnitude of pds associated with phloem cells punctures has already suggested for the repetitive pds (R-pds) produced by *Acyrtosiphon pisum*, *Brevicoryne brassicae* (Tjallingii and Gabrys, 1999), as well as *Periphyllus acericola* and *Drepanosiphum plantanoidis*. Similar to phloem-pds, R-pds occur just before the phloem phases and after many standard pds and their pd magnitudes were similar to the E1 potential drop. In apoplastic phloem loading plants like sugar beet (Gamalei, 1989), there

are few plasmodesmata between the SE/CC complex and surrounding plant tissue and this may result in a different electrical status between the SE/CC complex and surrounding tissues (van Bel and van Rijen, 1993). That could explain the difference in magnitude in the potential drop between standard pds and phloem phase, as well as phloem pds assuming that the latter are penetrations of the SE/CC complex. The difference appears to be due to greater electrical resistance between the substrate electrode and the electrically isolated SE/CC complex than between the substrate electrode and the more superficial leaf tissues. In sugar beet, there are few plasmalemma connections between the SE/CC complex and surrounding cells (Gamalei, 1989). The higher resistance during penetration of the SE/CC complex is probably due to the relative isolation of the SE-CC complex from the outer tissues in terms of plasmodesmata (van Bel and van Rijen, 1993). Therefore, the different features observed in the phloem-pd (reduced potential drop magnitude and reduction in number of intervals per second) are suggesting the occurrence of this unique type of pd, in a different tissue, likely the phloem cells. Chapter 7 of this dissertation confirms that phloem-pds are produced during penetration of the SE/CC complex.

Our findings from EPG experiments confirm the phloem-limited nature of BYV and that the phloem-pds produced by aphids actually occur in phloem tissues. Whereas no virus inoculation was observed when aphid probing was interrupted after standard pds produced either in epidermis or mesophyll cells (0/34), maximum inoculation efficiency occurred when aphids performed at least a single phloem-pd (20/25; 80%). Also, no virus acquisition occurred after aphids produced either a single standard pd (0/62) or five consecutive standard-pds (0/62) on BYV-infected source plants. Thus, the high inoculation rate that was observed during phloem-pds and the proximity of these intracellular stylet punctures to the E1 salivation phase, occurring at an average of 24 seconds before E1, strongly support the hypothesis that phloem cells are actually punctured during the occurrence of phloem-pd waveforms. The lack of systemic infection when viruliferous aphids produced a series of standard intracellular punctures suggest that BYV particles are released into epidermal or mesophyll cells by aphids but are unable to move cell-to-cell within the mesophyll and reach the phloem cells, and are kept confined within the non-vascular tissues. Our results show that only virus particles delivered into phloem cells (phloem parenchyma/companion or sieve element cells) allows the virus to circulate through the sieve tubes and consequently produce a systemic

infection. Although BYV has been demonstrated to be occasionally present out of phloem tissues in *B. vulgaris* in late stages of virus infection (Esau, 1960), our EPG-assisted transmission experiments confirmed the phloem-restricted nature of the virus under our experimental set-up.

Presumably phloem-pds could be very useful in further studies regarding compatible/non-compatible aphid-plant interactions. Also, this new pd type seems not to be a unique feature of *M. persicae*. EPG studies recently conducted by our research team have shown that the same type of phloem-pd is also produced by *Rhopalosiphum padi* in barley. Moreover, re-examination of EPG recordings of *Aphis gossypii* on *Cucumis melo* cv ‘Regal’ conducted in the past by our team also reveals the production of the same kind of phloem-pd, although not reported at that time (Garzo *et al.*, 2018). The E1 waveforms were also preceded by this unique phloem-pd waveform in both *R. padi* and *A. gossypii* recordings, sharing the same characteristics of lower potential drop magnitude and frequency of intervals in sub-phase II-2 as those observed for *M. persicae* in *B. vulgaris*. Therefore, it is interesting to study whether they possibly play an important role in aphid-plant interactions at the sieve element level, possibly triggering wound responses before the E1 salivation (Tjallingii and Hogen Esch, 1993; Garzo *et al.*, 2017; Medina-Ortega and Walker, 2015). Another interesting observation is the shorter duration of sub-phase II-1 of phloem-pds compared to that of standard pds. This suggests that the inoculation of BYV is not associated with the duration of subphase-II-1, but to the plant tissue where these phloem-pds are produced.

The retention site of BYV in the vector and how the virus is released for inoculation remain still unclear. Binding sites of other semipersistently transmitted closteroviruses such as *Lettuce infectious yellows virus* (LIYV, *Closterovirus*) have been already identified in the anterior foregut of *B. tabaci* (Chen *et al.*, 2011). *Citrus tristeza virus* (CTV, *Closterovirus*) was also localized in the foregut of *Toxoptera citricida* (Killiny *et al.*, 2016). Semipersistent viruses have been assumed to be likely foregut borne (Nault, 1997; Ng and Perry, 2004). Nevertheless, several works studying interference of BYV supported a hypothesis that BYV could be stylet borne (Brack and Polak, 1976; Vanderveken, 1968). Also, semipersistently transmitted CaMV is retained in the acrostyle, a specific area in the anterior common duct part of food and salivary canal (Uzest *et al.*, 2007). Finding BYV retention sites in the aphid mouthparts might also

provide a key to clarify the mechanism involved in dislodging viral particles, either egestion if BYV is retained in the foregut or watery salivation or egestion if it is retained in the common duct of the stylets. If the inoculation indeed occurs during the 1st sub-phase of the phloem-pd (subphase II-1) it would suggest that the virus is inoculated by salivation (as previously shown for non-persistent viruses). Conversely, if inoculation occurs during the 2nd sub-phase of the phloem-pd (sub-phase II-2) it would support that the virus is inoculated by another mechanism, very likely egestion (or a combination of both salivation and egestion); the old egestion-ingestion hypothesis proposed for non-persistent and semi-persistent virus transmission (Harris and Harris, 2001; Moreno *et al.*, 2012).

CHAPTER 7. LOCALIZATION OF *Myzus persicae* STYLET TIP DURING THE INTRACELLULAR PUNCTURE ‘PHLOEM-PD’ ASSOCIATED WITH THE INOCULATION OF THE SEMIPERSISTENT AND PHLOEM-LIMITED *Beet yellows virus* (BYV, *Closterovirus*)³

7.1 INTRODUCTION

Many plant viruses require insect vectors to be transmitted from infected plants to new uninfected host plants (Feres and Racciah, 2015). Therefore, the study of insect feeding activities in the plant and their relationship to virus acquisition and inoculation enhances our understanding of virus epidemiology and may contribute to developing strategies for controlling the spread of plant viruses. Numerous studies over many years have focused on the mechanisms of transmission of plant pathogens by insect vectors (Watson, 1938; Sylvester; 1956; Scheller and Shukle, 1986; Jiménez *et al.*, 2018). Among insect-transmitted pathogens, viruses vectored by aphids have been the most studied. Plant viruses transmitted by aphids account for more than 50% of the insect-transmitted viruses and approximately 40% of the 700 known plant viruses (Hogenhout *et al.*, 2008; Feres, 2016).

The Electrical penetration graph (EPG) technique (Tjallingii, 1978) has been key in the study of the feeding behaviour of insect vectors and its association with virus transmission (Prado and Tjallingii, 1994; Tjallingii and Prado, 2001; Martín *et al.*, 1997). Different aphid stylet activities in plants have been correlated with different EPG patterns (Tjallingii, 1978; 1985). Details of stylet penetration activities such as stylet tip position in different plant cells, salivation and ingestion can be detected in real time with EPGs. The first phase of aphid stylet penetration is referred to as "pathway phase".

³Manuscript accepted: Jiménez J., Garzo E., Alba-Tercedor J., Moreno A., Feres A., Walker G.P. 2019. The phloem-pd: a distinctive brief sieve element stylet puncture prior to sieve element phase of aphid feeding behaviour. *Arthropod-Plant Interactions*.

The dominant EPG waveform during pathway phase is waveform C, which is correlated with intercellular stylet penetration and is periodically interrupted by brief intracellular punctures that are detected in EPG recordings as "potential drops" (pds). "Phloem sieve element phase" (PSEP) activities occur when the stylet tips are in a phloem sieve element (or sometimes companion cell - Medina-Ortega and Walker, 2015; Peng and Walker, 2018) and within a few seconds after penetration of the cell, the aphids start secreting watery saliva into the sieve element (waveform E1). If the PSEP is successful, E1 is followed by passive phloem sap ingestion (waveform E2). Two types of aphid saliva are secreted into the plant at different stages throughout the feeding process. Watery saliva is secreted from the onset of aphid stylet penetration during pathway phase and also is secreted into penetrated cells during pds and into sieve elements during PSEP waveform E1 (Martín *et al.*, 1997; Moreno *et al.*, 2011). Another type of saliva, sheath saliva, is secreted in extracellular space during pathway phase waveform C; it gels shortly after secretion and forms a sheath enveloping the stylets (López-Abella *et al.*, 1988; Tjallingii, 2006).

