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# THÈSE présentée par :


## Souheyla KHECHMAR


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# Impact de la multi-infection de la betterave sucrière sur la transmission des virus de plante par puceron

**THÈSE dirigée par :**

| | |
|---|---|
| **Mme. BRAULT Véronique** | Directrice de recherche, INRAE Colmar |

**Codirigée par :**

| | |
|---|---|
| **M. DRUCKER Martin** | Directeur de recherche, INRAE Colmar |

**RAPPORTEURS :**

| | |
|---|---|
| **Mme. GERMAN-RETANA Sylvie** | Directrice de recherche, INRAE Villenave d'Ornon |
| **M. AMELINE Arnaud** | Professor, Université de Picardie Jules Verne Amiens |

**AUTRES MEMBRES DU JURY :**

| | |
|---|---|
| **M. HEITZ Thierry** | Directeur de recherche, IBMP-CNRS Strasbourg |
| **Mme. MARZACHI Cristina** | Directrice de recherche, CNR-IPSP Turin |

**“L’enseignement et la connaissance sont importants parce qu’ils définissent ce qui, à travers les siècles, a fait de nous des humains, et non parce qu’ils peuvent améliorer notre compétitive mondiale”**

Drew Gilpin Faust, présidente de l’Université de Harvard 2007-2018

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Finally, I would like to dedicate this PhD thesis to my nephew Hayou.

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# List of frequent abbreviations

BChV: beet chlorosis virus

BMYV: beet mild yellows virus

BtMV: beet mosaic virus

BWYV: beet western yellows virus

BYV: beet yellowing virus

CP: capsid protein

CPm: minor capsid protein

DAS-ELISA: double antibody sandwich - enzyme linked immuno sorbent assay

EPG: electrical penetration graph

HC-Pro: helper component proteinase

HsP70h: heat shock protein

Kb: kilo base

kDa: kilodalton

MP: movement protein

ORF: open reading frame

PD: plasmodesmata

QPCR : quantitative polymerase chain reaction

RT: readthrough protein

SABER: signal Amplification by exchange reaction

SEL: size exclusion limit

UTRs: untranslated regions

VY: virus yellows

# Introduction

## What is multi-infection?

Multi-infection with pathogens, which includes co-, super- or mixed infection, refers to the co-existence of more than one biological disease agent in one host. This phenomenon is found in both eukaryotes (including humans, animals, plants, fungi) and prokaryotes (bacteria, archea), where individuals can be infected with different pathogens such as bacteria, fungi, viruses or parasites **(Vigasova et al., 2021)**. Multiple infection is of particular importance since it can change the impact that a pathogen will have on a given host and on the co-infecting pathogens. Within singly infected hosts, virulence (i.e., pathogen's capacity to induce disease damage) is the outcome of the struggle between one host and one pathogen. Multiple infection means, in the first place, that the conflict is not only between host and pathogens but between the different pathogens as well **(Van Baalen & Sabelis, 1995)**. The infecting pathogens can interact with each other directly or indirectly, through the host's resources or immune system **(Dutt et al., 2022; Griffiths et al., 2011)**. Multi-infection is rather the rule than the exception and it can have huge consequences on disease outcome including aggravation, alteration, or reduction of disease symptoms **(Devi et al., 2021)**. Determining the biological mechanisms that are affected by co-infection and their consequences is important, in particular to improve the management strategies in plants, animals and humans. In this introduction, I will cover the topic of multi-infection in different hosts (eukaryotes and prokaryotes), and also discuss the different interactions underlying multi-infections and the impact on the host system

## Prokaryote multi-infection

For the prokaryotes, multi-infections are common, but are restricted to viruses. The two domains of prokaryotes, bacteria and archea, are susceptible to multi-infection by different viruses **(López-Leal et al., 2022)**. Archea can be infected by highly diverse viruses that exhibit different morphotypes **(Baquero et al., 2020)**. Information about viral multi-infection of archea is limited, compared to the more extensively studied multi-infection of bacteria by bacteriophages. Data-mining showed that most pathogenic and non-pathogenic bacteria in the biosphere seemed to be co-infected, because a single bacterial cell often harbors more than one lysogenic phage (prophage) integrated within its chromosome **(López-Leal et al., 2022)**. Indeed, phage DNA may constitute 20% of bacterial genomes (**Nepal et al., 2022; X. Wang et**

**al., 2010)**. Related and unrelated prophages can switch to lytic phase simultaneously, a phenomenon called polylysogeny, with important consequences for both bacteria and phages **(Azulay et al., 2022; Chevallereau et al., 2022; Davies et al., 2016; Silpe et al., 2023)**. Phages can also be beneficial for bacteria, by increasing their pathogenic potential or allowing genetic exchange via transduction **(Silpe et al., 2023)**. Several factors contribute to the effects of multi-infection such as the host environment that may favor the co-existence of different bacterial species, the possibility of horizontal gene transfer and the diverse microbial communities where the bacteria exist in complex communities, such as biofilms and microbiomes. Only a few bacteria-phages interactions have been well characterized. For the vast majority, the impact of individual phages, or multiple phages on bacterial ecosystems remains largely elusive **(Burns et al., 2015; Dougherty et al., 2023)**. For the cases where they have been reported, the outcome of their interactions varies. In some cases, prophages increase fitness and confer benefits to the host bacterium, for example by playing a major role in the ecology and evolution of pathogenic bacteria through magnifying the competitiveness, which may impact the severity of infectious diseases caused by bacteria **(Chevallereau et al., 2022)**. A well-described example is the case of the aggressive pathogen Liverpool epidemic strain (LES) of *Pseudomonas aeruginosa* that causes deadly chronic respiratory infections in individuals with cystic fibrosis (CF). Here co-infection of the bacterium with two phages confers a large fitness advantage over co-infecting bacteria carrying only one or no phage that are more susceptible to the phages, enhancing the competitiveness of the LES *in vivo* **(Burns et al., 2015; Davies et al., 2016)**. This raises the hypothesis that phages may have a crucial role in LES chronic lung infection **(Burns et al., 2015)**. Some other possible benefits conferred by multiple phage infection is the regulation of bacterial populations in various environments. It was shown that prophages can be beneficial for resisting to oxidative, osmotic and acidic stresses, for enhancing bacteria growth and promoting biofilm formation, which can enhance the pathogens' resistance to antibiotics and immune defenses. For instance, prophage CPS-53 proteins YfdK, YfdO and YfdS increased resistance to oxidative stress, prophages e14, CPS-53 and CP4-57 together in bacteria enhanced resistance to acid, and proteins from prophage e14 and rac increased early biofilm formation **(X. Wang et al., 2010)**. Additionally, the existence of prophages in bacteria can be accompanied with release of toxins that confer virulence potential to the host bacteria and can cause deadly outbreaks. For example, prophages have been found to encode toxins in *Corynebacterium diphtheriae* (diphtheria toxin), *Clostridium botulinum* (botulinum toxin), *Vibrio cholera* (cholera toxin), *Escherichia coli* O157:H7 (Shiga toxin), and *Salmonella enterica* (SopE effector protein) **(Nepal et al., 2022)**. Apart from toxins, prophages can also

harbor genes that encode multiple functions such as auxiliary metabolic genes, virulence factors, antimicrobial resistance genes and immune evasion genes, which are often present in genome clusters **(Nepal et al., 2022)**.

## Eukaryote multi-infection

### A. What kinds of eukaryote multi-infections are there?

In nature, eukaryotic organisms, including humans, animals, plants, and fungi can also be susceptible to infection with agents from more than one pathogenic microbial phyla **(Fig. 1)**, each with its own unique characteristics and effects on the host.

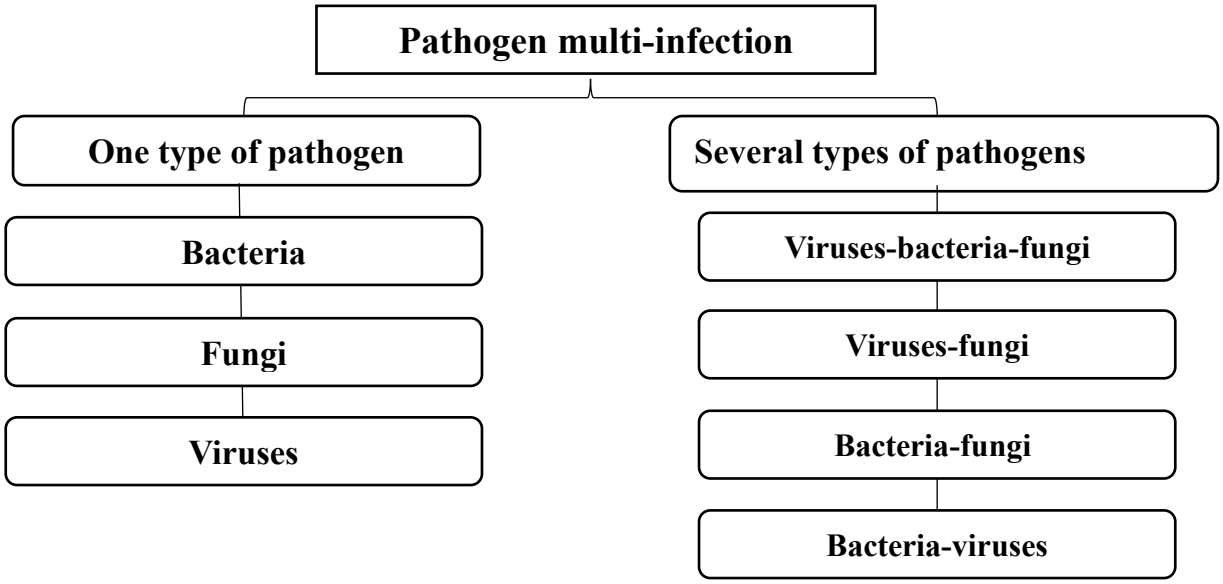



**Figure 1.** Types of pathogen multi-infections in eukaryotes.

1. Bacterium-virus and fungus-virus multi-infections

When multiple pathogens, such as viruses, bacteria and fungi, infect the same host, they can have a beneficial effect on each other. A pathogen can substantially help another pathogen by disabling cellular immune defenses (reviewed by **(Roe, 2022**) for human pathogens). Mixed-infections with bacteria and viruses, or fungi, are commonly identified in viral respiratory tract infections in animals and humans and this is often associated with greater severity of illness

and increased mortality. **(Bakaletz, 2017; Langford et al., 2020; Liu et al., 2021)**. For example, the "Porcine Respiratory Disease Complex" (PRDC) is due to a combination of infectious agents (viruses and bacteria) increasing the severity of the disease and leading to higher mortality in most cases **(Saade et al., 2020)**. There is also evidence that viral infection of the respiratory tract predisposes patients to secondary bacterial infection **(Bakaletz, 2017)**. Viruses can damage epithelial cells which is one of the causes leading to secondary infection. This was shown in the case of the primary influenza or RSV infections that induce epithelium damage and leads to a higher susceptibility to *Staphylococcus aureus* or *S. pneumoniae* in animal models. RSV and influenza viruses alter tight junctions resulting in loosened epithelial cells. This effect can be explained by the viruses targeting directly or indirectly the proteins involved in the formation of tight junctions, such as claudin, occludin, or ZO-1 **(Oliva & Terrier, 2021)**. Viruses can also induce modifications of airway function by increasing mucociliary clearance and enhancing bacterial adhesion in respiratory cells. As an example, RSV infection increases the adhesion and the virulence of *S. pneumoniae* on epithelial cells through direct binding of G glycoprotein to bacterial components. The G glycoprotein anchored at the cell membrane after infection acts as a bacterial receptor **(Oliva & Terrier, 2021)**. In addition to physiological mechanisms, another study demonstrated that virus infection reduced host defense that resulted in bacterial super-infection **(Paget & Trottein, 2019)**.

A range of viruses and bacteria (as well as parasites) can infect the human alimentary canal. Ten different bacterial groups, among them *E. coli, Shigella spp., Salmonella spp., Vibrio spp*. are associated with nine main virus groups (rotaviruses, Norwalk-like viruses, adenoviruses, astroviruses, Sapporo-like viruses, toroviruses, coronaviruses, picornavirus and herpesviruses) and have been identified in mixed infections in human gastroenteritis or have been used to study such mixed infections **(Marshall, 2002)**.

Heterogeneous mixed infection with pathogens from different phyla also widely exists in cultivated and wild plants, and can have significant impacts on plant health. However, there are only a few reports in the literature of plant disease complexes involving association of more than one pathogenic microbial phyla. As an example, we can cite brown apical necrosis of walnut fruit that is caused by mixed infection of different plant pathogenic fungi (*Cladosporium, Fusarium, Colletotrichum, Phomopsis* and *Alternaria*) and a bacterium, *Xanthomonas arboricola* **(Belisario et al., 2002)**. Another example is root rot disease complex of *Panax notoginseng* where a large number of plant pathogenic fungi (*Alternaria panax, A. tenuis, Cylindrocarpon destructans, C. didynum, F. solani, F. oxysporum, Phytophthora*

*cactorum, Phoma herbarum, and Rhizoctonia solani*) and bacteria (*Pseudomonas sp*. and *Ralstonia sp*.) was found **(Miao et al., 2006)**. The mechanisms of interaction that result in synergism of pathogens in these complex diseases is not clear in the above-described examples. Another, better studied example is the co-infection with two pathogens that alone cause the two major rice diseases in Africa: the rice yellow mottle virus (RYMV) and the bacterium *Xanthomonas oryzae pathovar oryzicola* **(Tollenaere et al., 2017)**. The outcome of the virus-bacterium interaction resulted in the alteration of the host phenotype by increasing bacterial-induced symptoms. This was moreover accompanied by a decrease of virus titer compared to mono-infection, and the authors found evidence for involvement of gene silencing in mediating these interactions **(Tollenaere et al., 2017)**. In another virus-bacterium pathosystem, cauliflower mosaic virus and *Pseudomonas syringae,* the increase of bacterial growth and symptoms was a result of suppression of oxidative burst and salicylic acid-dependent autophagy pathways caused by CaMV protein P6 in co-infected plants **(Zvereva et al., 2016)**. Further research is however needed to elucidate the specific effects of mixed infections involving bacteria, fungi, or viruses in plants.

### *2. Bacterium–bacterium multi-infection*

Multi-infection with different bacteria is common in various hosts and results in complex interactions that alter disease outcome and transmission dynamics. In plants, several important diseases of agricultural relevant crops are caused by co-infection with at least two bacterial species or strains where the damage caused by co-infection is often much more severe than the single infections with either bacterium. This is the case of the complex disease tomato pith necrosis that is caused by up to eight different pathogenic bacterial species: *Pseudomonas cichorii*, *P. corrugata*, *P. viridiflava*, *P. mediterranea*, *P. fluorescens*, *P. atrosepticum, P. carotovorum* and *Dickeya chrysanthemi*. Here, co-infection with two or more bacteria can drastically increase severity of the disease compared to mono-infection **(Lamichhane & Venturi, 2015)**. This is also true for *P. corrugata–P. marginalis,* or *P. corrugata–P mediterranea* co-infection **(Moura et al., 2005; Saygili et al., 2008)**. Another example, broccoli head rot disease, is caused by synergistic interactions between four different pathogenic bacteria, namely *Pectobacterium carotovorum, P. marginalis, P. fluorescens,* and *P. viridiflava* **(Canaday et al., 1991).** The mechanisms behind these synergistic interactions among the different bacterial species are currently unknown.