Aphid stylet activities and their associated EPG waveforms involved in non-persistent (NP) virus transmission have been well studied. Acquisition and inoculation of NP viral particles occur during brief intracellular stylet punctures in superficial plant tissues, primarily in epidermal or mesophyll cells (Martín *et al.* 1997) and often named "standard-pds" (Chen *et al.*, 1997; Jiménez *et al.*, 2018). These intracellular punctures are composed of three different phases (I, II and III). Within phase II, there are three subphases. Subphase II-1 is associated with injection of watery saliva into the cell and inoculation of non-persistent virus particles (Powell, 2005). Subphase II-3 is associated with uptake of cell cytoplasm and therefore acquisition of non-persistent viral particles (Powell *et al.*, 1995; Martín *et al.*, 1997). Subphase II-2 is associated with inoculation of the semipersistently transmitted *Cauliflower mosaic virus* (CaMV, *Caulimovirus*) (Moreno *et al.*, 2012). However, the mechanisms involved in CaMV particles dislodging from the acrostyle (binding site of CaMV at the apex of the maxillary stylets - Uzest *et al.*, 2007) during subphase II-2 remain unknown. Subphase II-2 could represent an additional salivation phase or possible egestion of cell content previously ingested (Moreno *et al.*, 2012).

Successful transmission of phloem-limited and persistently transmitted viruses, such as *Barley yellow dwarf virus* (BYDV, *Luteovirus*), has been associated mainly with aphid stylet activities in sieve elements: inoculation occurs during waveform E1 and acquisition during waveform E2 (Prado and Tjallingii, 1994). However, a few incidences of virus acquisition and inoculation were observed to occur prior to waveforms E1 and E2, suggesting that acquisition and inoculation also can take place during brief intracellular punctures in phloem parenchyma, companion or sieve element cells (PPC, CCs or SEs, respectively). Tjallingii and Hogen Esch (1993) demonstrated that penetration of sieve elements does not always result in the PSEP waveforms E1 and E2, but sometimes the aphids produce brief pds that the authors found indistinguishable from the standard-pds produced during penetration of epidermal and mesophyll cells.

The specific aphid stylets activities underlying semipersistent virus transmission are known for only CaMV and *Beet yellow virus* (BYV, *Closterovirus*). The term "semipersistently transmitted" refers only to retention time after acquisition; while other significant biological characteristics, such as tissues infected, retention site and stylet penetration behaviour associated with transmission differ among semipersistently transmitted viruses (Ng and Zhou, 2015). Some, like CaMV, infect all plant tissues (Palacios *et al.*, 2002), whereas others, like BYV or *Citrus tristeza virus* (CTV, *Closterovirus*), are limited to phloem tissues (Esau, 1969; Price, 1966). Stylet penetration behaviour associated with transmission also varies. CaMV is inoculated during intracellular punctures (pds) in outer leaf tissues (i.e., epidermis and mesophyll) (Moreno *et al.*, 2012), similar to NP viruses, whereas inoculation of the phloem-limited BYV by *Myzus persicae* occurs during specific intracellular stylet punctures that always precede PSEP (Jiménez *et al.*, 2018). These pds had some distinct differences from the standard-pds that are associated with non-persistent virus transmission, so the authors hypothesized these novel pds occurred during penetration of phloem cells, either into phloem parenchyma, companion or sieve element cells. They were tentatively named phloem-pds awaiting verification that they were specifically associated with intracellular punctures of phloem cells.

7.2 OBJECTIVE

The main objective of this work was to identify the type of cell penetrated by the stylet tips during these novel pds (phloem-pds) produced by *M. persicae*. Stylets were fixed *in*

situ in the plant tissue by cryofixation with liquid nitrogen during several specific EPG patterns including standard-pds and the novel phloem-pd. This was followed by observation of the stylets or salivary sheath tracks in the plant tissue by both confocal laser-scanning microscopy (CLSM) and micro-computed tomography (Micro-CT).

7.3 MATERIAL AND METHODS

The experiments in this work were conducted in three different laboratories. EPG monitoring of aphid probing behaviour followed by cryofixation of samples was performed at the Institute of Agricultural Sciences of The Spanish National Research Council (ICA-CSIC) (Madrid, Spain) and at the University of California Riverside (UCR) (USA). Samples obtained at UCR were analyzed by confocal laser-scanning microscopy (CLSM) at UCR's Center for Plant Cell Biology. For samples obtained at ICA-CSIC facilities, analysis by micro-computed tomography (Micro-CT) was conducted at the Department of Zoology of the University of Granada (Spain).

7.3.1 PLANTS AND APHIDS CULTURE FOR CRYOFIXATION

For cryofixation and later analysis by CLSM, the plants used were 5 week-old sugar beets from breeding line resistant to powdery mildew. Plants were maintained as described in Chapter 3 (section 3.2.1). Newly emerged apterae *M. persicae* adults (2-3 days old) reared in sugar beet were used for the experiments. Aphids were reared as indicated in Chapter 3 (section 3.2.2). For cryofixation and later analysis by Micro-CT, the plants used were 4 weeks old sugar beets var. Julietta reared as described in Chapter 3 (section 3.2.1). Newly emerged apterae *M. persicae* adults (2-3 days old) reared in sugar beet (following the procedures indicated in Chapter 3, section 3.2.2) were used for the experiments.

7.3.2 ELECTRICAL PENETRATION GRAPHS (EPG) SETUP

The aphid feeding process was monitored by EPG [for a detailed EPG set-up description and aphid wiring, see Chapter 3 (section 3.5)] and terminated by cryofixation in order to fix the stylets *in situ* in the plant during different EPG waveform patterns: I) At the end of subphase II-2 during the 10th or 11th standard-pd in the same probe, II) At the end of subphase II-2 during the first or second phloem-pd in a probe (phloem-pds are always preceded by multiple standard-pds), and III) 15-30 sec after the start of the first PSEP E1 waveform which was always preceded by both standard and putative phloem-pds. A

compromise of 10-11 standard-pds was considered for treatment I to allow aphids to penetrate deeper in the plant tissues for a successful cryofixation of the stylets, and minimizing the production of a phloem-pd if a higher threshold of pds were applied. When a phloem-pd was observed during the series of 10-11 consecutive standard-pds (treatment I) the aphids were not used for cryofixation and discarded. Standard-pds and putative phloem-pds are distinguished by several features that were initially described by Jiménez *et al.* (2018) and are illustrated in Fig. 7.1: potential drop magnitude (difference of the output voltage after 100x gain between the voltage in a stabilized C waveform two-three seconds before the potential drop and the beginning of subphase II-2), number of intervals in subphase II-2 (intervals are the distance between two sequential downward peaks - Fig. 7.1) as well as the number of intervals per second within subphase II-2; all three features are greater in standard-pds than in phloem-pds. The values of these features for each cryofixation sample in this study are given in Table 7.1.

Figure 7.1. Comparison of standard-pds and phloem-pds. All 3 subfigures are from the same phloem-pd and standard-pd. A) General features of both the standard and phloem-pd. Three different pd subphases, II-1, II-2 and II-3 are shown. Down arrow at the end of subphase II-2 in Fig. A indicates the exact point when samples were cryofixed in the cryofixation experiments. B and C) Main characteristics distinguishing standard and phloem-pds. B) Subphase II-2 of a standard-pd and a phloem-pd (down arrows indicate the first and last downward peak; up arrows, intervals in subphase II-2). C) Differences in the potential drop (ΔV) between standard and phloem-pds. Potential drop magnitude was measured as the difference in output voltage (after 100x gain) between a stable C waveform (2-3 sec before the potential drop) and the first peak in subphase II-2 of either standard or phloem-pd.

7.3.3 CRYOFIXATION OF *Myzus persicae* STYLETS IN SUGAR BEET LEAVES

Once the aphid produced the EPG waveforms previously described, liquid nitrogen was poured on the adaxial side of the leaf opposite where the aphid was feeding on the abaxial side, instantaneously freezing both leaf and aphid.

At UCR, the cryofixation protocol was the same as described by Walker and Medina-Ortega (2012) with one modification that greatly improved the success rate of fixing the stylets *in situ* in the leaf by minimizing movement of the leaf when impacted by the liquid nitrogen. The leaf was sandwiched between the leaf holder appressed to its abaxial side and a thin piece of plastic appressed to its adaxial side. The plastic had an opening the same size as the opening in the leaf holder. The holes were aligned and the unit (leaf holder, leaf and thin plastic) was held together by paper clips. Liquid nitrogen was poured on the leaf through the opening on the adaxial side directly opposite to where the aphid fed on the abaxial side. Aphids commonly dislodged during cryofixation but about 30-50% of the time, they broke off from their stylets, which remained inside the leaf tissue. If the stylets pulled out when the aphid dislodged, the samples were discarded. When the aphid remained attached to the leaf, a cold pair of forceps was used to break the aphid body from the stylets/labium+stylets, which remained in the leaf. Immediately after cryofixation, powdered dry ice was poured on the leaf to keep it frozen. The leaf was severed from the plant and immediately placed in a jar containing 95% ethanol previously chilled to dry ice temperature (-78.5 °C). The samples in ethanol were maintained at -78.5°C for two days and then at -20°C for other two days. Then samples were transferred to a 4°C refrigerator for one day and then placed inside a Styrofoam container to slowly reach room temperature.

At ICA-CSIC, EPGs were recorded from the aphids feeding on the down-facing abaxial side of leaves. The leaves were secured, abaxial side down, to a shallow dish (2 cm deep, 10 cm diameter) using a clip to secure the petiole of the leaf to the border of the shallow dish. Stabilizing the leaf in this manner helped prevent the leaf from moving away from the tethered aphid while liquid nitrogen was poured. When the aphid produced one of the desired EPG patterns (treatments I, II, or III), liquid nitrogen was poured (100-150 ml) over the leaf close to the feeding site (not directly on feeding point, to avoid aphid movement), instantaneously freezing and fixing the stylets in the plant tissue.

Immediately after cryofixation, the leaf was severed from the plant, and the dish containing the aphid+leaf was placed over dry ice powder (-78.5°C) to keep it frozen. The aphid's legs then were glued to the leaf with transparent nail polish (Fig. 7.2: A, B). If the aphid dislodged from the feeding site either during cryofixation or during application of glue to the legs, the sample was discarded. In successful samples, a 50mm^2 area of leaf containing aphid body was excised, and samples were then dehydrated in 96% ethanol at -78.5°C . Samples were maintained in ethanol following the similar temperature/time combinations described above for the UCR procedure.

7.3.4 CONFOCAL LASER-SCANNING MICROSCOPY OBSERVATION OF *Myzus persicae* STYLET TIP AND CORRELATION WITH EPG WAVEFORMS

After freeze substitution, samples were placed in a Petri dish containing 95% ethanol and the stylets were located by searching the abaxial side of the leaf tissue with a Leica MZ16 stereomicroscope and substage lightning. Once the stylets were found, samples were turned over and an electrolytically sharpened tungsten wire (Brady, 1965) was used to dissect away tissues (cuticle, epidermis, mesophyll, xylem and sometimes phloem) from the adaxial surface down to the stylets. The dissection had to be deep enough so that the stylet tips could be seen in substage lighting; otherwise, there would be too much overlying tissue for clear images to be seen later by confocal laser scanning microscopy. Care also had to be taken to avoid dissecting too deep and destroying the tissue where the stylet tips were located. An Mk1 micromanipulator (Singer Instruments, Roadwater, UK) was used to facilitate the dissection. After dissection, an approximately 50 mm^2 area containing the dissected area was cut out with a razor blade.

Samples were then placed in a small Petri dish (35 mm diameter) and covered by several drops of DiOC₇(3) (Invitrogen, Carlsbad California, USA) stain (0.1% in 95% ethanol). Lids were placed on the Petri dishes and several dishes were placed in a larger Petri dish in an ethanol-saturated atmosphere to retard evaporation of the staining solution. After approximately 1 hour of staining, the DiOC₇(3) solution was carefully blotted away from the samples and then replaced by three changes of 95% ethanol followed by three changes of water. Next, several drops of sulforhodamine 101 (Invitrogen, Carlsbad California, USA) (1% in water) were applied to the samples for about an hour. Then, the sulforhodamine solution was removed and replaced by three changes of water. A few

initial samples also were stained with aniline blue for staining callose in addition to DiOC₇(3) and sulforhodamine 101, but aniline blue staining was discontinued because it did not contribute substantially to studying the samples. The sulforhodamine 101 and DiOC₇(3) served as general stains. Although the Invitrogen handbook (Haugland, 2005) indicates that DiOC₇(3) is used primarily as a membrane stain, we found that on fixed material, it functioned more like a general stain, and stains even non-membranous structures such as forisomes (Walker and Medina-Ortega 2012; Medina-Ortega and Walker 2015). Finally, samples were transferred to a microscope slide and rinsed with a few drops of water to remove possible loose cells in order to get an unobstructed view of the dissected area. Water was carefully blotted away and samples were covered with an aqueous mounting medium (80 ml glycerol, 4 g propylgallate, 20 ml Tris [0.1M, pH 9]) and cover-slipped. Samples were observed using a Leica SP5 microscope using default settings for FITC/Rhodamine labeling (and DAPI for samples that were stained with aniline blue in addition to DiOC₇(3) and sulforhodamine 101). Different fluorescence channels (sulforhodamine, DiOC₇(3), aniline blue) or the merge fluorescence were applied to produce the images for further analysis of the penetrated cell. The cell type penetrated by the stylet tips could be identified in most samples; while in a few samples, only the tissue type could be positively identified.

7.3.5 MICRO-COMPUTED TOMOGRAPHY SCANNING OBSERVATION OF *Myzus persicae* SALIVARY SHEATH AND CORRELATION WITH EPG WAVEFORMS

Samples for Micro-CT examination were manipulated and studied using methodology similar to that used by Cornara *et al.* (2018) to study the tracks of *Philaenus spumarius* stylets in olive leaf tissues. After freeze substitution, the samples were stained with iodine (1% in 96% ethanol) for 24h at 4°C. After that time, iodine was then carefully blotted away and the sample was dried with hexamethyldisilazane (HDMS) for 24h inside a fume hood at room temperature. The stylet position in each sample was examined by micro-computed tomography (micro-CT) with a Bruker SkyScan 1172 high-resolution desk-top microtomograph (Bruker, Kontich, Belgium) upgraded with a Hamamatsu L7902 100/250 X-ray source and a Ximea (SHT) 11 megapixels camera (Ximea GmbH, Münster, Germany). To obtain the rendered images, 360° rotation scans were performed, with the scanning parameters setup as follows: for Fig. 7.4 A and 7.4 D: isotropic voxel size: 0.54 µm, voltage = 59 kV, current = 49 µA, image rotation step = 0.5°; for Fig. 7.4

B and 7.4 C: isotropic voxel size: 1 μm , voltage = 44 kV, current = 49 μA , image rotation step = 0.5°, and for Fig. 3D: isotropic voxel size: 1.08 μm , voltage = 59 kV, current = 49 μA , image rotation step = 0.5. The Bruker micro-CT's Skyscan software (NRecon, DataViewer, CTAnalyser) was used for primary reconstructions and the “cleaning” process to obtain the datasets of “slices” as described in detail by Alba-Tercedor (2014). Amira's Software 6.4.0 Amira 3D Visualization and Analysis Software v.6.4 (Hillsboro, Oregon, USA: FEI; 2017, now distributed by ThermoFisher Scientific, Waltham, MA) was used to obtain volume rendering reconstructions images in Fig. 7.4 (A-D). For micro-CT scanning, samples were glued with cyanocrilate to the tip of a 200 μm diameter nylon fishing line (Fig. 7.2 A, B, C), inserted in a piece of plasticine at the end of the sample holder (Fig. 7.2 A, D) and protected with a plastic straw to prevent possible movements during the cooling process by ventilation during scanning (Fig. 7.2 E). Only images produced from samples where the aphid remained fixed in place after cryofixation and dehydration, with no visible displacement, were considered.

Figure 7.2: For micro-CT scanning, samples were glued with cyanocrilate to the tip of a 200 μm diameter nylon fishing line (3) (A, B, C), inserted in a piece of plasticine (4) at the end of the sample holder (5) (A, D) and protected with a plastic straw tube (6) to prevent possible movements in the scanning process due to the cooling process by ventilation (E) (in A, arrows point to the gold-wire (2) glued with silver paint to the dorsum of the aphid (1) used to monitor the aphid feeding behaviour by electrical penetration graphs (EPG) technique).

Whereas using CLSM we could identify the tip of the aphid stylets, the analysis of cryofixed samples by Micro-CT was conducted by following the track of the salivary sheath from the salivary flange below the aphid's labium to its terminal end. Longer probes have a greater chance of producing long and multiple salivary sheath tracks that would be difficult to analyze. To avoid this problem for treatments II and III (cryofixation during phloem-pds and E1 waveform, respectively), if a probe produced 35-40 standard-pds without producing the desired waveform, that probe was terminated and the aphid was manually moved with a small paint brush to another section of the leaf to start a new probe. Also, when the aphid finished a probe without any desired EPG pattern produced (e.g., no ten consecutive pds for treatment I, or more than 10 pds without any phloem-pd or E1 waveform for treatments II and III respectively) aphid was also moved to another section of the leaf. As a result of these manipulations, only a single and not excessively long salivary sheath was located in sugar beet tissues when the sample was examined.