### *3. Fungus–fungus multi-infection*

Mixed infection involving fungal pathogens is also a common phenomenon in animals and plants, and again, in many cases, mixed fungal infections result in enhanced disease symptoms. Interaction between co-infecting fungal pathogens varies from competition, cooperation to coexistence **(Abdullah et al., 2017; Dutt et al., 2022)**. Synergistic interactions in co-infections inducing decline in plant vigor, while the corresponding mono-infections were less severe, have been studied in some depth. The young grapevine decline disease (YVD) is a good example of the consequences of mixed fungal infection resulting in decline and death of grapevine within few years after planting. It is caused by single or multiple fungal pathogens belonging to different species: *Ilyonectris* sp., *Phaeomoniella chlamydospora*, *Togninia* sp., and *Botryosphaeriaceae* sp. **(Mugnai et al., 1999; Whitelaw-Weckert et al., 2013)**. For co-infection of wheat with different strains of the fungus *Zymoseptoria tritici*, hyphae of the various strains co-existed in the apoplast space, resulting in competition for host resources and reduced growth rates. This resulted, depending on the strain and the wheat genotype, in decreased transmission, as scored by decreased pycnidia production. However, no obvious link between transmission and virulence was observed and the outcome of mixed infections was also dependent on infection stage **(Barrett et al., 2021)**.

### *4. Virus-virus multi-infection*

Viruses are obligate parasites that must replicate inside the cells of their host. They can modify the host cellular environment through different mechanisms that modulate the function of the host cell and promote the invasion and replication of the virus. Viruses are genetically the most diverse pathogens infecting all phyla including plants, animals and humans **(Mehetre et al., 2021)**. Viral genomes are of different nucleic acid nature: single strand, double strand, linear, circular, minus sense, plus sense, ambisense, DNA or RNA. This genomic material is surrounded by a protective proteic coat and, for many, especially animal viruses, a lipid envelope **(Gelderblom, 1996)**. Single-stranded RNA (ssRNA) viruses are the most important group infecting eukaryotes and are known to have a rapid evolution rate compared to many DNA viruses, allowing them to adapt quickly to changes in their hosts. They are part of a group of pathogens responsible for emerging and reemerging diseases in plants and animals. Most disease-causing viruses of plants and many viruses of animals depend on vectors for spread and survival (so-called vector-borne viruses) (S. M. Gray & Banerjee, 1999). For humans, these include Dengue, Chikungunya, Japanese encephalitis, Zika, and yellow fever viruses, leading

to millions of infections worldwide **(Weaver et al., 2018)**. Vectors can carry at the same time different viruses that may interact with each other in the vector. Studies on hosts infected by several viruses have shown that co-infection can affect viral traits such as accumulation, symptoms and transmission, either by direct virus-virus interaction or indirectly by competition for host resources or by tampering host pathways to the profit of one or both viruses. Therefore, multi-infection may have a profound effect on viral evolution, diversity, propagation and pathogenicity. Like for the other pathogens described in the previous sections, there can be multi-infections by closely related or unrelated viruses, with consequences that will be described in the next sections **(Leggewie et al., 2023; Lin et al., 2023)**.

### *a) Multi-infection with closely related viruses*

Closely related viruses refer to virus species that share a high degree of genetic similarity and structure, and sometimes cause the same disease. Closely related viruses are formed through a process over time known as divergent virus evolution, where viruses undergo genetic variation through mutations, which are irrespective of co-infection (i.e., mutations are introduced into virus genomes no matter whether it is in a co-infection or mono-infection context). On the other hand, evolution through genetic exchange (reassortment and recombination), relies on co-infection **(LaTourrette & Garcia-Ruiz, 2022; Momtazmanesh & Rezaei, 2022; Pérez-Losada et al., 2015)**. These genetic exchanges can also increase the fitness of viruses by repairing defective viral genomes or efficiently removing deleterious mutations caused by error-prone replication. The recombination frequencies vary extensively among viruses. In co-infected hosts, genetic exchange by reassortment or recombination occurs usually if the two viruses infect and replicate within the same cell and when both viruses exhibit molecular compatibility (McDonald et al., 2016a; H. Wang et al., 2022). Studies have identified instances of recombination and reassortment of viruses in humans, animals and plants. RNA recombination is explained by copy-choice model where the viral RNA polymerase (rdrp) switches templates during synthesis of the nascent strand. Genome reassortment between viruses is a form of genetic rearrangement exclusively seen in viruses with segmented genomes, regardless of whether they are single- or double-stranded. Genome reassortment occurs by exchange of genome segments, giving rise to new segment combinations that are encapsidated in one virion. It has been documented in families with 2 (*Arenaviridae* and *Birnaviridae*), 3 (*Bunyaviridae*), 6-8 (*Orthomyxoviridae*), or 10-12 (*Reoviridae*) genome segments (McDonald et al., 2016a). A special case of segmented viruses are multipartite viruses where each segment is encapsidated in a proper particle and reassortment is achieved by shuffling the various

virions. This can have as consequence that co-infection of cells is not required for reassortment because virions from different cells can contribute to formation of reassorted genomes, as observed for nanoviruses **(Sicard et al., 2019)**. Segments exchange may result in the production of more virulent reassortants as in the case of bluetongue virus strains **(Nomikou et al., 2015)**. Another example where reassortment changes virulence and host specificity significantly, is influenza A virus. For instance, the 2009 H1N1 influenza pandemic was the result of consecutive reassortment events involving human, swine, and avian influenza A viruses (McDonald et al., 2016a; Pérez-Losada et al., 2015).

Also DNA viruses are subject to reassortment and recombination, but the mechanisms of recombination may be different from RNA viruses **(D. P. Martin et al., 2011; Pérez-Losada et al., 2015; Weller & Sawitzke, 2014)**. Recombination rates are high in some dsDNA and ssDNA viruses **(Pérez-Losada et al., 2015)**, and emergence of recombinants can have agricultural and medical significance. Well-known examples are recombinant geminiviruses **(García-Andrés et al., 2007)**, herpes simplex virus and phage λ **(Weller & Sawitzke, 2014)**. An example of a plant dsDNA virus with a high recombination rate is cauliflower mosaic virus. In this particular case, the recombination sites can be equally distributed along the genome and might arise during reverse transcription of the genome **(Froissart et al., 2005)**. For sugar beet-infecting viruses, recombination between the different poleroviruses has been shown, but its impact on emergence of new viruses remains unclear **(Kozlowska-Makulska et al., 2015)**. Recombination in polerovirus co-infection concerns often large fragments and could be a key driver in emergence/generation of new virus species **(Boulila, 2011)**. Interestingly, recombination was evoked as the mechanism for vector switch of an emerging polerovirus, pepper whitefly-borne vein yellows virus, that is whitefly-transmitted, contrarily to all other species of the genus that are aphid-transmitted (Ghosh et al., 2019a).

### *b) Multi-infection with unrelated viruses*

Multi-infection with unrelated viruses refers to multi-infection involving viruses that do not share similarity of their genetic structure. However, it is possible that they share similarities in the function of structural or non-structural proteins or domains, which can result in virus-virus interactions. As for closely related viruses, interactions between two unrelated viruses in multi-infection can affect disease progression to varying degrees. Co-infections involving viruses belonging to different genera (*Rotavirus*, *Norovirus*, *Astrovirus*, *Adenovirus*, and *Enterovirus*) cause acute gastroenteritis, are frequent (20-50% of cases), and can exacerbate disease

**(Makimaa et al., 2020)**. Mixed infection between non-related viruses increasing the severity of symptoms has been reported in some crop plants. For example, maize chlorotic mottle virus (MCMV, genus *Machlomovirus*) in co-infection with wheat streak mosaic virus (WSMV, genus *Tritimovirus*) causes corn lethal necrosis. The interactions between these two viruses result in a significant increase (up to >10-fold) of the MCMV concentration in plants **(Scheets, 1998)**. In addition, WSMV infection is considerably enhanced by the presence of MCMV both in terms of infection rates and intensity, which is rather unusual, because in most co-infections involving a member of the *Potyviridae* family the latter does not profit. Another example is blackberry yellow vein disease (BYVD) complex which is caused by the cooperation between different viral species **(R. R. Martin et al., 2013)**. More specifically, tobacco ringspot virus (a nepovirus), raspberry bush dwarf virus (an idaeovirus), and blackberry yellow vein-associated virus (a crinivirus) are involved. No disease symptom was caused by each of the virus in single infections while symptoms clearly become visible in mixed infections. The BYVD disease severity correlated with accumulation of the viruses present in the plant. However, there was evidence that the titre of different virus species infecting the plants was likely to be more important than their type.

### *c) Timely aspects of multi-infection: Virus-virus co-infection vs super-infection:*

The outcome of multi-infections can be greatly influenced by the sequence of infection. Does virus A infect a cell or host before virus B or vice-versa, or do they infect it simultaneously?

#### (1) Virus-virus super-infection

Viral super-infection occurs when a host previously infected by one virus becomes subsequently over-infected with another virus. This occurs in many cases. However, in nature, several viruses evolved different mechanisms to prevent super-infection. This is called super-infection exclusion (SIE; also called super-infection inhibition, resistance or homologous interference). SIE inhibits subsequent infection by a second virus within the same cell or a distant cell of the same tissue or organism **(Syller & Grupa, 2016)**. The second virus can be either a closely related virus (homologous SIE) or even a totally different virus species (heterologous SIE). This phenomenon can be an active virus-controlled function that protects the virus from related competing secondary virus. It helps maintaining the stability of the viral sequences and counteracts creation of new variants by recombination and reassortment. SIE of a challenger virus is associated with multiple mechanisms acting at different levels of the virus

life cycle, including interference with the entry of the challenging virus, competition between the primary and secondary viruses for host resources, inhibition of capsid uncoating, inhibition of translation and replication of the challenging virus, and (especially for plant and insect viruses) induction of RNA silencing by the primary virus that leads to degradation of the challenging virus RNA. The first virus can induce multiple defense mechanisms that are sometimes complementary and interdependent and, in some cases, act independently. Although SIE has been observed for many animal and plants viruses, the molecular bases remain in many cases a mystery.

### (2) Virus-virus co-infection

Viral co-infection pertains to the simultaneous infection of a host by multiple virus species. This can result, at the cellular level, in individual cells being infected only by one or the other virus, or being simultaneously co-infected with both viruses. The last possibility consists of cells initially infected by a single virus being super-infected with another virus later on **(H. Wu et al., 2024)**. Co-infection can be studied at the organism level or at the cellular level, and spatial as well as timely aspects can be important for co-infection. For instance, in the case of influenza A virus infection, it was found that SIE is operational when two spatially independent infection foci meet but not within infection foci in which neighboring cells become co-infected. **(B. E. Martin et al., 2020; Sims et al., 2023)**. As indicated above, co-infection of cells can impact production of viral progeny and generate new viral genotypes with different fitness by recombination or reassortment between different viruses.

## Highlight on plant virus multi-infection

### B. What effect can multi-infections have on host, vector and co-infecting viruses?

Also for plants, viral multi-infections are very common in nature and can lead to diverse outcomes. As mentioned in the previous section, the outcome of any co-infection or super-infection can be affected by the intra-host interactions between the different infectious agents as well as their interactions within the host and the vector **(Dutt et al., 2022)**. Virus-virus interactions in animal as well as in plant hosts can be synergistic, i.e. the presence of one virus may facilitate or aggravate the infections by other viruses, neutral, i.e. the presence of one virus does not affect infections by other viruses, or antagonistic, i.e. the presence of one virus may

inhibit the infections by other viruses **(Abdullah et al., 2017; Dutt et al., 2022)**. The most studied mixed viral infections are synergistic interactions **(Mukasa et al., 2006; Singhal et al., 2021)**. Virus-virus interactions and virus-plant interactions in multi-infection can alter the fitness of co-existing viruses by changing virus replication, accumulation (i.e. boost or impede accumulation of one or both viruses), movement or tissue tropism in the plant. Interference with plant defenses, in particular with post-transcriptional gene silencing has been suggested **(Moreno & López-Moya, 2020)**. Multi-infections can influence plant phenotype (symptoms severity) including development, chemical and physiological profile of the infected plants. Besides effects on plant fitness parameters (biomass, survival, seed production etc.), multi-infection can influence vectors in ways that can affect virus acquisition and transmission **(McLaughlin et al., 2022)**.

### *1. Synergistic interactions*

Synergistic interactions often manifest as increase of virus titer of at least one virus in co-infected plants. Often, viral suppression of the RNA-silencing plant defense mechanism by one virus results, amongst others, in increased replication of the other virus. For example, in the co-infection of the RNA cucumovirus cucumber mosaic virus (CMV) and the DNA begomovirus abutilon mosaic virus (AbMV), AbMV benefits from the viral interplay and replicates better in co-infected *Nicotiana benthamiana* and tomato, which results in increased symptom development in co-infected plants. The beneficial effect on AbMV accumulation was shown to be linked to the activity of CMV protein 2b as an RNA-silencing suppressor. In addition to a higher titer, the phloem-restriction of AbMV was alleviated in co-infected plants and the virus was able to infect non-phloem tissue **(Wege & Siegmund, 2007)**. Another example for RNA silencing suppression-based synergism is the interaction between a potyvirus, turnip mosaic virus (TuMV), and a crinivirus, lettuce infectious yellows virus (LIYV). In this specific combination, the titer of the crinivirus increased in double-infected wild type plants and in LIYV-infected transgenic plants and protoplasts expressing the RNA silencing suppressor P1/HC-Pro of TuMV. Taken together, these data suggest that the potyvirus-mediated RNA silencing suppression is one of the molecular causes that increases accumulation of LIYV in co-infected plants **(J. Wang et al., 2009)**. In most examples of co-infections with a potyvirus, the potyvirus has synergistic effects on the co-infecting virus, but does not profit from it. Often P1/HC-Pro was shown to be involved in the interaction likely because of its strong RNA silencing suppressor activity (Pruss et al., 1997; Savenkov & Valkonen, 2001a). Other silencing

suppressors have been shown to be involved in synergistic virus-virus interactions. For example, the mechanism of synergistic interaction among the sweet potato feathery mottle virus (SPCSV, genus *Crinivirus*) and sweet potato chlorotic stunt virus (SPFMV, genus *Potyvirus*) has been investigated and shown to be mediated by the SPCSV encoded RNase3 protein which functions as suppressor of RNA silencing **(Cuellar et al., 2009)**. Interestingly, this is one of the rare cases where a potyvirus profits from co-infection. Although the exact mechanism has not been clearly elucidated, it was suggested that it may target a specific host component via interference with small-RNA biogenesis **(Cuellar et al., 2015)**. Synergisms can also affect other parameters of the virus cycle, more difficult to address than the easily measured increased viral load **(Moreno & López-Moya, 2020)**. For instance, in soybean (*Glycine max*) exhibiting extreme resistance against soybean mosaic virus due to the R protein Rsv3, it was demonstrated that in double infection by bean pod mottle virus (BPMV) and soybean mosaic virus, viral synergism suppressed the Rsv3-mediated resistance by tampering with defense pathways downstream of Rsv3 **(Alazem et al., 2023)**.