7.3.6 STATISTICAL ANALYSIS

Potential drop magnitude (ΔV), number of intervals (No. Ints) in subphase II-2 and the frequency of intervals in this subphase (Ints/s) were compared between standard and phloem-pds using paired difference tests with the SPSS Statistics v25.0 package (IBM SPSS Statistics, 2017). Raw data, and if needed, log (x+1) or sqrt (x+1) transformations were first tested for normality with the Shapiro Wilk W test. If the raw data were normal, then Student's paired t-test was used to compare standard-pds and phloem-pds; if neither the raw data or any of the transformations were normal, then the non-parametric Wilcoxon test was used.

7.4 RESULTS

7.4.1 STRUCTURE OF STANDARD VERSUS PHLOEM POTENTIAL DROPS

Structural features of the standard-pds and phloem-pds during which samples were cryofixed are given in Table 7.1. For Treatment II, structural features are given for both the phloem-pd during which the samples were cryofixed and the last standard-pd preceding the phloem-pd. In Treatment II, all three variables used to distinguish the two types of pd were significantly greater for standard-pds compared to phloem-pds: the potential drop magnitude ($p < 0.001$, $t = 9.19$), number of intervals during subphase II-2

($p=0.005$; $Z=2.814$), and frequency of the intervals during subphase II-2 ($p<0.001$; $t=14.62$) (Table 1).

7.4.2 LOCALIZATION OF *Myzus persicae* STYLET TIPS BY CONFOCAL LASER-SCANNING MICROSCOPY AND CORRELATION WITH EPG WAVEFORMS

The cell punctured by the stylet tips at the time of cryofixation was successfully identified in 15 samples: four samples with the aphid fixed during the 10th or 11th consecutive standard potential drop (standard-pd) in a probe (treatment I), seven samples cryofixed during the first or second phloem-pd in a probe (treatment II) and four samples cryofixed during the first E1 phase in a probe (treatment III) (Table 7.1). Mesophyll or the bundle sheath cells were punctured in four samples cryofixed during the 10th or 11th standard-pd in a probe (Table 7.1, Fig. 7.3 A). In an additional sample cryofixed during the 10th standard-pd, stylet tips were located in the periphery of the vascular bundle, presumably puncturing a bundle sheath or phloem parenchyma cell, but the type of cell could not be determined. Samples fixed during phloem-pds showed the intrusion of aphid stylets into phloem tissue. In four samples in this treatment, stylets punctured a sieve element cell (Table 7.1, Fig. 7.3 B), and in three samples, a companion cell was punctured (Table 7.1, Fig. 7.3 C). In another sample also cryofixed during a phloem-pd, the stylets were found in the phloem tissue, probably puncturing a SE or CC cell, but the type of cell could not be identified. The stylet tips were in sieve elements in the four samples cryofixed during the first E1 phase produced by the aphid (Table 7.1, Fig. 7.3 D). Two more samples were cryofixed during the first E1 waveform, with stylet tips in phloem, but images were not clear enough to determine the cell type.

Table 7.1. EPG data and identity of the penetrated cell for samples cryofixed during a standard-pd (S-pd) (Treatment I), a phloem-pd (P-pd) (Treatment II) or during PSEP waveform E1 (Treatment III). "No. standard-pds" and "No. phloem-pds" are the numbers of standard and phloem-pds in the probe during which the sample was cryofixed (for treatments I and II, these numbers include the pd during which the sample was cryofixed). For treatments I and III, ΔV is the potential drop magnitude (after 100x gain) of the pd or E1 during which the sample was cryofixed. For Treatment I, No. Ints, is the number of intervals and Ints/s is the number of intervals per second of subphase II-2 of the pd, during which the aphid was cryofixed. For treatment II, ΔV , No. Ints, and Ints/s are given for both the phloem-pd during which the sample was cryofixed and the last standard-pd prior to cryofixation, and % ΔV is the relative difference in the potential drop magnitude between the two ($(\Delta V[\text{P-pd}] \div \Delta V[\text{S-pd}] * 100)$). For treatments I and III, last column refers to the penetrated cell by the aphid stylets during the 10th or 11th standard-pd and first E1 phase, respectively. For treatment II, last column refers to the penetrated cell by the aphid stylets during the phloem-pd.

Treatments	^a Sample	No. S-pds	No. P-pds	ΔV potential drop (mV)			No. Ints Subphase II-2		Ints/s Subphase II-2		^b Pun. Cell
I) Standard-pd				Last standard-pd terminated by cryofixation							
	CLSM1	10	–	5,371			10		6.25		M
	CLSM2	10	–	5,649			5		5.19		M
	CLSM3	10	–	3,456			9		5.92		BS
	CLSM4	11	–	2,742			5		6.67		BS
	MCT1	10	–	5,103			4		6.45		M
	Mean±SE	10.2±0.2	–	4,464.2±575.2			6.6±1.2		6.1±0.3		
II) Phloem-pd				Last standard-pd (S-pd) and the phloem-pd (P-pd) terminated by cryofixation							
				S-pd	P-pd	% ΔV	S-pd	P-pd	S-pd	P-pd	
	CLSM1	29	1	2,987	1,902	63.68	7	2	5.38	2.38	SE
	CLSM2	24	2	3,315	2,562	77.29	11	2	5.64	2.60	SE
	CLSM3	25	2	3,979	2,765	69.49	8	2	5.16	2.94	SE
	CLSM4	16	2	3,364	2,820	83.83	8	2	5.26	2.82	SE
	CLSM5	15	2	3,361	2,806	83.49	5	2	5.88	2.67	CC
	CLSM6	14	2	3,865	3,487	90.22	10	2	5.21	2.94	CC
	CLSM7	8	1	4,259	3,276	76.92	7	3	4.46	2.78	CC
	MCT1	20	2	3,231	2,353	72.83	5	2	4.46	2.04	SE
	MCT2	32	1	4,244	3,085	72.69	8	2	4.49	1.67	SE
	MCT3	22	1	3,761	2,596	69.02	7	2	5.78	2.11	SE
	Mean±SE	20.5±2.3	1.6±0.2	3,636.6±140.7 ^{*1}	2,765.2±144.3 ^{*1}	75.95±2.6	7.6±0.6 ^{*2}	2.1±0.1 ^{*2}	5.2±0.2 ^{*1}	2.5±0.1 ^{*1}	
III) E1				Last phloem-pd prior to E1							
	CLSM1	8	1	2,849			2		2.82		SE
	CLSM2	14	2	2,244			3		2.80		SE
	CLSM3	19	2	2,550			2		2.72		SE
	CLSM4	18	2	2,804			2		2.86		SE
	MCT1	15	3	2,795			2		2.78		SE
	Mean±SE	14.8±1.9	2.0±0.3	2,648.4±113.8			2.2±0.2		2.8±0.02		

^aSamples with the label CLSM were examined by confocal laser-scanning microscopy and those with the label MCT were examined by micro-computed tomography (micro-CT)

^bPunctured cell; Abbreviations: M: mesophyll cell; BS: bundle sheath cell; CC: companion cell; SE: sieve element cell.

^{*1}Significant differences according to a Paired Student's t-test (non-transformed data) ($p \leq 0.05$).

^{*2}Significant differences according to a Wilcoxon test (non-transformed data) ($p \leq 0.05$).

Figure 7.3. Confocal laser-scanning microscope (CLSM) micrographs of sugar beet leaf tissues penetrated by *M. persicae* stylets. A) Apex of the salivary sheath containing the stylets located in a mesophyll from a sample cryofixed during the tenth consecutive standard-pd in a probe. B) Apex of the salivary sheath containing the stylets in contact with a sieve element from a sample fixed during a phloem-pd. Inset: image from a previous slice in the Z-stack showing the stylets encased in the salivary sheath; the SE, CC and SP* in the inset are the same cells and sieve plate labeled SE, CC and SP in the main figure. C) Apex of the salivary sheath containing the stylets in contact with a companion cell from a sample fixed during a phloem-pd. D) Stylet tips inserted in a sieve element cell from a sample fixed during an E1 waveform. Images A and D are from just the DiOC₇(3) channel whereas B and C are blends of the DiOC₇(3) and sulforhodamine 101 channels. SS (aphid stylets encased in the salivary sheath), St (stylet tip), M (mesophyll), SE (sieve element), SP (sieve plate), CC (companion cell), BS (bundle sheath).

7.4.3 LOCALIZATION OF *Myzus persicae* SALIVARY SHEATH TIP BY MICRO-COMPUTED TOMOGRAPHY AND CORRELATION WITH EPG WAVEFORMS

The cell punctured by the stylet tips was successfully identified in five samples: a single sample with the aphid fixed during the 10th standard-pd in a probe (treatment I), three samples cryofixed during the first or second phloem-pd in a probe (treatment II) and a

single sample cryofixed during the first E1 phase in a probe (treatment III) (Table 7.1). Micro-CT rendered images of sugar beet tissues containing aphid stylets are shown in Fig. 7.4.