### *2. Antagonistic interactions*

Contrary to synergism, antagonism is a form of interaction where only one of the viruses is likely to be the beneficiary, and its existence and activity lowers the fitness of the second virus. There are fewer examples for antagonistic than for synergistic interactions. This is expected because attenuation of virus fitness instead of exacerbation is likely to remain unnoticed. Antagonism in virus-virus interactions have been described mainly for closely related viruses. A well-known type of antagonism is cross protection **(Ziebell & Carr, 2010)** or otherwise referred to as homologous interference or super-infection exclusion (SIE, see also previous section). This type of interaction occurs when a primary infection with one virus prevents or interferes with subsequent infection with a homologous second virus. Super-infection exclusion between viruses has been proposed to be caused by induction of RNA silencing, which is a major antiviral defense mechanism in plants. While this is true for some virus-plant pathosystems **(Ratcliff et al., 1999)**, there is lots of evidence that other mechanisms cause cross protection as well. For example, there is no direct evidence that mutations specifically disrupting RNA silencing affect SIE in some viruses such as citrus tristeza virus (CTV) and turnip crinkle virus (TCV) **(Folimonova, 2012; Zhang et al., 2018)**. For the latter virus, inhibition of replication of super-infecting TCV by the viral replication-associated protein p28

has been proposed as the principal mechanism **(Zhang et al., 2018)**. For pepino mosaic virus (PepMV) and CTV, it was shown that cross-protection occurs only between variants belonging to the same strain but not when they belong to different strains **(Agüero et al., 2018; Folimonova et al., 2010)**. However, antagonism can also occur between non-related viruses. For instance, in the case of tomato plants infected with tomato torrado virus (ToTV) and PepMV, the severity of ToTV-induced symptoms was unaffected in co-infected plants but the titer of ToTV slightly increased in the early phase of the infection, whereas PepMV accumulation was reduced at all time points, including a pronounced decrease at later times **(Gómez et al., 2010)**. Another example where antagonism affect virus titers is co-infection of *Nicotiana benthamiana* plants with two tobamoviruses, hibiscus latent Singapore virus (HLSV) and tobacco mosaic virus (TMV). HLSV level was reduced in co-infected plants compared to mono-infection, whereas TMV levels remained unaltered **(Z. Chen et al., 2012)**.

### C. Effect of multi-infection on virus vector transmission

Most plant viruses rely on vectors for their transmission and spread. The largest and the most versatile vectors are hemipterans such as whiteflies and aphids, responsible for the transmission of nearly 50% of the plant virus species described to date **(Bragard et al., 2013; Ng & Perry, 2004)**. Virus transmission by vectors consists of at least three stepwise processes, which starts with (i) vector landing and taking up the virus on an infected plant (acquisition), (ii) retention of acquired virions within the vector, followed by (iii) dispersion of the viruliferous vector towards non-infected plants for virus inoculation **(Ng & Perry, 2004)**. Plant viruses can directly or indirectly influence vector physiology and behaviour to increase their chances of efficient transmission **(Gutiérrez et al., 2013)**. This is achieved through changing foliar symptoms and surface properties, as well as the chemical and physiological profile of the infected plants. Virus infection can also modify the metabolite composition of the plant (primary and secondary metabolites) and affect emission of volatile organic compounds (VOCs). Because vectors use color, VOCs, metabolite composition as cues to locate and accept plants, modification of these traits will affect vector preferences **(K. E. Mauck et al., 2016)** and consequently transmission efficiency.

Virus multi-infection of plants will induce different changes in plants, compared to mono-infected plants with consequences on vector preference and feeding behavior, with potential

subsequent effects on virus transmission. One example is mixed infection with a begomovirus and a crinivirus, which lowered crinivirus but not begomovirus accumulation in plants and in whitefly vectors **(Gautam et al., 2020)**. Another example showed that plants co-infected with a potyvirus and a polerovirus increased the fecundity of the vector *M. persicae* and its preference for co-infected plants, compared to mono-infected plants, thereby facilitating virus transmission from co-infected plants **(Srinivasan & Alvarez, 2007)**. In addition, synergistic or antagonistic interactions in multi-infected plants can modify virus accumulation and hence their availability and acquisition by the vector. In synergistic interactions, a virus might facilitate the transmission of the co-infecting virus. This process, which naturally happens in specific virus complexes, is frequently referred to as "helper" dependence or trans-complementation, **(Erickson & Falk, 2023)**. In such infections, the co-infecting "helper" virus encodes a protein for which the "dependent" virus is deficient in and that can be used by the latter to complete its infection cycle. These proteins usually facilitate within host movement or transmission between hosts. One example is natural co-infection of plants with the potexvirus potato aucuba virus (PaMV) and the potyvirus potato virus C (PVC) where PaMV, a mechanically transmitted virus, uses PVC encoded protein HC-Pro, which binds potyvirus virions to aphid stylets **(Pirone & Blanc, 1996)**, for aphid transmission **(Kassanis & Govier, 1971)**. Another example is the co-infection of potato with different strains of PVY where strain 1 can use HC-Pro of strain 2. It was shown that PVY 2 was transmitted by PVY 1 HC-Pro more efficiently than by its own HC-Pro. It was suggested that this may result in competition for virus binding and/or displacement in aphid stylets, concentration alteration of virus or HC-Pro in the source material, or release efficiency **(Mondal, Wintermantel, et al., 2021)**. An even more extreme example of complementation in mixed infections is found in the case of groundnut rosette disease (GRD) complex which combines the polerovirus groundnut rosette assistor virus (GRAV), the umbravirus groundnut rosette virus (GRV, does not encode a proper coat protein), and either one of the two tombusvirus-like associated satellite (sat) RNAs (tlaRNA) **(Erickson & Falk, 2023)**. The inter-dependence and interactions among the three agents responsible of rosette disease are complex. GRAV replicates autonomously in plants and is transmitted only by aphids, mainly *Aphis craccivora* (Homoptera: Aphididae). By contrast, sat RNA depends on GRV for replication. GRV and sat RNA depend on CP of GRAV for their encapsidation and subsequent transmission by an aphid vector **(Erickson & Falk, 2023; Murant & Kumar, 1990)**. In nature, all three agents must occur together for rosette disease to be successfully transmitted by the aphid vector. Thus GRV and its sat RNA are dependent for their survival not only on each other but also on GRAV **(Naidu et al., 1998; Taliansky et al., 2000)**. This strict

dependence probably explains why the sat RNA has been found in all naturally occurring GRV isolates. Very similar results have been obtained for tobacco bushy top disease, a complex of the umbravirus tobacco bushy top virus (TBTV), the polerovirus tobacco vein distorting virus (TVDV) and TBTV and TVDV sat RNAs **(X. Chen et al., 2022)**.

Multi-infection can also induce virus competition for receptors in the vector with ultimate consequences on virus transmission. For instance, the co-infection by two propagative and circulative cytorhabdoviruses, rice stripe mosaic virus (RSMV) and rice gall dwarf virus (RGDV), significantly increased the acquisition and transmission efficiency of RSMV and promoted its propagation in the planthopper vector *Recilia dorsalis*, while RGDV parameters remained unchanged **(Jia et al., 2022)**.

Finally, changes in virus tissue tropism in multi-infected plants can also affect transmission parameters. An expansion of the plant tissue infected by one virus in multi-infected plants may lead to an increased number of infected cells and therefore a raise in virus titer and a larger surface available for virus acquisition by a vector, both potentially resulting in a direct effect on virus transmission. This specific case is the double infection with the phloem-restricted polerovirus potato leafroll virus (PLRV) and the systemic umbravirus pea enation mosaic virus 2 (PEMV2). PLRV can benefit from the systemic infection of PEMV2, by potentially using PEMV2 cell to cell movement proteins (MPs) to alleviate its phloem limitation, invade the entire leaf and become mechanically transmissible **(Ryabov et al., 2001)**.

In some cases, it is difficult to explain how multiple viral infections affect virus transmission. A recent study showed that a co-infection with a begomovirus and a crinivirus resulted in a synergistic effect on accumulation of both viruses, increased accumulation of the begomovirus in upper leaves and increased accumulation of the crinivirus in lower leaves of infected plants. Whiteflies acquired the begomovirus faster from co-infected plants, without impacting its transmission efficiency. However, transmission rates were 100% from mono-infected plants under the experimental conditions, so any increase in transmission from co-infected plants would have been unnoticed **(Li et al., 2021)**. This example shows also that the experimental set-up is important to avoid bias in results.

### D. Effect of virus co-infection on expression of plant genes

The systemic colonisation of host plants by plant viruses is accompanied by massive gene expression modifications that might cause changes in metabolism and symptom expression **(Whitham et al., 2006)**. The infecting virus appropriates cellular functions to allow and facilitate translation, replication, movement within the plant and suppression of host defences. In this complex interaction, plants try to accommodate their metabolism in order to restrict virus infection and to counteract the adverse effects caused by the obligate-biotrophic colonisation. Reports describing transcriptomic responses to mixed infections are rather rare, and the few studies available showed that virus co-infection can lead to significant stronger deregulation of plant gene expression, compared to single virus infection. One example of transcriptomic analyses is tomato plants singly or mixed-infected with two PepMV isolates of two strains that showed higher gene alterations occurring at early infection times. Moreover, it was shown that each of the viral strains modulated the host transcriptome differentially. Mixed infections caused transcriptomic alterations corresponding, at early infection times, to the sum of the transcriptomic deregulations occurring in single infections, but the transcriptome responses differed clearly at later times of infection **(Alcaide et al., 2022)**.

## Viral multi-infection in sugar beet

### E. What is known about sugar beet multi-infection?

Viral multi-infection is also a very common phenomenon in sugar beet plants. One of the most economically important viral diseases of sugar beet is virus yellows (VY), caused by a complex of different virus species: two poleroviruses (family *Solemoviridae*), beet mild yellowing virus (BMYV) and beet chlorosis virus (BChV), a closterovirus (family *Closteroviridae*) beet yellows virus (BYV), and a potyvirus (family *Potyviridae*) beet mosaic virus (BtMV). A third polerovirus, beet western yellows virus (BWYV), is currently restricted to the USA and Asia and has so far not been detected in Europe (Stevens et al., 2004b; Wintermantel, 2005a; Xiang et al., 2008). All these viruses are transmitted by aphids; the main vector species being the green peach aphid, *Myzus persicae* **(Kozłowska-Makulska et al., 2009; Limburg et al., 1997)**. These viruses can cause important damage due to intense leaf yellowing reducing photosynthetic activity of leaves, interfering with assimilates transport as well as exerting massive metabolic deregulations **(Clover et al., 1999)**. This results in considerable yield losses

and reduction of sugar content. The most severe losses are registered when infection occurs earlier in the season (Smith & Hallsworth, 1990a).

BYV is a closterovirus with a genome of 15.5 kb encoding nine ORFs. The untranslated regions (UTRs) at the 5' and 3' extremities of the monopartite single-stranded positive sense RNA are of 107 and 141 nucleotides length, respectively. ORF 1a is cleaved into the leader-protease L-Pro and a replication-associated protein containing Met and Hel domains, ORF 1b encodes the RdRp polymerase. Downstream of the replication genes are ORF 2 (P6), ORF 3 (HsP70h), ORF 4 (P64) and ORF 5 (CPm) which form the closterovirus hallmark gene module **(Dolja, 2003)**. ORFs 2, 3 and 4 encode proteins that are involved in cell-to-cell movement of the virus during infection **(Dolja, 2003)**. The deletion of ORF 3 resulted in the complete arrest of BYV cell-to-cell movement **(Peremyslov et al., 1999).** ORF 5 and ORF 6 encode the minor (CPm) and major (CP) capsid proteins with molecular weights of 24 and 22 kDa, respectively. While CP encapsidates the genomic RNA and forms the core of the filamentous virus particle, CPm, HSP70h and p64 are incorporated specifically at one end of the particle, the so-called tail. ORF 7 encodes a protein of 20 kDa which is involved in long distance transport, and is a tail component, via interaction with HSP70h. ORF 8 encodes p21, a protein that has an important role in RNA replication, and in the suppression of RNA silencing **(Ye & Patel, 2005)**. CPm of other members of the family *Closteroviridae* plays a role in transmission, probably by binding the virus to receptors in the oesophagus of the aphid, whitefly or mealybug vectors, thus CPm of BYV is thought to have the same function **(A. Y. S. Chen et al., 2011; Killiny, Harper, Alfaress, El Mohtar, et al., 2016; Prator & Almeida, 2020)**.

BtMV is a potyvirus. Its genome consists of a positive sense single-stranded RNA molecule of approximately 9.5 kb in size, encoding a single ORF that begins at nt 166 and terminates at nt 9423.The deduced genome organization is typical for a member of the genus *Potyvirus* and includes 10 proteins derived from the polyprotein precursor: P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb and the coat protein (CP). A polymerase slippage gives rise to an alternate RNA encoding a polyprotein from which protein P3N-PIPO, is cleaved **(Chung et al., 2008)**. I describe here the functions of potyviral proteins in general, but they should apply to BtMV as well. HC-Pro has been shown to be multifunctional. It acts as a suppressor of RNA-mediated gene silencing and, in addition, it is required for aphid transmission, by interacting with virions and aphid stylets **(Valli et al., 2018)**. CP is a protein that, besides being involved in encapsidation of the viral RNA into filamentous particles, is also involved in aphid transmission, cell-to-cell and systemic movement, and in the regulation of viral RNA

amplification. As for other potyviruses, CI and 6K2 encode proteins that contribute mainly to virus replication. NIa is found as a whole protein whose functions are not well known, and is processed into NIaPro, a protease liberating HC-Pro from the polyprotein precursor, and VPg that is covalently linked to the 5' end of the genome is presumed to possess RNA-binding and proteolytic activities **(Gadhave et al., 2020)**. It was shown that it interacts with several viral and host factors such as elF4E and it is assumed to be a multifunctional protein involved in all essential steps of the virus infection cycle, translation, replication, and movement **(Tavert-Roudet et al., 2017)**.

BChV and BMYV are poleroviruses consisting of icosahedral particles of 25 nm of diameter, which contain the RNA genome. The genome structure of the two viruses resembles that of other poleroviruses; it is composed of a single-stranded positive sense RNA molecule with a size of approximately 5.7 kb (Kozlowska-Makulska et al., 2015a; Stevens et al., 2004b), currently acknowledged to contain 6 ORFs in addition to the UTRs at the 5' and 3' extremities. P0 is the silencing suppressor (Pazhouhandeh et al., 2006a). Sequence analysis of the two viruses showed differences in the P0 sequence, while the CP sequence is highly conserved **(Hauser et al., 2002a)**. P0's RNA silencing suppressor activity acts by enforcing degradation of most AGO proteins and impeding formation of RISC complexes containing viral small interfering RNAs (vsiRNA) **(Baumberger et al., 2007; Bortolamiol et al., 2007)**. By analogy with other poleroviruses, P1 and P2 encode replication genes and the VPg. ORF3 codes for the viral coat protein while ORF4 is involved in virus movement in a host-specific manner **(Boissinot et al., 2020)**. ORF5 of *Solemoviridae* is expressed as a P3-P5 fusion protein (RT Readthrough protein) by translational readthrough of the coat protein UAG termination codon. The RT protein plays a role in virus movement and both structural proteins, CP and a truncated version of the RT protein, are required for aphid transmission **(Brault et al., 1995, 2000)**. Protein P3a, expressed from a non-canonical start codon, is involved in long-distance movement (Smirnova et al., 2015a), and corresponding putative ORFs are found in the genomes of BMYV and BChV.

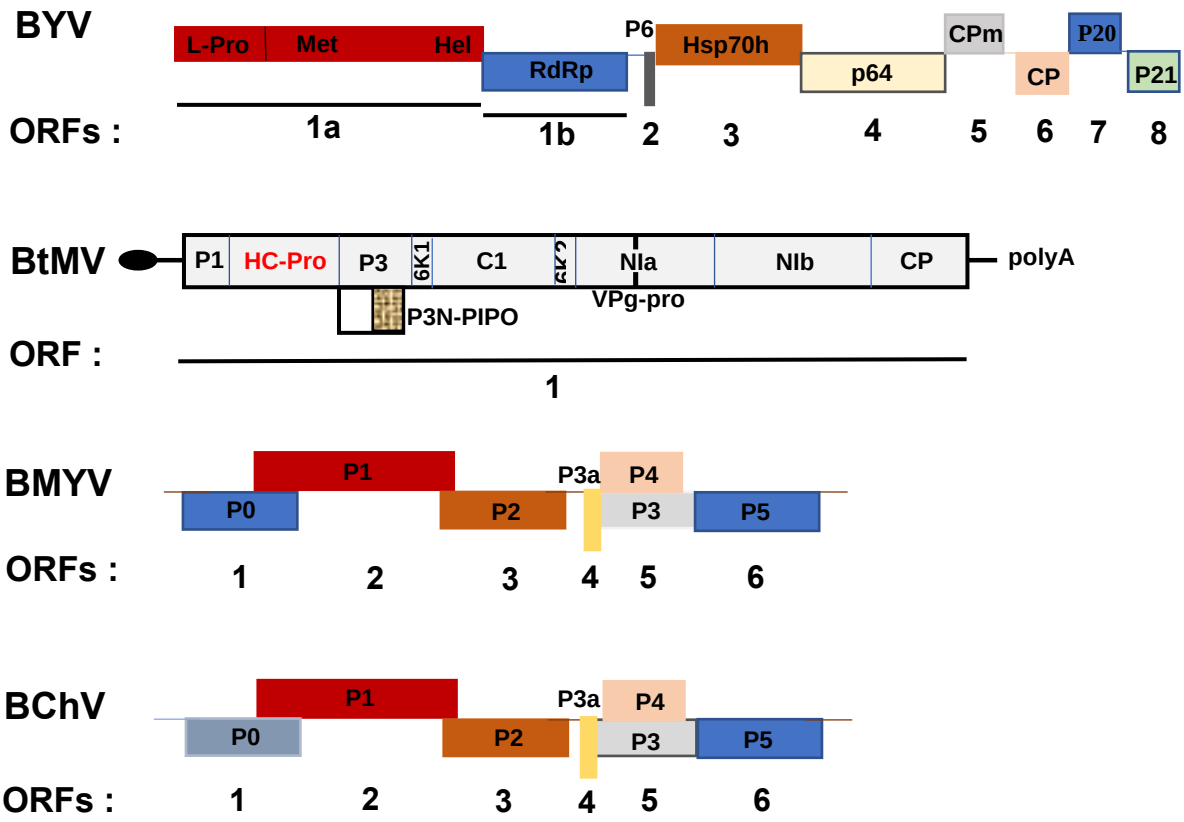



**Figure 2.** Genome organization of sugar beet viruses (BYVV, BtMV, BMYV & BChV).

The different viruses mentioned above can be found in the field alone or in various combinations in the same beet plant (Hossain et al., 2021a). The prevalence of a given virus and the percentage of co-infections vary from year to year and from region to region and also depend on the climate. Indeed, aphid vectors are more numerous following mild winters and warm springs, which increases the percentage of multi-infected plants (Hossain et al., 2021a). The impact of single and multiple sugar beet infections on the severity of leaf yellowing and/or yield has been studied for some viral combinations. In single infections, BYV is generally regarded as the most devastating sugar beet virus causing yield loss between 10-50% or more, followed by polerovirus infections. BtMV infection is considered as the least damaging (Clover et al., 1999; Hossain et al., 2021a; Smith & Hallsworth, 1990a; Stevens, 2007). Mixed infections are less well examined. In one study, co-infection of sugar beet with BtMV, BYV and BWYV was addressed (Wintermantel, 2005a). In this study, sugar beet lines were inoculated with either one, two, or all three viruses. Faster appearance of symptoms and more severe stunting were observed in mixed infections with beet BYV and BtMV, compared to mono-infections.

Co-infection with BYV and poleroviruses resulted in slightly more pronounced symptoms and yield loss, compared to mono-infections (Hossain et al., 2021a; Wintermantel, 2005a). A recent report showed that BtMV barely reduced sugar yield in mixed infections with BYV or BChV, compared to mono-infections, but had a rather strong negative effect on yields in co-infection with BMYV (Borgolte et al., 2024a). It thus seems that effects of mono-infections and mixed infections depend on virus isolates, sugar beet cultivars and field conditions. Sugar beet was also shown to be co-infected by BChV and BMYV. Because the two viruses have important sequence homology, they might be subject to recombination when they co-infect a plant, a driver for emergence of new viruses (see previous section). Indeed, analysis of infected sugar beets indicated two recombination events (in a total of 24 samples analyzed) between BChV and BMYV (Kozlowska-Makulska et al., 2015a). The recombination occurred at the end of the highly conserved coat protein sequence upstream of the readthrough domain, which is involved in aphid transmission. The presence of such recombinants in co-infected plants could therefore affect the aphid transmissibility of the viruses. However, it should be mentioned that the identified BMYV/BChV recombinants could be artifacts caused by template switch of the polymerase during the cDNA synthesis.

### F. Replication, tropism and transmission of sugar beet viruses

As mentioned above, tropism refers to the ability of a virus to infect specific cells or tissue types. Generally, in human and animal viruses, tropism is related to the ability of virus particles to bind to specific receptors on the host cell surface and replicate in the cell **(Cann, 2008)**. For plant viruses, initial entry into the host cells relies on physical breaks of the cell wall (through mechanical inoculation) or on subtle damages induced by vector punctures. Many viruses such as the potyvirus BtMV are capable of infecting all cell types. However, other plant viruses such as the poleroviruses BChV and BMYV and the closterovirus BYV display tissue tropism and are limited to phloem. These viruses are typically inoculated directly into the phloem by vectors such as aphids and cannot be inoculated mechanically. Whereas BChV and BMYV are strictly restricted to phloem tissues (Boissinot et al., 2017; Esau & Hoefert, 1972; Mutterer et al., 1999a), BYV can colonize epidermal and mesophyll cells at a late stage of infection **(Dolja, 2003)**.

The causes for phloem limitation of viruses are not known but several hypotheses have been proposed:

- **Phloem restriction could be due to the lack of expression of viral factors in non-phloem cells:** For instance, the closterovirus CTV was able to overcome partially its phloem restriction and invade other cells in transgenic plants expressing its silencing suppressor p23 under control of the 35S promoter **(Fagoaga et al., 2011).**

- **Phloem restriction could be a virus-controlled function allowing the virus to maximize its titers at the feeding sites of vectoring insects:**

  Phloem restriction was shown to rely on the C-terminal part of the RT protein of PLRV. Deletion of this domain from the PLRV genome resulted in phloem escape of the mutant virus but only in a specific host (*Solanum saccharoides*) after pruning **(Peter et al., 2009)**.

- **Phloem restriction could be regulated by plant host immune system:** In the case of CTV, deletion of p33 protein induces phloem escape which was accompanied by a perturbation of the xylem tissue resulting in severe stem pitting **(Tatineni & Dawson, 2012)**. A follow-up study showed that p33 is an effector protein inducing ROS-mediated defense responses in virus-infected and adjacent cells. Due to the strong defense reaction, the virus remains confined to phloem cells and is unable to move beyond. Deletion of p33 alleviates plant defense responses and allows the virus to escape from the phloem, invade the xylem and perturb the vasculature structure **(Sun & Folimonova, 2019)**.

## Virus transmission mode by aphids

Most animal and human viruses use host plasma membrane receptors and endocytosis mechanisms to cross epithelia, for example lung alveoli for airborne viruses, and to enter and infect a new host after their passage from the previous one. Plant cells are surrounded by rigid cell walls and cuticles insolating them from their environment. Therefore, plant viruses cannot use host internalization systems as a convenient means for initial cell penetration. Rather, most plant viruses rely on vectors that inoculate viruses into a new host. Vectors are found mostly among nematodes, fungi and arthropods, especially hemipterans such as aphids, whiteflies and plant and leafhoppers (Gallet et al., 2018; S. M. Gray & Banerjee, 1999). This is due to their delicate piercing-sucking feeding behavior that allows for uptake or deposit of viruses with their needle-like stylets without inflicting major damage to the visited tissues and cells. I will

concentrate here mostly on aphid transmission of viruses, because the viruses studied in this thesis are transmitted by these pests.

Viruses use different modes of transmission, classified according to two parameters: by the time required for acquisition, latency, retention and inoculation, or by the type of interaction between virus and vectors. The first classification distinguishes between persistent viruses that are transmitted by their vectors during long time spans, sometimes lifelong, non-persistent viruses that are transmitted during short time spans only, and semi-persistent viruses (somewhere in between). The second classification distinguishes between viruses that are retained in the vector mouthparts (=non-circulative) and those that are internalized by their vector (=circulative). Most often, persistent viruses are circulative, and non-circulative viruses are non-persistent or semi-persistent. There are important differences between persistent/circulative transmission mode (as for BMYV and BChV) and semi-persistent/non-circulative (like for BYV) or non-persistent/non-circulative **(Bragard et al., 2013)** (concerns BtMV) transmission modes as shown in **Fig. 2**.

Viruses transmitted in the persistent and circulative mode pass from the insect's digestive tract into the hemolymph and then accumulate in the salivary glands, ending up in the saliva from which they are inoculated into new plants when the aphids feed on them. Circulative transmission is characterized by long phases of virus acquisition (hours to days) on the infected plant, and latency (the time between the start of acquisition until the vector can inoculate new hosts successfully). The retention time during which the vector retains the ability to transmit the virus can be life-long for circulative persistent viruses since the virus is not lost through molting. The inoculation time, the time required to introduce virus particles into a new host, is usually several hours. Circulative viruses are further classified as circulative non-propagative or circulative propagative, depending on whether the acquired virus replicates within the vector.

Semi-persistent and non-persistent non-circulative transmission modes are characterized by virus retention outside aphid cells. Non-persistent viruses, also called stylet-borne viruses, attach to the vector’s stylets or anterior digestive tract. Non-persistent viruses are characterized by short acquisition and inoculation times (in the range of seconds to minutes) and by short retention times (minutes to hours). Semi-persistent viruses are also stylet-borne, the only difference is the longer retention time (hours to some days), compared to non-persistent viruses. There is no latency time for non-persistent and semi-persistent viruses and both are lost from the vectors after molt **(Bragard et al., 2013; Brault et al., 2010)**.

Several viral and aphid proteins play a direct role in the transmission of plant viruses by aphids. Identifying and characterizing these proteins is crucial for understanding and possibly disrupting virus-vector interactions. Virus capsid proteins of non-circulative viruses like CMV, or of *Closteroviridae* and capsid-binding proteins like potyviral HC-Pro and cauliflower mosaic virus (CaMV) P2 are all involved in virus retention in vector mouthparts. The vector-binding domains of these but not of many other virus proteins are rather well characterized **(Blanc et al., 2014; Gadhave et al., 2020)**, whereas the receptors in the mouthparts remain elusive, with the notable exception of the aphid stylet protein stylin-1 in the aphid acrostyle, a distinct region in the stylet tips to which P2 and HC-Pro bind **(Deshoux et al., 2018; Webster et al., 2018)**. Vector-binding determinants of circulative viruses are mostly capsid proteins, for example RT of *Solemoviridae* **(Brault et al., 2010)**. Their receptors in the digestive tract of vectors are known for very few viruses, for example for two members of the *Solemoviridae*, turnip yellows virus and pea enation mosaic virus. The RTs of these viruses bind to the ephrin receptor and to alanyl aminopeptidase N, respectively (Linz et al., 2015; Mulot et al., 2018a). Other aphid candidate receptors are GAPDH3 and RACK (receptor for activated C kinase) that were found to bind to BWYV virions in far-western blot assays **(Seddas et al., 2004)**, but their exact role in transmission is not elucidated.

In addition to proteins that bind virions to the vector, there are also virus proteins indirectly helping in transmission (effector proteins). Examples are virus proteins interacting with vector saliva proteins, or, in the case of circulative propagative viruses, proteins modifying the vector nervous system **(Ray & Casteel, 2022)**. For plant viruses like rice stripe virus (RSV, family *Phenuiviridae*, genus *Tenuivirus*) that can replicate in their insect vectors, viral proteins produced in the salivary glands may act as effectors to promote virus transmission through specific molecular interactions. So RSV nucleocapsids are assumed to bind a vector salivary carbonic anhydrase that upon inoculation in the plant inhibits callose deposition, a plant defense response, thus facilitating infection of the new host plant **(Zhao et al., 2023)**. A recent study on leafhoppers revealed that the expression of a saliva calcium-binding protein is inhibited by rice gall dwarf virus, thus causing an increase of cytosolic $Ca^{2+}$ levels in infested rice and triggering callose deposition and $H_2O_2$ production. This increases the frequency of insect probing, thereby enhancing viral inoculation into the rice phloem **(W. Wu et al., 2022)**. Circulative propagative aphid-transmitted viruses have not been studied in this aspect.