Figure 7.4. Micro-CT volume rendering reconstruction images of aphid stylets in sugar beet leaf tissues for samples cryofixed during three EPG waveforms. A) Sample cryofixed during the tenth standard-pd in a probe; salivary sheath tips are in the mesophyll. B) Cross section of sugar beet leaf tissue cryofixed during a phloem-pd; salivary sheath tips are in contact with a sieve element. C) Longitudinal section of same sample as B. D) Sample cryofixed during PSEP waveform E1; salivary sheath tips are in contact with a sieve element. L (aphid labium), St (aphid stylets), SS (salivary sheath), SSt (salivary sheath tip), M (mesophyll), SE (sieve element), CC (companion cell), X (xylem vessels).

Mesophyll and bundle sheath cells seem to be damaged by ice formation and also likely the shrinkage caused by the drying process with hexamethyldisilazane (HDMS); however, companion cells and sieve elements are well preserved due to the cryoprotectant effect of their high sucrose content. Xylem tracheary elements also are well preserved

due to thick lignified cell walls and their lack of protoplasm. The sample cryofixed during the 10th standard intracellular puncture revealed the tip of the salivary sheath in mesophyll tissue, thus indicating puncture of a mesophyll cell (Fig. 7.4 A). Three samples were cryofixed during a phloem-pd, and the tips of the salivary sheaths were in contact with a sieve element in all three samples (cross section; Fig. 7.4B; longitudinal view: 7.4 C). The salivary sheath tip was also in contact with a sieve element cell in the single sample cryofixed during the first E1 waveform (Fig. 7.4 D).

7.5 DISCUSSION

In Chapter 6 we described a distinctive type of pd (named phloem-pd) during which the phloem-limited virus BYV was inoculated by *M. persicae* into sugar beet plants (Jiménez *et al.*, 2018). Inoculation of a phloem-limited virus is a strong indication that these pds are intracellular punctures in phloem cells. Using anatomical evidence, the current study confirms that these distinctive pds are penetrations of phloem cells. Specifically, during phloem-pds, aphid stylets penetrated sieve elements and companion cells.

For *M. persicae* feeding on sugar beet, PSEP is always immediately preceded by one or more phloem-pds, and their voltage drop is the same magnitude as the voltage drop at the start of PSEP, both of which are less than the voltage drop magnitude of standard-pds (Table 7.1; Jiménez *et al.*, 2018). The similar magnitude of the voltage drop between phloem-pds and PSEP is further evidence that phloem-pds are punctures of SEs or CCs. SEs and CCs are highly interconnected symplastically via numerous specialized plasmodesmata, called pore-plasmodesmata units (PPUs) (van Bel and Kempers, 1997; van Bel, 2003); this results in SEs and their associated CCs having the same electrical status (specifically the membrane potential that produces the V-drop during pds and PSEP) (van Bel and van Rijen, 1993; Hafke *et al.* 2005; Hafke *et al.* 2013). However, in plants that load phloem apoplastically (such as sugar beet; Gamalei, 1989), the SE/CC complex has very few symplastic connections with surrounding cells, which likely results in surrounding cells, including phloem parenchyma, having a different electrical status than the SE/CC complex (van Bel and van Rijen, 1993). Consequently, the similarity in voltage drop magnitude of phloem-pds and PSEP and the difference from standard-pds is supporting evidence that phloem-pds represent stylet punctures into the SE/CC complex while standard-pds are not associated with penetration of the SE/CC complex.

Another defining feature of phloem-pds is their distinctive subphase II-2: it has fewer intervals and a lower frequency of intervals compared to standard-pds (Table 7.1; Fig. 7.1). Little is known about what occurs during this specific pd subphase. Inoculation of the semi-persistently transmitted CaMV occurs during subphase II-2 of standard-pds (Moreno *et al.*, 2012), but the specific behaviour during subphase II-2 that results in inoculation is unknown. In contrast, non-persistently transmitted viruses are inoculated via salivation during pd subphase II-1 (Martín *et al.*, 1997, Powell 2005). The retention site of CaMV in the aphid vector is the acrostyle where the food and salivary canals merge at the apex of the stylets (Uzest *et al.*, 2007), and although the acrostyle is in the path of salivary secretion into the plant, CaMV is not inoculated during the salivation that occurs during subphase II-1 (Moreno *et al.*, 2012). Therefore, the likely aphid behaviour during subphase II-2 when CaMV is inoculated is either egestion (Harris and Harris, 2001) or perhaps secretion of a different composition of saliva.

The pd subphase as well as the aphid mechanism responsible for BYV inoculation during phloem-pds has not yet been identified. This question might be answered using the methodology that Martín *et al.*, (1997) and Moreno *et al.*, (2012) used to identify subphase II-1 and subphase II-2 as the subphases when non-persistent viruses and CaMV, respectively, are inoculated. Additionally, the retention site of BYV in the aphid vector has not been determined. The retention site of *Lettuce chlorosis virus* (LCV), a whitefly-transmitted *Crinivirus* (also family *Closteroviridae*) is in the foregut of its whitefly vector (Chen *et al.*, 2011). However, attempts to identify the retention site of BYV in *M. persicae* using the same methodology of Chen *et al.* (2011) have so far been unsuccessful (unpublished). The foregut, cibarium, precibarium and stylet food canal are not in the path of saliva excretion and consequently egestion would be the only feasible behaviour responsible for inoculation if any of those is the retention site. Virions retained in the acrostyle where the food and salivary canals converge at the apex of the stylets potentially could be dislodged and inoculated by either egestion or salivation.

BYV is a phloem-limited virus and the finding that it is inoculated during phloem-pds rather than PSEP differs from all previous EPG studies on inoculation of phloem limited viruses. Inoculation of the aphid-transmitted *Barley yellow dwarf virus* (BYDV, *Luteovirus*), two whitefly-transmitted criniviruses (LCV and ToCV), and one whitefly transmitted geminivirus (TYLCV) were associated primarily with PSEP, specifically in

most cases with PSEP salivation waveform E1 (Prado and Tjallingii, 1994; Johnson *et al.*, 2002; Maluta *et al.*, 2017; Jiang *et al.*, 2000). In these studies, feeding behaviour of viruliferous insects on uninfected plants was monitored by EPG and was terminated artificially by pulling the insect off the plant either before or after the PSEP salivation waveform E1. Successful inoculation was determined by the plants eventually developing infection. Successful inoculation occurred primarily in the treatment where the insects produced PSEP waveform E1, and only a few of those where feeding was terminated prior to PSEP developed infection. However, in these studies, there were no criteria to distinguish pds associated with SE/CC penetration versus penetration of other cells. The present study and Jiménez *et al.* (2018) show that at least in the case of *M. persicae* feeding on sugar beet, waveform E1 was preceded by a single or several phloem-pds, occurring very close in time (24.28 ± 0.48 s) to waveform E1. Therefore, it is very possible that the majority of the treatments in the other studies where feeding was terminated before PSEP not only lacked PSEP but also lacked pds that penetrate the SE/CC complex. Consequently, we are currently examining potential phloem-pds produced by *Rhopalosiphum padi* and their possible role in inoculation of BYDV.

Finally, other studies also have found that some pds prior to PSEP are produced by penetration of sieve elements (Tjallingii and Hogen Esch, 1993; Tjallingii and Gabrys, 1999; Walker and Medina-Ortega, 2012). In the case of *Aphis fabae* feeding on *Vicia faba*, sieve element-penetrating pds could not be distinguished from standard-pds (Tjallingii and Hogen Esch, 1993). However, in the case of r-pds (penetrations of SEs or CCs - Walker and Medina-Ortega, 2012) of *Acyrtosiphon pisum*, *Brevicoryne brassicae*, *Drepanosiphum platanoidis* and *Periphyllus acericola*, SE/CC-penetrating r-pds were easily distinguished from standard-pds (Tjallingii and Gabrys, 1999). Similar to the phloem-pds in the current study, the r-pds always had the same voltage drop magnitude as in the subsequent PSEP, both of which were less than the voltage drop magnitude in the preceding standard-pds. Also, the duration of the subphase II-2 intervals (inverse of intervals/sec in Table 1) in r-pds is distinctly longer than that in standard-pds for *A. pisum* and *D. platanoidis* (Figs. 2 and 5 in Tjallingii and Gabrys 1999; Walker, unpublished data). Furthermore, the phloem-pd produced by *M. persicae* could be the same or similar biological feature as the X-waveforms reported long ago for aphids when using alternating current EPG (AC-EPG). When studying the feeding behaviour of the English Grain aphid *Sitobion avenae*, a series of X-waveforms always preceded the continuous

phloem ingestion phase (Ip waveform) and also resulted in successful inoculation of the phloem-limited and persistently transmitted BYDV (Scheller and Shukle, 1986).