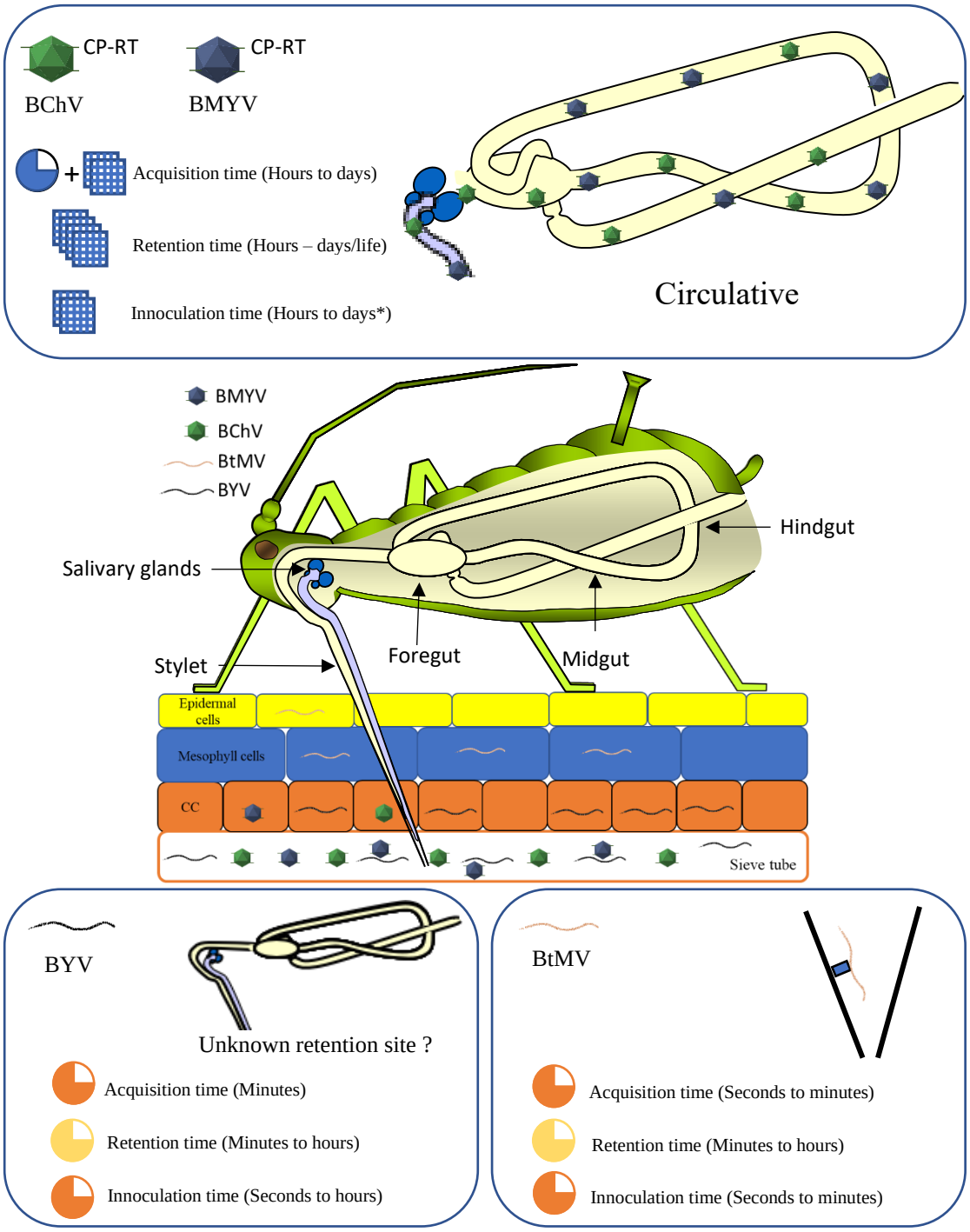



**Figure 3.** Schematic representation of the different transmission modes of BChV, BMYV, BYV and BtMV. The time frames of the different steps of the transmission process are indicated with sectors on clock faces or with calendar pages. The viruses are represented by simplified drawings of virus morphology (icosahedral, flexuous).

## Aphids as vectors:

Aphids belong to two superfamilies, namely *Aphididea* and *Phylloxeroidea* (Order: *Homoptera*). The majority of plant viruses including sugar beet viruses treated in this thesis are transmitted by aphids that belong to the *Aphididea* family. In this family the four aphid species *Myzus persicae, Macrosiphum euphorbiae*, *Aphis craccivora* and *Aphis gossypii* are the most important species by the number of virus species they can transmit and the economic impact they have **(Jayasinghe et al., 2022)**. In nature, aphids can reproduce asexually and sexually **(Emden & Harrington, 2007)**. Many aphid species reproduce from spring to autumn through the asexual, or parthenogenetic reproduction, giving rise to many all-female generations that are exact copies (clones) of their parents. This is followed by a single sexual generation in autumn that mates, with their offspring overwintering as eggs that hatch the following spring. Aphid parthenogenesis is generally associated with viviparity and telescoping of generations (where the granddaughters of a female aphid are already developing within the daughters inside her). This confers a high demographic potential (i.e they can produce massive numbers of individuals under the right conditions). Sexual reproduction occurs in autumn and it is induced by short day length and cool temperatures. During sexual reproduction, mothers pass only half of their genes to their daughters, diminishing their legacy. The sexual phase can occur either on the same plant species as the asexual reproduction or on a different plant species (in host-alternating aphids). *Myzus persicae*, the aphid studied here, follows this typical life cycle and overwinters on Prunus, but there are many variations of it and it is also possible that aphids reproduce under certain environmental conditions only clonally, for example in continuous long-day conditions. Aphids display an amazing level of polyphenism (i.e. discrete phenotypic variation within a genotype) because sexual and asexual forms, winged and wingless individuals, and soldiers can have the exact same genotype. Some species, populations or individual lines have lost the sexual phase, becoming obligate parthenogens, also called 'asexual lines' **(Emden & Harrington, 2007; Simon et al., 2002)**. Aphids are very efficient vectors thanks to the polyphagous aspect of some species (e.g. *M. persicae*). In addition, they are equipped with very efficient piercing-sucking mouthparts (called stylets) that allow them to puncture plant cells without inducing much damage **(Bragard et al., 2013; Brault et al., 2010)**.

## Virus manipulation of plant and aphid:

Plant virus infection can induce plant modifications that can have repercussions on vector behavior and ultimately on viral transmission dynamics, a phenomenon that is commonly referred to as "plant manipulation" or "host mediated effect**" (Eigenbrode et al., 2018; K. Mauck et al., 2012; K. E. Mauck et al., 2016)** and previously described for parasites **(Poulin, 2010)**. Viruses can indeed indirectly affect vector orientation preference, feeding behavior and performance. Some of the phenotypic changes in the plant that induce vector changes are well known, such as the alteration of quantity and composition of plant volatile organic compounds (VOC) and of symptoms such as yellowing, mosaics and leaf curling that act as cues for host recognition and selection by aphids (and other vectors). Sugar and amino acid content of phloem sap can also have strong influence on aphid settling and feeding behavior **(Nalam et al., 2021)**. For example, it was shown that populations of the poplar leaf aphid move from older leaf to younger leaf on their hosts, tracking the higher amino acid content in younger or "sink" tissue **(Gould et al., 2007)**. Similar results were obtained with green peach and potato aphids that performed better on young than on old plants **(Karley et al., 2002)**. Viral infection can alter amino acid composition in the plant and consequently aphid performance. For example, infection with barley yellow dwarf virus increased the total amino acid content, mainly alanine and glutamine in spring wheat leaves **(Ajayi, 1986)**. Similarly, an effect, possibly favorable for transmission, on amino acids content and VOCs of Arabidopsis was observed on TuYV-infected plants **(Claudel et al., 2018; Krieger et al., 2023)**.

The effects of viruses on host plant physiology can be variable, depending on the transmission mode and tropism of a virus (persistent *vs* non-persistent; phloem-limited *vs* non-phloem-limited) **(K. E. Mauck et al., 2018; K. E. Mauck & Chesnais, 2020)**. A persistent virus should attract and arrest insect vectors, favoring settlement and reproduction to ensure maximum dissemination. A non-persistent virus, in contrast, should attract but then repel vectors, because its acquisition is fast and retention extremely short. It was shown that potato leafroll virus-infected potato plants emitted VOCs that attract and arrest the non-viruliferous vector *M. persicae*, compared to uninfected plants or plants infected with potato virus X or potato virus Y **(Eigenbrode et al., 2002)**. A similar effect was seen in the *Rhopalosiphum padi*/wheat/barley yellow dwarf virus pathosystem where non-viruliferous aphids preferentially responded to volatile cues of virus-infected plants compared to non-infected plants **(Eigenbrode et al., 2002; Ingwell et al., 2012; Jiménez-Martínez et al., 2004)**. Additionally, plant viruses (persistent,

semi or non-persistent) may alter the number and probing time of the insect vector, as shown for persistent rice dwarf virus and semi-persistent cauliflower mosaic virus **(Verdier et al., 2023; W. Wu et al., 2022)**. Non- or semi-persistently transmitted plant viruses are transmitted during brief probing events, whereas persistently transmitted plant viruses require longer phloem feeding **(Fereres & Moreno, 2009)**. Thus, non- or semi-persistent viruses tend to manipulate their vector by increasing short probing events to enhance their transmission. In contrast, persistently transmitted plant viruses tend to optimize their transmission by increasing phloem feeding time **(Eigenbrode et al., 2018; K. Mauck et al., 2012)**. One well-described example is the non-phloem limited propagative tomato spotted wilt virus (TSWV) which increases probing events of its vector, the Western flower thrips, resulting in an increased transmission rate and virus spread **(Stafford et al., 2011)**. Similarly, the phloem-limited tomato yellow leaf curl virus (TYLCV) manipulates phloem feeding behavior of its vector *Bemisia tabaci* Mediterranean (MED), resulting in increase of phloem feeding time **(Moreno-Delafuente et al., 2013)**.

The previous paragraphs dealt with plant-mediated indirect effects of viruses on their vectors. However, there are also direct effects of viruses on vectors, which are much less studied/understood. This concerns primarily circulative propagative viruses that can express, in the vector, factors that impact vector behavior or performance (see above). An example of a direct effect of a circulative non-propagative virus barley yellow dwarf virus (BYDV), where the aphid *R. padi*, after acquiring BYDV during feeding on artificial medium, preferred non-infected wheat plants, while non-viruliferous aphids fed on artificial medium without virus preferred BYDV-infected plants. The mechanisms behind this choice modification are not known **(Eigenbrode et al., 2018; Gutiérrez et al., 2013; Ingwell et al., 2012)**. Another example is non-circulative cauliflower mosaic virus: aphids having fed on artificial medium containing viral P2 that binds to a specific region in the aphid stylets – the acrostyle – increase the frequency of test punctures. Because this virus is acquired during these punctures, this might be an example of a direct vector manipulation **(Verdier et al., 2023)**.

Beside the manipulation, additional effects such as fitness parameters, in particular survival, fecundity and wing formation, could also facilitate/increase transmission. For instance, satellite RNA (satRNA) of cucumber mosaic virus (CMV) accelerates wing formation in its vector *Myzus persicae* **(Jayasinghe et al., 2022)**.

## Main objectives of the PhD thesis

Our study focused on the impact of multi-infection of sugar beet with the four viruses (BMYV, BChV, BYV and BtMV) on host-virus-vector interactions, with particular emphasis on the consequences of multi-infection on virus transmission by aphids. We first carried out a battery of transmission tests to compare the efficiency of virus transmission from mono-infected plants versus co- or multi-infected plants. Where differences in transmission were observed, we sought to identify the causes. To do this, we studied the impact of multi-infection on the following parameters, all of which could be related to virus acquisition:

1) Virus cellular localization (tropism) in infected plants. We investigated whether multi-infection or, initially, co-infection could modify (i) the intracellular localization of viruses or (ii) affect tissue tropism, in particular by overcoming the phloem limitation of BMYV, BChV and BYV, since this change in localization could affect the accessibility of the virus(es) to the vector.

2) Virus accumulation. The question was whether interactions between viruses resulted in synergy, antagonism or neutral effects in terms of viral titer, which could also explain increased or decreased virus transmission.

3) Aphid feeding behavior on multi-infected plants. We hypothesized that single and multiple viral infections would have different effects on plant phenotype, such as metabolism, with consequences for aphid feeding behavior. Since feeding behavior is important for virus acquisition, its modification by single or multiple plant infection could have consequences for acquisition and transmission.

# Chapter 1: Impact of sugar beet co-infection on aphid virus transmission efficiency and selection of co-infections for further study

Multi-infection of sugar beet with different viruses might directly affect the transmissibility of the four viruses (BChV, BMYV, BtMV and BYV) by aphids. First, metabolic and other changes induced by multi-infections *vs* single viral infection may affect plant cell or phloem sap composition and thus impact aphid feeding behavior, which will have a direct impact on virus acquisition. Second, co-infecting viruses can have synergistic or antagonistic interactions that can, besides impacting symptoms, modify their accumulation, and/or change virus tissue tropism and intracellular distribution. In particular, aphids could acquire phloem-limited viruses from cells (epidermis or mesophyll) that do not naturally contain these viruses in mono-infections. Virus acquisition time could then be reduced from a few hours to a few minutes.

To study the outcome of multi-infection on aphid transmission, experiments were carried out to test virus transmissibility from plants infected by one of the 15 possible combinations of the four viruses and using plants mono-infected as controls. We have developed a transmission assay to test all possible virus combinations to identify those for which virus transmission efficiency is affected. For this, various parameters were optimized such as acquisition and inoculation times, and the number of aphids on source and test plants. The vector species *M. persicae* was used because it transmits all the viruses considered. The optimized transmission protocol is outlined in **Fig. 1**. In brief, aphid virus transmission experiments were performed using 3 detached sugar beet leaves from co-infected plants on which aphids acquired viruses during 24 h. This step was followed by a 72 h virus inoculation on test plants using 3 viruliferous aphids per plant and 15 test plants. Three weeks later, plants were analyzed by DAS-ELISA or immunocapture-reverse transcription-polymerase chain reaction (IC RT PCR) to detect and identify viruses (**Fig. 1**). The transmission efficiency of each co-infecting virus was compared to that of transmission of each virus from mono-infected plants.

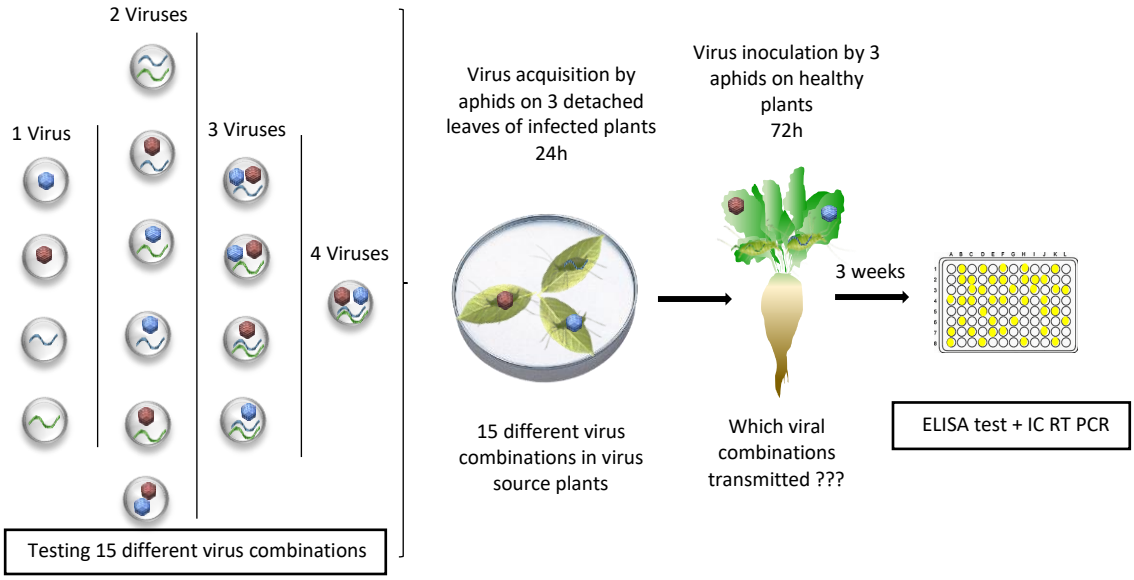



**Figure 1. Methodological approach for transmission assays using multi-infected or mono-infected plants**

Because of the difficulties to obtain multi-infected source plants and in particular the 15 possible virus combinations at the same time, we started our transmission experiments by studying only a few combinations at a time. The experiments were repeated up to six times and the results of the 15 different combinations are presented in **Tables S1-S4**.

The 15 tested combinations consisted of the 4 mono-infection controls and the 11 multi-infections **(Fig. 1)**. Five of the multi-infections yielded statistically significant results, for the other six combinations we did not obtain exploitable results either because results were not significant due to high variability or because we could not do enough repetitions. The five combinations are divided into three groups. In the first group, co-infection of the closterovirus BYV with one or two poleroviruses (BYV/BChV, BYV/BMYV, BYV/BMYV/BChV), in most cases leads to the drop of transmission of the poleroviruses. In the second group, co-infection of the two poleroviruses, showed a mutual synergism in regards to their transmission. In the last group, co-infection of the potyvirus BtMV with unrelated viruses BYV or BMYV, showed different outcomes: a drop of BYV transmission, or an increase of BMYV transmission in preliminary experiments.

In the first group, representing co-infection of BYV with poleroviruses, we selected BChV/BYV for further study, because BChV is highly prevalent in France and also because BChV and BMYV are very closely related viruses that are expected to give similar results in co-infection with BYV. Another reason to select this combination is that closterovirus/polerovirus co-infection has not been studied in detail before. We also studied in depth co-infection of the two poleroviruses, BChV and BMYV, because of their close

genetic relatedness unlike the other combinations, and because we observed a synergism regarding their transmission by aphids. Finally, in the third group BtMV/BYV co-infection was very interesting to study because it is rarely reported for a potyvirus to 'win' against a co-infecting virus. The three combinations - BChV/BYV, BChV/BMYV and BtMV/BYV - are presented in **Chapters 2**, **3** and **4**, respectively. The partial results obtained with two other co-infections - BYV/BMYV and BYV/BMYV/BChV - are summarized hereafter, while we chose not to show BtMV/BMYV co-infection because of the very preliminary nature of the results.

- **Co-infection with BYV/BMYV**

As with BYV/BChV co-infection, we observed a drop in BMYV transmission from co-infected plants (aphid transmission rate of 28 % from mono-infected plants and 10 % from BYV/BMYV co-infected plants, **Fig. 2B),** and no effect on BYV transmission (75 % *vs* 80 % for mono-infections *vs* co-infections, respectively, **Fig. 2A)**. Aphids feeding on co-infected plants transmitted both viruses together at 10 % transmission rate, which was lower than expected, based on the theoretic virus co-transmission rate (25 % expected co-infection rate, calculated as the product of the transmission rates using mono-infected source plants, **Fig. 2C**).

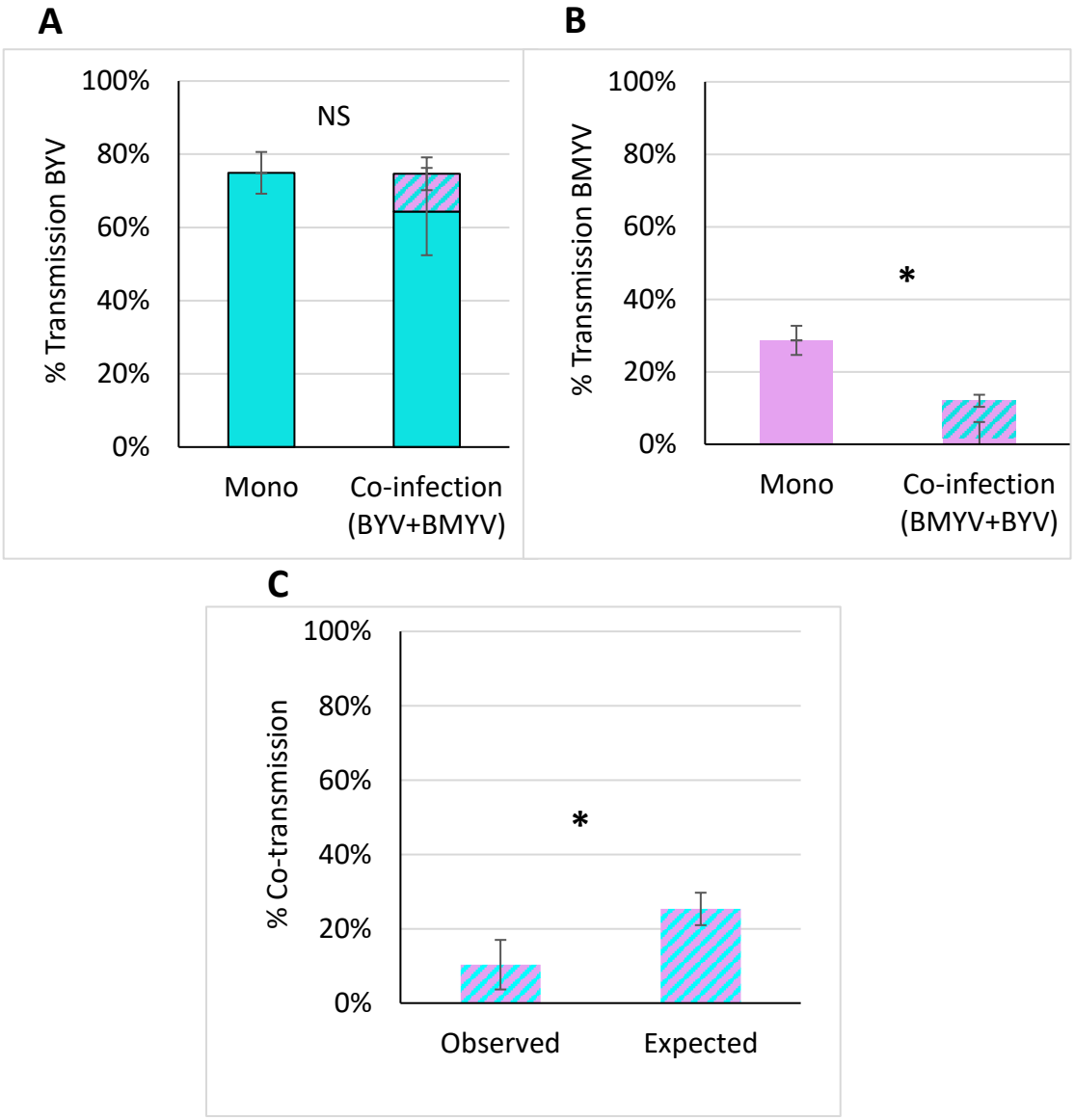



**Figure 2. Aphid transmission efficiency of (A) BYV and (B) BMYV from mono or co-infected plants.** *Myzus persicae* acquired virus from mono or co-infected leaves for 24 h. Then, three aphids were transferred per test plant for 72 h inoculation. A DAS-ELISA assay was performed 3 weeks later to detect infection. The percentage of infected plants is indicated (% Transmission). The difference was statistically significant for BChV but not for BYV transmission (GLMER, BYV: p-value =0.592; BMYV: p-value=0.0140; n = 60, four independent experiments; df = 1;). NS = not significant, * = p-value < 0.001.

## Virus localization in BYV/BMYV co-infected plants

To assess if co-infection affected tissue localization of BMYV or BYV, SABER-FISH was performed on mono- and co-infected plants at 21 dpi. We visualized the presence of both viruses in leaves and roots and found that the BMYV infected more phloem cells in roots than in leaves.

In cross-sections, initial results suggested cellular exclusion of both viruses in both leaves and roots; these results need to be confirmed by further experiments. Both viruses were observed exclusively in phloem cells as expected, and with similar labeling intensity **(Fig. 3)**. We estimated the percentage of co-infected cells and mono-infected cells in co-infected leaves and roots. Ten to eleven sections from co-infected plants were observed in two independent experiments **(Table 1-2)**. Depending on the experiment, we noticed a different percentage of cells infected either with BMYV or BYV in co-infected leaves . Five percent to 50 % of infected cells contained only BMYV and 50 % to 95 % of infected cells contained only BYV **(Table 1**). In infected roots the percentage of both viruses was more homogenous **(Table 2)**.

**Table 1: Percentage of cells infected by one or both viruses in sections of leaves co-infected with BMYV and BYV**

| | **Exp. 1** | **Exp. 2** |
|---|---|---|
| Virus(es) observed | Plant #1 | Plant #1 |
| **BMYV** | 5 %[a] | 50 % |
| **BYV** | 95 % | 50 % |
| **BYV+BMYV** | 0% | 0% |

**Table 2: Percentage of cells infected by one or both viruses in sections of roots co-infected with BMYV or BYV**

| | **Exp. 1** | **Exp. 2** |
|---|---|---|
| Virus(es) observed | Plant #1 | Plant #1 |
| **BMYV** | 40 %[a] | 27.5 % |
| **BYV** | 59 % | 47.5% |
| **BYV+BMYV** | 0% | 0% |

[a] Percentage of cells labeled for the indicated virus/total number of labeled cells in 10 to 11 sections

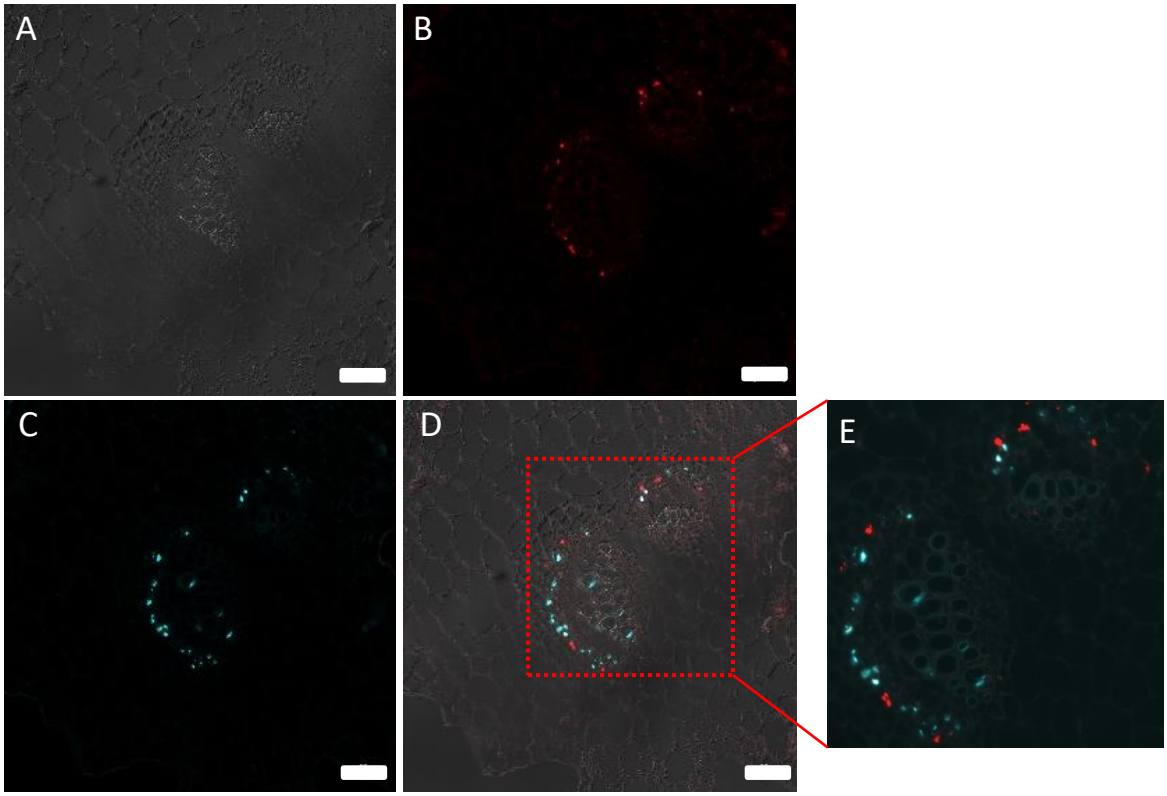



**Figure 3. Tissue distribution of BMYV and BYV in co-infected leaves**. Leaves were processed three weeks after inoculation by SABER-FISH for detection of BMYV and BYV. (A-D) Representative images of a transversal section showing (A) the bright field acquisition, (B) BMYV label (red), (C) the BYV label (turquoise), and (D) a merge. (E) Magnification of the region outlined in (D) showing virus distribution of the two viruses.

## Conclusion:

We found that co-infection of sugar beet with the closterovirus beet yellows virus (BYV) and the unrelated polerovirus beet mild yellowing virus (BMYV) lowered aphid transmission of BMYV, but not of BYV. Similar results are presented in **Chapter 1** where BYV/BChV co-infection reduced transmission of BChV. Unexpectedly, virus localization showed cell exclusion of BYV with BMYV co-infection, which was not observed with BYV/BChV co-infection. The ratio of cells infected by one or the other virus was very variable **(Table 1)**. This might be due to unequal inoculation by aphids conditions or patchy tissue distribution of the viruses.

- **Co-infection with BYV/BMYV/BChV**

The results of BYV/BMYV/BChV triple infection showed a decrease in BMYV and BChV transmission and no major change in BYV transmission. BMYV aphid transmission rate

dropped from 31 % from mono-infected plants to 18 % from triple infected plants and BChV transmission dropped from 36 % to 20 % from mono-infected and triple-infected plants, respectively **(Fig. 4A).** There was no effect on BYV transmission (73 % *vs* 75 %, from mono or multi-infected plants respectively, **Fig. 4B)**. Aphids feeding on triple-infected plants transmitted BMYV&BYV together at 3 %, BChV&BYV at 3 % and BChV&BMYV at 1 % transmission rate, which were lower than expected **(Fig. 5 A-D)**. For triple infection, there was no effect on transmission of the three viruses between observed and expected transmission rate. These results suggest that BYV abolished the synergistic effect on transmission of both poleroviruses in dual infections.