We recently reviewed some previous EPG recordings and have detected characteristic phloem-pds in three more aphid-plant combinations besides *M. persicae/B. vulgaris*: *R. padi/Hordeum vulgare* and *Aphis fabae/Chenopodium album* (unpublished data) and *Aphis gossypii/Cucumis melo* (EPG recordings from Garzo *et al.*, 2018). Consequently, penetration of the SE/CC complex during pds prior to PSEP is likely a common phenomenon for aphids, and has important implications for interpreting EPG data. The EPG variable "time to first phloem sieve element phase" is sometimes interpreted as being affected entirely by plant qualities encountered by the aphid in non-sieve elements. However, the period preceding the first PSEP likely includes earlier penetrations of the SE/CC complex, and thus the time to first PSEP may be influenced by factors in sieve elements in addition to non-sieve element cells. The ability to distinguish SE/CC pds from non-SE/CC pds may provide insights into the location of plant factors that affect successful feeding. The novel results provided in this work add to our understanding of aphid stylet activities into phloem tissues, since first phloem contact occurs in a time slightly earlier than previously thought. The role of the newly described phloem-pd in other aphid-plant-pathogen interactions, such as plant defense responses before the E1 waveform, or their role in the inoculation of persistently transmitted phloem-restricted viruses require further investigations.

CHAPTER 8. GENERAL DISCUSSION

The increase of world population represents a great challenge for the future of agriculture, requiring a major increase in crop production to assure global food security. The present PhD Thesis provides new and key information in the field of mechanisms of transmission of plant viruses as well as their interactions with their insect vectors. We revisited and investigated different aspects regarding virus-plant-aphid interactions that remained unknown or not thoroughly studied. We particularly focused on the transmission of noncirculative (NC) viruses by aphids, and whose infection may cause significant crop losses in key crops such as vegetables, cereals and woody plants.

Our findings indicate that a pre-acquisition fasting period of aphids does not universally favor the transmission of non-persistent (NP) viruses (Chapter 4). Fasted and non-fasted *Aphis gossypii* individuals showed similar ability to acquire, retain and transmit *Cucumber mosaic virus* (CMV, *Cucumovirus*). Previous studies historically reported that a pre-acquisition fasting period of aphids universally favored the acquisition of non-persistent particles by either i) removing inhibitory plant compounds from the aphid cuticle that could interfere in virus retention (Watson, 1938; Wang and Pirone, 1996) or ii) enhancing the probing behaviour of the aphid (Bradley, 1956). The first postulate was supported by the fact that aphids fed on artificial diets did not display the pre-acquisition fasting effect, while the aphids maintained in the plants did (Wang and Pirone, 1996). The second postulate reflected the widely reported influence of fasting on aphid probing behaviour (Bradley, 1952; 1956). Fasted aphids sample and ingest plant sap faster than non-fasted aphids and tend to produce longer and more frequent intracellular punctures (Powell, 1993) and more importantly, produce a longer sub-phase II-3 of the pd which is known to be associated with NP virus acquisition (Collar and Fereres, 1998). Acquisition of NP virus particles is known to occur during the last phase of the potential drop (II-3) and thus ingestion of cell content is presumed to occur (Powell *et al.*, 1995). Despite the lack of a fasting effect on *A. gossypii* transmitting CMV-Fny, we also found that, at least for CMV, the occurrence of a fasting effect is affected by the virus isolate and its interaction with a particular vector species, since fasted *A. gossypii* individuals transmitted significantly better than non-fasted one of the six CMV isolates studied

(CMV-V698). However, when using *Myzus persicae* as the vector species, fasted individuals enhanced CMV-Fny transmission compared to non-fasted. Interestingly, the lack of a pre-acquisition fasting effect in CMV was again confirmed when *A. gossypii* was used as a vector (Chapter 5). Undoubtedly, in case of *M. persicae* and *A. gossypii* transmitting CMV-Fny, the postulates of inhibitory compounds interfering with virus retention and changes in behavioural aspects of the aphids would not explain the results obtained. Physiological or anatomical differences among aphid species that modulate the interaction with virions or helper proteins could explain their ability to retain and transmit NP viruses (Ferreles, 2016). For instance, *Tobacco etch virus* (TEV, *Potyvirus*) transmissions by *M. persicae* and *Myzus ascalonicus* were not similar between aphid species even though the occurrence and duration of pds was similar (Wang *et al.*, 1998). Also, the chemical composition of the inner surface of the maxillary stylets could differ between aphid species (Uzest *et al.*, 2010). Studies performed with *A. gossypii* and *M. persicae* feeding on melon carrying the *Vat* resistance supported differences in aphid saliva composition as only *A. gossypii* triggered hypersensitive response after superficial intracellular stylet punctures (Chen *et al.*, 1997; Villada *et al.*, 2009). Differences in aphid saliva composition or chemical composition of the cuticular lining may explain the differences in CMV transmission between *A. gossypii* and *M. persicae* rather than the presumable physiological differences. The anatomical structure of the common duct is apparently well conserved among aphids as observed for the acrostyle structure in several aphid species (Uzest *et al.*, 2010). However, specific putative receptors for NP viruses could vary between aphid species, as it is the case of CMV. Proteins of family RR-1 [named for the presence of a motif described by Rebers and Riddiford (1988)] present in *M. persicae* cuticle of the common duct were suggested as putative candidates for the interaction with the CP of CMV (Liang and Gao, 2017; Webster *et al.*, 2018), whereas proteins within RR-2 family were suggested as receptors in case of *Acyrtosiphon pisum* (Webster *et al.*, 2017). The putative CMV receptors in *A. gossypii* and likely chemical implications in the virus retention process are unknown and need further investigations.

Attempts to identify virus retention sites and putative virus receptors in the aphid stylets have been paramount recently in the field of plant-virus transmission (Liang and Gao, 2017; Webster *et al.*, 2017; Webster *et al.*, 2018; Deshoux *et al.*, 2018; Fereres and Perry, 2019). However, few works were performed and little information was available at the beginning of the present PhD Thesis (2015). Currently, *Cauliflower mosaic virus* (CaMV,

Caulimovirus) is the only virus known to be retained in the common duct of the aphid maxillary stylets and whose retention sites have been successfully identified within the comma-like structure area called ‘acrostyle’ (Uzest *et al.*, 2010). Therefore, we attempted to elucidate putative retention sites of NC virions in aphid mouthparts by sequential virus acquisition experiments or from a mixed virus-infected plant to determine virus transmission efficiencies and possible competition/interference between NC viruses (Chapter 5). The exposure of aphids to several virus-infected source plants is quite common in nature, since mixed infections are commonly found in host plants rather than a single viral infection (DaPalma *et al.*, 2010). Also, in most of such mixed infections, the viruses are known to interact sometimes synergistically (Palukaitis and García-Arenal, 2003; Wege and Siegmund, 2007; Mascia and Gallitelli, 2014).

Recently, new advances in the identification of peptides of aphid stylets interacting with different NC viruses have been achieved. For instance, in very recent work, the protein *stylin-01* from the RR-1 subfamily (Rebers and Riddiford, 1988) was postulated as a candidate for receptor for the transmission of NC viruses (Webster *et al.*, 2017). In fact, the *in vivo* silencing of *stylin-01* gene through RNA interference was shown to have a negative effect in the CaMV transmission efficiency by *M. persicae* (Webster *et al.*, 2018). The *stylin-01* was actually the same Mpcp4 cuticular protein identified by Dombrovsky *et al.* (2007), interacting with the coat protein of CMV (Liang and Gao, 2017). Such Mpcp4 protein was in fact the same as the *stylin-01* described by Webster *et al.* (2018). However, during our experimental procedure, no interference was observed when *M. persicae* acquired CaMV or P2 from plants or artificial diet, respectively, prior to CMV acquisition, suggesting that CaMV particles are dislodged during subsequent CMV acquisition. In fact, the persistence of CaMV in *M. persicae* has been reported to be much shorter than in other vector species of CaMV such as *B. brassicae* (Moreno *et al.*, 2005). Nevertheless, competition experiments supported the hypothesis that *stylin-01* may act as a receptor for both the P2-CaMV and CMV (Rodrigo Almeida, unpublished data). He found that there was a decrease in CMV transmission when *A. gossypii* individuals fed on an artificial diet containing both CMV virions and the P2-P3-CaMV complex. Therefore, there might be competition between CMV and CaMV for the same retention sites within the aphid stylet's but receptors may have a higher affinity for the retention of P2-CaMV than for CMV explaining why we found no interference in the sequential transmission experiments (Chapter 5). Also, *M. persicae* retains CaMV for

shorter periods of time than its main vector *B. brassicae* (Moreno *et al.*, 2005). Therefore, the lower affinity for the receptors or shorter retention of the CaMV virions to *M. persicae* stylets could lead into free receptors during the subsequent acquisition of CMV. Whether CMV interaction to Mpcp4 (*Stylin-01*) in yeast observed by Liang and Gao (2017) represents a true binding in the aphid stylets needs further investigation since attempts to successfully attach CMV particles to Mpcp4 have failed so far (Webster *et al.*, 2018). However, the literature on the modes of retention of CaMV to its different vectors and the affinity of CMV for the CaMV receptors are in agreement with the results obtained in the competition and interference studies (Chapter 5). Conversely, no competition or interference was observed between CaMV and *Turnip mosaic virus* (TuMV, *Potyvirus*), suggesting that the cuticular proteins (CuPs) involved in TuMV binding to the stylets of *Brevicoryne brassicae* are different to the *stylin-01* reported for CaMV (Webster *et al.*, 2018).