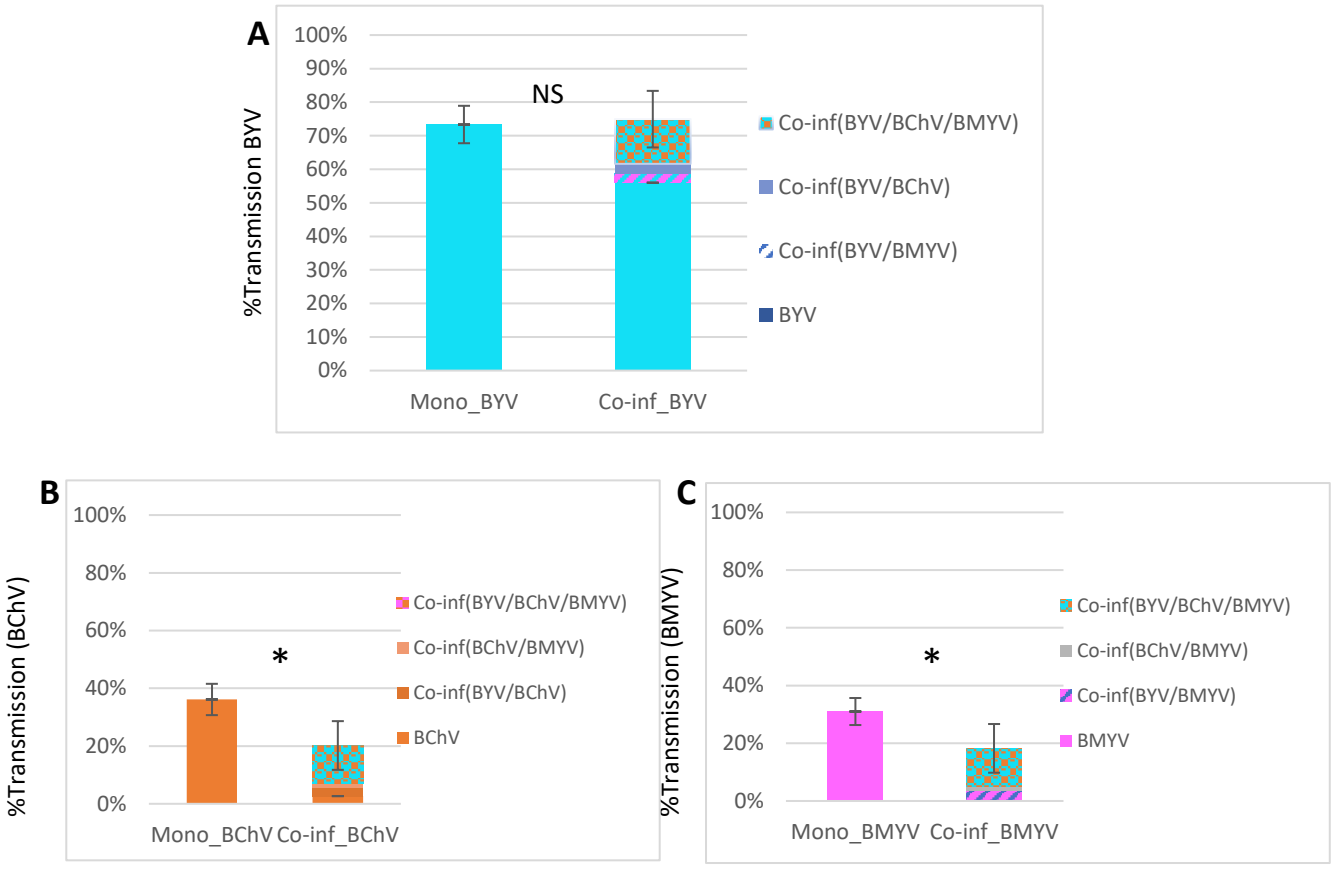



**Figure 4. Aphid transmission efficiency of (A) BYV, (B) BChV and (C) BMYV from mono or co-infected plants.** *Myzus persicae* acquired virus from mono or co-infected leaves for 24 h. Then, three aphids were transferred per test plant for 72 h inoculation. A DAS-ELISA assay and IC RT PCR was performed 3 weeks later to detect infection. The percentage of infected plants is indicated (% Transmission). The difference was statistically significant for BChV and BMYV but not for BYV transmission (GLMER, BYV: p-value =; BMYV: p-value< 0.001; BMYV: p-value< 0.001; n = 75, five independent experiments; df = 1;). NS = not significant, *** = p-value < 0.001.

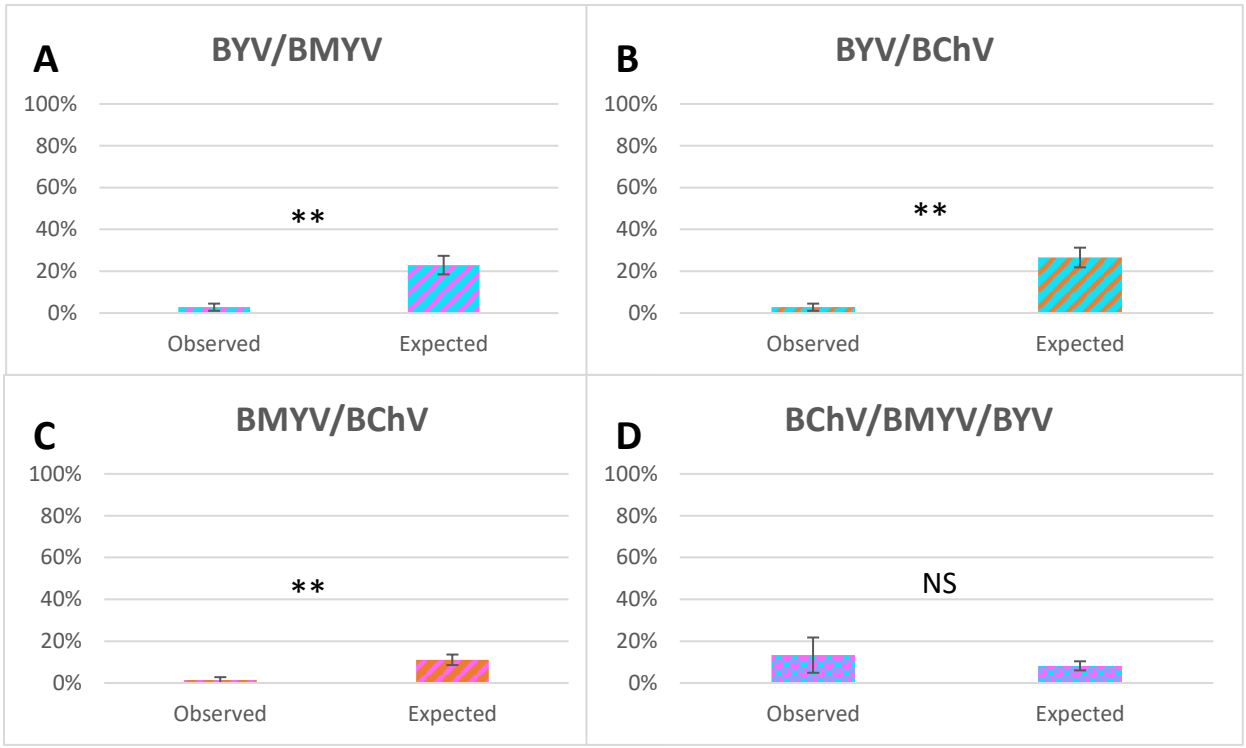



**Figure 5. Expected aphid transmission efficiency of BYV, BMYV and BChV from triple-infected plants.** *Myzus persicae* acquired virus from multi-infected leaves for 24 h. Then, three aphids were transferred per test plant for 72 h inoculation. A DAS-ELISA assay and IC-RT PCR was performed 3 weeks later to detect infection. The percentage of expected and observed co- or multi-infected plants is indicated. Differences were statistically significant (p-value <0.001; n=75, five independent experiments; df=1; chi-squared($\chi$2)).

### Virus localization in BYV/BMYV/BChV co-infected plants

With regard to cellular localization, results showed that all viruses were confined in the phloem and multi-infection did not alter tissue distribution of the three viruses. We observed mono- double- and triple-infected cells, this suggests that there is no exclusion between the three viruses at the cellular level and all possible combinations can exist **(Fig. 6)**.

We estimated the percentage of co- or multi-infected cells with two or three viruses and mono-infected cells in triple-infected plants. Ten to eleven sections from multi-infected plants were observed in two independent experiments **(Table 4)**. We noticed a very low percentage of cells infected with BMYV (18.0 % of cells containing only BMYV or co-infected) compared to BChV (45.85 % of cells containing only BChV or co-infected). A very high percentage of cells was infected with BYV (70.05 % of cells containing only BYV or co-infected) **(Table 4)**. One percent of all infected cells were co-infected with BChV and BMYV, 8 % with BChV and BYV and 5 % co-infected with BMYV and BYV. Triple-infected cells represented 10 % of all infected cells. The label intensity of each virus seemed to be similar in co-infected cells.

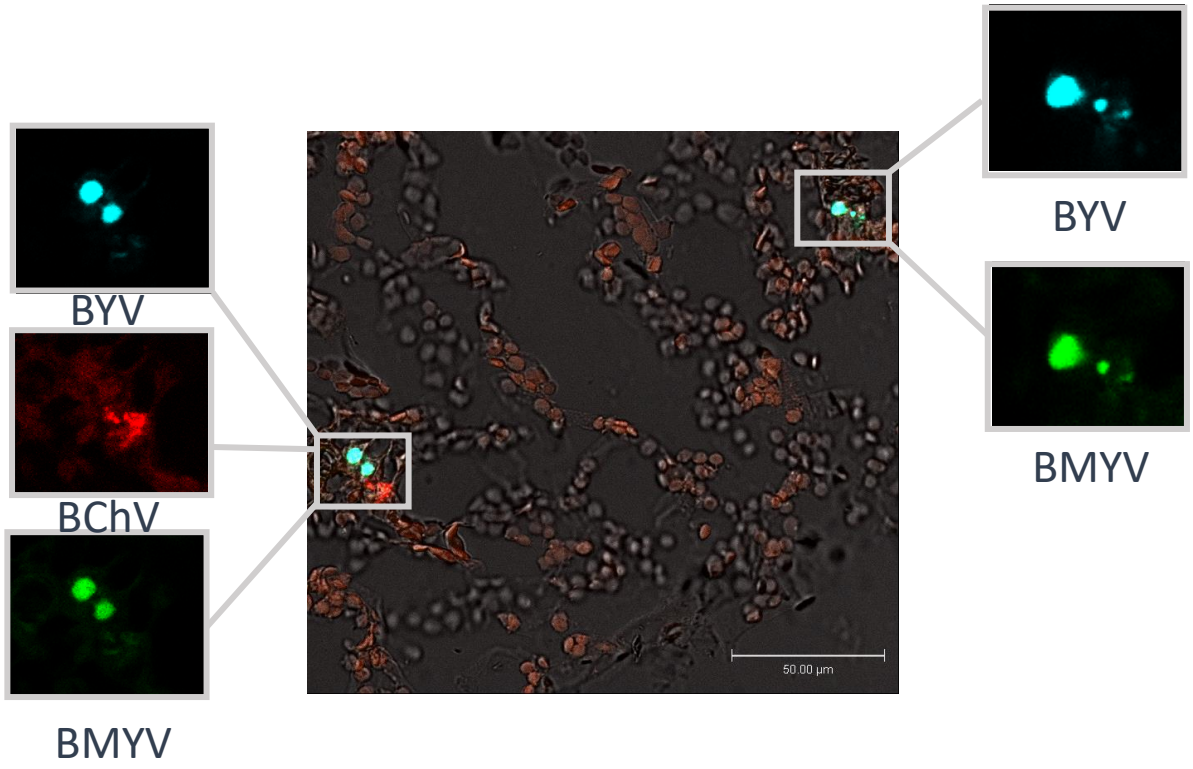



**Figure 6. Tissue distribution of BMYV, BChV and BYV in co-infected leaves**. Leaves were processed three weeks after inoculation by SABER-FISH for simultaneous detection of BMYV, BChV and BYV. Representative image showing a transversal section with BYV label (turquoise), BChV label (red), BMYV (green) and the merge of the three. Magnifications of the regions outlined show double- and triple-infected cells.

**Table 3: Number of cells infected by one, two or three viruses in sections of leaves multi-infected with BChV, BMYV and BYV.**

[a] Number of cells observed on 10 to 11 sections of each plant showing a fluorescent signal for the indicated virus.
[b] Percentage of cells labeled for the indicated virus/total number of labeled cells in 10 to 11 sections.
[c, d] Percentage of cells containing one or both viruses/total number of cells observed in the experiments.

| | Exp1 | | Exp2 | | |
|---|---|---|---|---|---|
| Virus(es) observed | Plant #1 | Plant #2 | Plant #1 | Plant #2 | %[d] |
| **BChV** | 24[a] (25%[b]) | 12 (20 %) | 11 (22.9 %) | 18 (37.5 %) | 26.35 % |
| **BMYV** | 0 (0 %) | 3 (5 %) | 0 (0 %) | 3 (6.25 %) | 2.81 % |
| **BYV** | 58 (60.4 %) | 30 (50 %) | 21 (43.75 %) | 15 (31.25 %) | 46.35 % |
| **BChV/BMYV** | 0 (0 %) | 2 (3.30 %) | 0 (0 %) | 0 (0 %) | 1% |
| **BChV/BYV** | 1 (1 %) | 6 (10 %) | 6 (12.5 %) | 4 (8.33 %) | 8% |

| | | | | | |
|---|---|---|---|---|---|
| **BMYV/BYV** | 0<br>(0 %) | 0<br>(0 %) | 6<br>(12.5 %) | 4<br>(8.33 %) | 5.20% |
| **BChV/BMYV/BYV** | 13.5<br>(13.54%) | 7<br>(11.66 %) | 4<br>(8.33 %) | 4<br>(8.33 %) | 10.5% |

**Conclusion:**

BMYV, BYV & BChV triple infection had no significant effect on BYV transmission and caused a drop of the transmission of both poleroviruses (BMYV & BChV). The presence of BMYV lowered the percentage of BYV/BChV co-infected cells, compared to BYV/BChV double-infected plants, while the presence of BChV cancelled the cellular exclusion between BYV and BMYV, observed in BYV/BMYV co-infected plants.

# Chapter 2: Interplay between a polerovirus and a closterovirus decreases aphid transmission of the polerovirus

Souheyla Khechmar [1,2], Quentin Chesnais [1,2], Claire Villeroy [1], Véronique Brault [1,2]*, Martin Drucker [1,2]*

[1] SVQV, UMR 1131, INRAE Centre Grand Est, Colmar, France

[2] Université Strasbourg, Strasbourg, France

* Corresponding authors: martin.drucker@inrae.fr, veronique.brault@inrae.fr

Short title: Polerovirus-closterovirus interplay

Keywords: plant virus, virus co-infection, virus localization, aphid transmission, virus accumulation, aphid behavior, plant-virus-virus-vector interactions.

**ABSTRACT**

Multi-infection of plant by viruses is very common and can change drastically infection parameters such as virus accumulation, distribution and vector transmission. Sugar beet is an important crop that is frequently co-infecting by the polerovirus beet chlorosis virus (BChV) and the closterovirus beet yellows virus (BYV), both vectored by the green peach aphid (*Myzus persicae*). These phloem-limited viruses are acquired while aphids ingest phloem sap from infected plants. Here we found that co-infection decreased transmission of BChV by ~50 %, but had no impact on BYV transmission. The drastic reduction of BChV transmission was not due to lower accumulation of BChV in co-infected plants, nor to reduced phloem sap ingestion by aphids from these plants. Using the SABER-FISH *in situ* hybridization technique on plants, we observed that 40 % of the infected phloem cells were co-infected and that co-infection caused redistribution of BYV in these cells. The BYV accumulation pattern changed from distinct intracellular spherical inclusions in mono-infected cells to a diffuse form in co-infected cells. There, BYV co-localized with BChV throughout the cytoplasm, indicative of virus-virus interactions. We propose that BYV-BChV interactions could restrict BChV access to the sieve tubes and reduce its accessibility for aphids and present a model of how co-infection could alter BChV intracellular movement and/or phloem loading and reduce BChV transmission.