Although great advances have been achieved on the knowledge of CaMV and CMV putative receptors in the aphid mouthparts, the first study to identify cuticular proteins in *M. persicae* binding to NC viral particles was conducted many years ago by Dombrovsky *et al.* (2007) with *Zucchini yellow mosaic virus* (ZYMV, *Potyvirus*). They found that proteins of RR-2 family specifically interacted with the HC-Pro of ZYMV in *M. persicae*. Therefore, ZYMV receptors would be different to those suggested for CMV also in *M. persicae* (*stylin-01*). However, virus transmission efficiencies presented in Figure 5.3 (Chapter 5) show evidences that first acquisition/retention of ZYMV by *A. gossypii* interferes with subsequent acquisition/retention and transmission of CMV. Even though peptide arrays within RR-1 domain were suggested for CMV interaction in *M. persicae* (Liang and Gao, 2017; Webster *et al.*, 2018), peptide arrays of RR-2 protein family showed interaction with CMV binding in *A. pisum* (Webster *et al.*, 2017). CMV actually interacts with both RR-1 proteins in yeast, and with RR-2 peptides *in vitro*, with both playing a role in virus retention (Deshoux *et al.*, 2018). The interference observed in our experiments suggests that both ZYMV and CMV are using same receptors in *A. gossypii* stylets. Whether peptides of RR-1 or RR-2 family proteins are involved in the retention of both viruses needs additional biochemical and functional validation. However, ZYMV transmission was not influenced when the reverse sequential transmission test was conducted (first CMV acquisition/retention and subsequent ZYMV acquisition and transmission), challenging our initial hypothesis of same receptors for ZYMV and CMV

in *A. gossypii* mouthparts. However, CMV binding to the aphid mouthparts is mediated by the CP strategy (Chen and Francki, 1990; Perry and Fereres, 2019), whereas ZYMV binding is mediated by an intermediate bridge molecule, the helper component protein (HC-Pro) (Pirone and Blanc, 1996). Our results suggested that CMV particles were probably released while aphids were subsequently allowed to probe on a ZYMV-infected source. Thus, when CMV is acquired first there is no interference with subsequent acquisition and retention of ZYMV. Overall our results suggest that HC-Pro can remain attached to the stylets even after release of the virions of ZYMV to the plant and that the binding of a *Potyvirus* (or its HC-Pro) to its receptor sites interferes with subsequent retention and transmission of CMV. Thus, we can postulate that HC-Pro-stylet binding is more stable than CMV-stylet binding.

The idea that all viruses transmitted in a NP manner are retained in the acrostyle has been already proposed (Pelletier *et al.*, 2012; Boquel *et al.*, 2013). However, identification of the virus retention sites and CuPs involved in virus retention is a difficult procedure as several factors are influencing the retention process. Virion-labeling studies are sometimes unspecific due to short periods of NP virions retention to the cuticle and also the presence of transient particles in the common duct in areas different from specific retention sites (Ammar *et al.*, 1994). Also, the size of the aphid common duct is a quite small structure composed of a complex network of chitin fibers and heavily cross-linked proteins always in contact with aphid saliva or plant compounds that interfere with virion retention (Webster *et al.*, 2017).

Once we revisited the influence of the pre-acquisition fasting effect in the transmission of CMV (Chapter 4) and later studied the interference of NC viruses for retention sites in the vector (Chapter 5), we continued to investigate one of the main aspects still unknown in the field of NC virus transmission by aphids: the specific aphid stylet activities in the plant associated with the transmission of semipersistently transmitted, phloem-limited viruses (Chapters 6 and 7). Still key information such as virus retention sites in the vector and mechanisms of transmission of several SP, concretely with phloem-limited category remain unknown (Ng and Zhou, 2015; Fereres, 2016; Uzest and Blanc, 2016). Even though several studies have focused on this type of viral transmission, e.g. *Beet yellows virus* (BYV, *Closterovirus*) (Sylvester, 1956; Limburg *et al.*, 1997) and *Citrus tristeza virus* (CTV, *Closterovirus*) (Herron *et al.*, 2006), no studies have focused on the

identification of key aphid behavioural feeding patterns involved in the transmission of this type of viruses.

We described a new EPG pattern, the phloem-pd that plays a key role in the transmission of BYV (Chapter 6). Previous works that focused on the transmission of the persistently transmitted, phloem limited *Barley yellow dwarf virus* (BYDV, *Luteovirus*) also reported the possibility that virus inoculation may occur during aphid activities prior to stylet insertion into sieve elements (Scheller and Shukle, 1986; Prado and Tjallingii, 1994). When using *Rhopalosiphum padi* as a vector, transmission of BYDV was mainly associated to waveforms E1 (inoculation) and E2 (acquisition) but some transmission was also observed in recordings where the aphid did not perform either E1 or E2 (Prado and Tjallingii, 1994). Studies using *Sitobion avenae* as a vector, also showed BYDV transmission prior to phloem ingestion (Ip), specifically during the X-waveform, which consists of repetitive intracellular punctures (Scheller and Shukle, 1986). Using histological techniques, authors demonstrated that *S. avenae*'s stylets were in the phloem during the X-waves. Therefore, brief stylet intracellular punctures, presumably occurring in phloem cells prior to continuous salivation into sieve elements are involved in the transmission of BYDV and also presumably in the transmission of all phloem-limited viruses. Our study represents the first attempt to positively identify the transmission of this type of virus in association with a newly identified and characterized EPG pattern ('phloem-pd').

Other distinctive intracellular punctures were also proposed as aphid stylets intrusions into phloem cells. For instance, the repetitive pds (R-pds) produced by *A. pisum* and *B. brassicae* were suggested to be phloem penetrations due to the similar magnitude of potential drops observed in comparison with the subsequent E1 waveform (Tjallingii and Gabrys, 1999). Even though results from Chapter 6 strongly suggest the intrusion of aphid stylets into phloem cells, the EPG technique was not enough to determine what kind of cells are punctured during the occurrence of a phloem-pd. Therefore, we attempted to identify the type of phloem cells [phloem parenchyma (PPC), companion (CC) or sieve element cell (SE)] punctured by *M. persicae* during the novel 'phloem-pd' pattern (Chapter 7). Cryofixation of aphid stylets during the phloem-pd and later observation by two different microscopic techniques showed that companion cells or sieve element cells were penetrated by *M. persicae* stylets. Although several studies have shown the

occurrence of brief intracellular punctures into phloem cells (Scheller and Shukle, 1986) our work reflected the first attempt to identify the specific phloem cells punctured by the aphids prior to the continuous salivation phase into sieve elements and previously associated to the transmission of SP, phloem-limited virus. Also, the precise phloem cells punctured (PPC, CC, SE) during the X-waveform performed by *S. avenae* in barley were never determined by S&S. Moreover, we have observed the same ‘phloem-pd’ patterns in several other aphid-plant combinations, such as *R. padi*/*Hordeum vulgare*, *Aphis fabae*/*Chenopodium album* (unpublished data) and *Aphis gossypii*/*Cucumis melo* (EPG recordings from Garzo *et al.*, 2018), suggesting that this new type of waveform is a general phenomenon in many aphid-plant interactions.

Although we successfully identified the key EPG patterns associated with BYV transmission (Chapter 6) and determined the type of cells penetrated during this newly EPG pattern (Chapter 7), we could only hypothesize about the mechanisms involved in virus release and delivery from the retention sites. So far, CTV has been the only aphid-vectored *Closterovirus* whose retention sites have been successfully identified; the retention site is in the foregut of its vector *Toxoptera citricida* (Killiny *et al.*, 2016). Also, retention sites of two other SP viruses have been found in the foregut of their specific vector: *Lettuce infectious yellow virus* (LIYV, *Closterovirus*) in the whitefly *Bemisia tabaci* (Chen *et al.*, 2011) and *Maize chlorotic dwarf virus* (MCDV, *Waikavirus*) in the black-faced leafhopper *Graminella nigrifrons* (Wayadande *et al.*, 1993). However, the location of the retention sites of BYV in the vector is still unknown.

Information provided in the present PhD Thesis can be used as the basis for interesting future experimentation that will complete the knowledge of the transmission of NC viruses by aphids. Results from the four chapters do not represent a closed episode within each specific virus-vector interaction studied, but all that information opens a new venue to develop future studies.

Results from Chapter 4 showed that the transmission efficiency of all viruses transmitted in a NP manner are not positively influenced by a fasting period prior to acquisition (e.g. CMV and *A. gossypii*). However, if the transmission of some other viruses transmitted in a NP manner is not enhanced by a pre-acquisition fasted period need to be studied. Also, future studies on the anatomical structure between different aphid species and

identification of proteins present in the cuticle lining could explain the differences in CMV transmission observed by fasted *A. gossypii* and *M. persicae* individuals in our experiments (Chapter 4).

On the other hand, results from Chapters 5 and 6 reflected the great field to be investigated about the localization of retention sites of NC viruses in the vector as well as the specific peptides arrays in the aphid mouthparts involved in their retention. Even though the binding strategies of different viruses transmitted in a NP manner are well known, the specific CuPs involved in the binding are so far known only for CaMV (Webster *et al.*, 2018). A major question that still needs an answer is: where are the retention sites of BYV in *M. persicae*? The answer to this question will be essential towards the identification of the aphid mechanisms involved in the release and delivery of virus particles. If BYV is stylet-borne, then either salivation or egestion of the aphid could be the mechanism involved in virus dislodging from the mouthparts. However, if BYV is retained in the foregut of the vector, same as CTV (Killiny *et al.*, 2016), then only egestion (regurgitation of content previously ingested by the aphids) could be the mechanism involved in the delivery of virus particles. Also, to identify and associate the different activities produced by the aphid to the different subphases of the phloem-pd will be of interest. Another study of interest will be to determine if systemic infection can also occur when the virus is delivered during a phloem-pd in either a companion cell or a sieve element, since the phloem-pd represent stylets penetrations in both type of cells (Chapter 7). The studies proposed above will be key to understand the mechanisms of transmission of SP, phloem-limited viruses by aphids.

Moreover, the characterization of the new phloem-pd pattern produced by *M. persicae* (Chapter 6) will lead to future studies to investigate additional roles of the phloem-pd in aphid-virus-host interactions. For example, these unique intracellular punctures may trigger plant defenses against certain pierce-sucking intruders as the phloem-pd represents the first contact of aphid stylets in the SE/CC complex by *M. persicae* (Chapter 7). The production of the phloem-pd by several other aphid species needs to be investigated as well as their likely role in the transmission of persistently transmitted, phloem-limited viruses (e.g. transmission of luteoviruses by *R. padi*). This will fill knowledge gaps still existing in the transmission of persistent, phloem-limited viruses. In fact, transmission of BYDV was rarely observed prior to waveform E1, however primary

inoculation of BYDV was not associated to any specific brief intracellular puncture (Prado and Tjallingii, 1994). This new information will allow us to expand our knowledge about the transmission mechanisms of non-circulative and circulative phloem-limited viruses by aphids.

The future of agriculture demands new strategies to interfere with the transmission of plant viruses, while also reducing the excessive use of chemical compounds in the field. Several strategies have been developed in order to disrupt the transmission of viruses by their vectors. Due to the specificity of virus transmission by vectors, there are several steps that represent a good target to interfere or disrupt the transmission process (Whitfield and Rotenberg, 2016). This is the case of the VLPs (virus-like proteins), which represent such a novel biotechnological tool to interfere with the spread of plant viruses (González-Gamboa *et al.*, 2017). These VLPs show the same virion-arrays of peptides responsible for the binding to the retention sites in the vector or with the virus helper proteins thus preventing retention of the complete virus particles to the mouthparts. The same mechanism has been described for the recombinant VAP (viral attachment proteins) interfering with viruses transmitted persistently by i) blocking virus binding to the vector and ii) reducing vector population using the viral protein to deliver toxic cargo to the insect (Whitfield and Rotenberg, 2016). Inhibition-based approaches have been conducted by using viral CPs that bind to whitefly guts and prevent *Tomato yellow leaf curl virus* (TYLCV, *Begomovirus*) from circulating through the vector (Wang *et al.*, 2014). Also, the knowledge of *Pea enation mosaic virus* (PEMV, *Enamovirus*) CP binding and movement through the insect gut was used to develop a recombinant CP fused to non-viral toxin peptides that appeared to have aphicidal effects (Bonning *et al.*, 2014).

Another strategy well studied and applied to agriculture is the use of resistant cultivars showing different mechanisms that negatively impact aphid feeding behaviour. Those strategies represent an effective approach to interfere with virus transmission and constitute an environmentally friendly way of combating viral diseases (Stevens and Lacomme, 2018). Several natural characteristics of specific plant varieties could be exploited as host-plant resistance, such as plant colour, palatability, waxiness, mechanical (hardness of plant surface), glandular trichomes, toxins or production of local necrosis ('hypersensitive response') (Hull, 2014; van Emden, 2018). The use of genotypes

resistant to viruses and to the aphid vectors has been paramount in deploying strategies against virus transmission. For instance, the TGR-1551 melon genotype had a negative effect on the feeding behaviour of *A. gossypii* (Garzo *et al.*, 2002). Also, TGR-1551 melon showed total resistance to the transmission of several isolates of CMV and ZYMV by *A. gossypii* (Soria *et al.*, 2003). Moreover, resistant cultivars to CMV and *Papaya ringspot virus* (PRSV, *Potyvirus*) have been incorporated in commercial melon cultivars, as well as potato cultivars resistant to potato viruses (Stevens and Lacomme, 2018).

There are several additional strategies that have been applied in the management of virus diseases in the field. Whereas some are focused in avoiding the entry of virus (elimination of virus sources, weed control eradication of volunteer plants or plant spacing) some others focus on the prevention or reduction of virus spread (e.g. control of vector by chemicals, oil sprays, biological control of vectors or barrier crops) (Stevens and Lacomme, 2018; Chang *et al.*, 2018).

More importantly, aphids and virus-spread dynamics in the future are uncertain within the horizon of an agriculture strongly influenced by future climate, with an increase in the global temperature and as well as in carbon dioxide (CO₂) concentration, which is forecasted to double by the end of this century (Trebecki *et al.*, 2017). Elevated CO₂ and higher temperatures have shown to increase both the virus titer (Nancarrow *et al.*, 2014; Trebecki *et al.*, 2015), the transmission efficiency by their vectors (Anhalt and Almeida, 2008) and natural virus incidence in the field (Trebecki *et al.*, 2017).

The future agriculture will demand even more the employment of biotechnological tools and the use of resistant varieties that interfere with aphid vectors and virus spread, contributing to a sustainable agriculture respectful with the environment. Results from the present PhD Thesis provide insights into factors influencing the transmission of non-circulative viruses as well as their interactions with the receptors present in the cuticle of the aphid mouthparts. All that knowledge will be of interest in the new lines of research focused on the development of innovative strategies against plant virus transmission by aphids.

CONCLUSIONS

1. A pre-acquisition fasting period altered the probing behaviour of *Myzus persicae* Sulzer and *Aphis gossypii* Glover, encouraging the aphid activities in the plant reported to enhance the acquisition of viruses transmitted in a non-persistent manner.
2. The transmission of *Cucumber mosaic virus* (CMV, *Cucumovirus*) was not enhanced by fasted *A. gossypii* individuals in comparison to non-fasted, proving that the pre-acquisition fasting effect is not universally promoting the transmission of all viruses transmitted in non-persistent manner by aphids.
3. The lack of competition or interference between first acquisition of *Cauliflower mosaic virus* (CaMV, *Caulimovirus*) and subsequent acquisition of *Turnip mosaic virus* (TuMV, *Potyvirus*) indicated that both virus species do not share the same receptors in the cuticle of their vector *Brevicoryne brassicae* L.
4. The transmission efficiency of CMV was not reduced after first acquisition of CaMV, suggesting that both virus species do not share the same receptors in the vector *M. persicae*.
5. First acquisition of *Zucchini yellow mosaic virus* (ZYMV, *Potyvirus*) interferes with subsequent acquisition and transmission of CMV. This suggests that both viruses bind to the same receptors in the cuticle of its vector *A. gossypii*. Consequently, receptors of potyviruses and cucumoviruses seem to be different to those of CaMV.
6. A new intracellular puncture pattern produced by *M. persicae* -termed phloem-pd- different from the standard pd was characterized and associated with the inoculation of BYV, a phloem-limited virus transmitted in a semipersistent manner.

7. Microscopic studies revealed that the phloem-pd in fact represents penetrations of *M. persicae* stylet tips into the phloem tissues, specifically in companion cells or sieve element cells.

General conclusion:

The transmission of NC viruses is a complex process, influenced by the vector species and virus isolate. Besides, certain NC virus particles do interfere for their binding during the acquisition process by the vector, proving their specificity for the receptors in the aphid cuticle. Also, the finding of a newly described brief intracellular puncture produced by aphids in phloem tissues and associated to the inoculation of semipersistently transmitted, phloem-limited viruses will contribute to studies concerning persistently transmitted, phloem-limited viruses as well as aphid-host plant interactions. Such knowledge could help to develop strategies to interfere or block virus retention and transmission.

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