**IMPORTANCE**

Mixed infection by two or more pathogens is the rule rather than the exception, but it remains understudied. Yet, mixed infections can have huge impact on the outcome of infection, for example, through synergistic or antagonistic effects exacerbating or attenuating symptoms in the host, altering the distribution and accumulation of co-infected pathogens, or tampering with pathogen spread. Here, we focused on the effect of mixed infection by two phloem-limited plant viruses on vector transmission. We found that co-infection of sugar beet with the closterovirus beet yellows virus (BYV) and the unrelated polerovirus beet chlorosis virus (BChV) lowered aphid transmission of BChV, but not of BYV. Titers of both viruses were unchanged by co-infection, ruling out lower accumulation of BChV as the reason for lower transmission. Likewise, altered ingestion of phloem sap, from which BChV is taken up, by aphids feeding on co-infected plants did not explain reduced transmission of BChV, because aphids fed for equal or even longer duration on co-infected plants. Co-infection of plants resulted in about one-third of the infected cells being infected by BChV, one-third by BYV and one-third being co-infected. This shows that BChV and BYV do not exclude each other and that replication of one virus does not interfere with the replication of the other virus. Co-infection induced

redistribution of BYV in phloem cells. In single-infected cells, BYV was found in distinct spherical cytoplasmic inclusions while in co-infected cells it colocalized with BChV uniformly throughout the cytoplasm. This is indicative of direct or indirect BYV-BChV interaction that might reduce the transmission of BChV. We propose that BYV inhibits the release of BChV into the sieve tubes, either by interfering with transport of BChV particles to plasmodesmata or by hindering their passage through them. Taken together, our results show that BChV-BYV interaction is neutral and has little or no effect on the virus or host with the exception of a negative effect on BChV transmission. The latter finding might have epidemiological consequences for the propagation of the two viruses in the field.

## INTRODUCTION

Historically, studies on diseases in both plants and animals focused mainly on simple interactions implicating only one host and one pathogen. However, in nature, a host is often subjected to a multitude of pathogens simultaneously, a scenario referred to as co-infection, multi-infection or mixed infection, and which might be the rule rather than the exception **(Abdullah et al., 2017; Dutt et al., 2022)**. The development of molecular tools such as high throughput sequencing further highlighted the high occurrence of mixed infections compared to mono-infection **(Dutt et al., 2022)**. Mixed infections can be heterogeneous (*i.e.,* implying different pathogens such as bacteria, fungi or viruses), or homogeneous (*i.e.,* with similar microorganisms) **(Devi et al., 2021)**. Multi-infection can occur through the simultaneous inoculation of several pathogens or sequentially, causing co-infection or super-infection, respectively. The outcome of a co-infection or super-infection will be affected by the intra-host interactions between the different infectious agents as well as by their interaction with the host or, in the case of vector-borne pathogens, with the vector **(Dutt et al., 2022)**.

Pathogen interactions, plant viruses in our case, within hosts can be synergistic, *i.e.,* the presence of one pathogen facilitates the infection of one or more additional pathogens. For example, synergistic interactions between tomato chlorosis virus (ToCV) and tomato infectious chlorosis virus (TICV) led to an increased TICV accumulation in *Nicotiana benthamiana* **(Wintermantel et al., 2008)**. Interactions between pathogens are considered neutral when they do not, or only marginally, interfere with each other, as demonstrated for example for tomatoes infected with three begomoviruses, tomato yellow mottle virus, tomato leaf curl Sinaloa virus and tomato yellow leaf curl virus. There, the accumulation of each virus was not strongly affected by the presence of the others although the triple infection induced stronger symptoms

**(Maliano et al., 2022)**. Finally, in antagonistic pathogen interactions, the presence of one pathogen impacts the infection of the other pathogen negatively **(Abdullah et al., 2017; Dutt et al., 2022; Gautam et al., 2020b; Syller & Grupa, 2016)**. An example is the co-infection of two tobamoviruses, hibiscus latent Singapore virus (HLSV) and tobacco mosaic virus (TMV), in *N. benthamiana* plants where HLSV levels decreased, compared to mono-infection, whereas TMV levels remained almost unaltered **(Z. Chen et al., 2012)**. As in this former example, and unlike many synergistic interactions, antagonisms tend to occur mainly between closely related viruses, inducing fitness costs for one or both competitors **(Syller & Grupa, 2016)**.

Virus-virus and virus-host interactions in a multi-infection context can impact different aspects of the virus cycle, such as virus replication, virus movement and virus localization in plant cells or tissues, or by interfering with plant defenses **(Mukasa et al., 2006; Singhal et al., 2021)**. The outcomes of the viral multi-infection cannot only alter the fitness of co-existing viruses but have also a direct impact on the fitness of the plant itself by affecting the severity of foliar symptoms, plant development, and plant chemical and physiological profiles **(Moreno & López-Moya, 2020)**. Plant traits modified by infection or multi-infection can have consequences on virus transmission because they can change the nutritional quality of the plant or modify visual traits and production of volatiles that play important roles in vector-plant interactions. These alterations of the multi-infected plants can therefore influence vector behavior and subsequently virus transmission **(Mauck et al., 2012; Mauck et al., 2016; McLaughlin et al., 2022; Moreno & López-Moya, 2020)**. Studies on mixed viral infections with regard to vector interaction and transmission are scarce. Ontiveros et al. reported that tomatoes co-infected with the begomovirus tomato yellow leaf curl virus (TYLCV) and the crinivirus ToCV were more attractive for the whitefly vectors than ToCV mono-infected plants **(Ontiveros et al., 2022)**. Co-infection with the reovirus raspberry latent virus and the closterovirus raspberry leaf mottle virus changed aphid vector preferences for infected raspberry plants, but not their feeding behavior **(Lightle & Lee, 2014)**.

The viral and host factors mediating these synergistic or antagonistic effects have only been identified in a few cases. For instance, the synergistic interaction between the crinivirus sweet potato feathery mottle virus (SPCSV) and the potyvirus sweet potato chlorotic stunt virus (SPFMV) is mediated by the SPCSV-encoded RNase3 protein **(Cuellar et al., 2009)**. RNase3 functions as a viral suppressor of RNA silencing (VSR) that might target a specific host component by interfering with small-RNA biogenesis **(Cuellar et al., 2015)**. Another example is the potyviral silencing suppressor HC-Pro that increases the titer of potato leafroll virus

(PLRV) in co-infection **(Savenkov & Valkonen, 2001b)**. One example on the host side is the salicylic acid-responsive gene PR-P6 whose expression correlates with virus titer and symptoms severity in tomatoes mono- or co-infected with TYLCV and ToCV **(Ontiveros et al., 2022)**.

Sugar beet (*Beta vulgaris*) is an economically important crop that is often co-infected in the field by several viruses **(Hossain et al., 2021; Wintermantel, 2005)**, causing alone, or in combination, leaf yellowing, yield reduction and low sugar content. Among the viruses detected are: the poleroviruses beet western yellows virus (BWYV-USA) so far not reported in Europe, beet mild yellowing virus (BMYV) and beet chlorosis virus (BChV), the closterovirus beet yellows virus (BYV) and a potyvirus, beet mosaic virus (BtMV) all occurring worldwide **(Hossain et al., 2021; Stevens et al., 2004)**. The impact of multiple sugar beet infections on the severity of leaf yellowing has been studied for BYV, BtMV and BWYV-USA **(Wintermantel, 2005)**. In this study, sugar beet lines were inoculated with either one, two, or all three viruses. Faster appearance of symptoms and more severe stunting were observed in mixed infections with BYV and BtMV, compared to mono-infections (Wintermantel, 2005). Acceleration of symptom development and greater yield losses were observed in sugar beets co-inoculated with BChV and BYV **(Hossain et al. 2021)**.

In this work, we focused on the co-infection of sugar beet with BYV and BChV. BYV is mainly transmitted by the two aphid species *Myzus persicae* and *Aphis fabae* **(German-Retana et al., 1999)**. The main vector species for BChV is *M. persicae* but this virus can also be transmitted efficiently by *Macrosiphum euphorbiae* **(Kozłowska-Makulska et al., 2009; Limburg et al., 1997b)**. BChV and BYV use two different modes of transmission: circulative and persistent for BChV, and non-circulative and semi-persistent for BYV. Viruses transmitted in the persistent and circulative mode are acquired during vector feeding and pass through the insect's digestive tract to be released into the haemolymph. From there, they reach the salivary glands where they accumulate and are inoculated, as a saliva component, when aphids feed on new plants **(Dáder et al., 2017)**. This transmission mode is characterized by long phases of virus acquisition (hours to days) on the infected plant, and latency (the time it takes for the virus to reach the aphid's salivary glands). The time required for aphids to inoculate the virus is also long (several hours), and aphids remain viruliferous for the rest of their lives **(Bragard et al., 2013)**. In the case of semi-persistent transmission, the virus attaches to areas of the mouthparts or anterior digestive tract. Maximum transmission is achieved after a few hours of acquisition phase, and aphids transmit viruses for only a few hours to a few days **(Bragard et al., 2013; Brault et al., 2010;**

**Jiménez et al., 2018)**. The two viruses differ also in their cellular tropism. BChV is restricted to phloem tissues **(Boissinot et al., 2017; Esau & Hoefert, 1972; Mutterer et al., 1999)**. BYV is also a phloem-limited virus, but can colonize epidermal and mesophyll cells at a late stage of infection **(Dolja, 2003; Dolja & Koonin, 2013; Esau et al., 1967)**.

Here, we studied mixed viral infection of sugar beet and examined its effect on vector transmission. Our results show that co-infection of sugar beet with BChV and BYV had no impact on BYV transmission, but did decrease transmission of BChV by 50 %. We present evidence that the decrease in transmission is due to specific BYV-BChV interactions in co-infected cells and not to changes in virus accumulation or vector-feeding behavior on co-infected plants.

## MATERIALS AND METHODS

### Plants, viruses and aphids

The virus-susceptible sugar beet variety "Auckland" was used in this study. Seeds were kindly provided by the seed company SESVanderHave (Tienen, Belgium) via the Technical Institute of Sugar Beet (ITB, Paris, France). One week after germination, plantlets were transplanted into individual pots and grown in a climate chamber at 22 to 25°C with a day/night cycle of 16 h of light and 8 h of darkness.

The *Myzus persicae* biotype WMp2 (local code NL) was used in this study (Reinink et al., 1989a). Aphids were maintained on Chinese cabbage (*Brassica rapa* subspc. *Pekinensis* var. Granaat) in a growth chamber under controlled conditions at 20±1 °C with a day/night cycle of 16 h of light and 8 h of darkness.

The BYV_BBRO_UK isolate used in this study was a kind gift from Prof Mark Varrelmann of the Institut für Zuckerrübenforschung (IfZ, Göttingen, Germany) but was originally sampled in the UK **(Stevens et al., 1997)**. The isolate was maintained on sugar beet plants and propagated by aphid transmission.

We used the BChV-2a isolate collected in East England (UK) with the GenBank accession number AF352024 **(Hauser et al., 2002b)**. BChV particles, prepared as described in **(Van den Heuvel et al., 1991)**, were maintained at -80 °C before being used as a virus source in an artificial medium for aphid acquisition (Bruyère et al., 1997a).

For all experiments, we used the systemically infected third upper leaf at 21 days post-inoculation (dpi).

**Virus detection by DAS-ELISA**

BChV and BYV were detected in leaves of sugar beets at 21 dpi by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA **(Adams & Clark, 1977)** with turnip yellows virus antibodies that recognize BChV as well (our observation) and beet yellows virus-specific antibodies, respectively (Loewe Biochemica, Sauerlach, Germany). Samples were considered infected when the $OD_{405\ nm}$ was twice the mean value of non-infected control plants (three technical replicates) plus three times the standard deviation.

**Establishing mixed infection in source plants**

Non-viruliferous aphids were fed on BYV-infected plants, or purified BChV particles for 24 h, before being transferred to 2-week-old healthy sugar beet plants for a 3-day inoculation access period. Aphids were then removed manually. The third upper leaves (just below the two youngest leaves) were always used as the virus sources. After aphid inoculation, the plants were maintained in a growth chamber under controlled conditions (22-25 °C and 16/8 h light/dark photoperiod) for three weeks before being analyzed by DAS-ELISA to verify infection.

**Aphid transmission experiments**

Aphids acquired viruses for 24 h from detached source leaves (three leaves from three independent infected source plants that were placed on 1 % agarose in petri dishes), followed by virus inoculation feeding on 15 test plants using three aphids per plant during 72 h. Plants were treated three days later with pirimicarb insecticide to kill aphids. After 3 weeks, plants were analyzed by DAS-ELISA, and the transmission efficiency of each virus from co-infected plants was compared to that of transmission of each virus from mono-infected plants.

**Localization of viruses by SABER-FISH**

For virus localization, we used signal amplification by exchange reaction fluorescent *in situ* hybridization (SABER-FISH), a multiplexable nucleic acid-based detection method. It includes a signal amplification step enabling highly sensitive target revelation. We used the protocol that was originally developed for frozen retinal tissue as described by Kishi **(Kishi et al., 2019)** with some adaptations to optimize the technique for plant tissues. For the detection of BYV and BChV, we designed 25 (BYV) and 16 (BChV) primers with OligoMiner **(Beliveau et al., 2018)**.

A TTT and a concatemer sequence were added to the 3'-end of each primer (concatemer 27 for BChV and 26 for BYV, see **S1 Table**).

Concatemers were added to the 3'-end of the probes by *in vitro* primer exchange reaction (PER) to obtain probes of 300-400 nucleotides length **(Kishi et al., 2019)**. The product of the PER reaction is a probe consisting of two parts: a specific sequence that recognizes the target and the concatemer part that enables target detection and amplification of the signal by binding a concatemer-specific fluorescent imager oligonucleotide. After concatemer extension, probes were purified and concentrated using MinElute PCR purification columns (Qiagen, Courtaboeuf, France). Probe concentrations after purification were determined with a Nanodrop 2000 spectrometer (Thermo Fisher Scientific, Illkirch-Grafenstedt, France) using ssDNA setting. A final concentration of 800 ng/µl of the probe was used for hybridization.

To prepare paraffin-embedded tissues, sections of leaf samples were immersed in 10 ml ice-cold 4 % paraformaldehyde solution in PBS. Samples were fixed by applying a vacuum (~720 mm Hg) overnight at 4 °C. After dehydration in a graded series of ethanol/water, followed by a graded series of ethanol/xylene and xylene/paraffin, the samples were embedded in paraffin (Paraplast, Leica, Nanterre, France). Sections of 10 µm were cut using a rotary microtome and immobilized on Superfrost+ slides. After de-waxing and rehydration, the fixed tissue samples were processed for SABER-FISH as described (Kishi et al., 2019). Briefly, slides were first prehybridized in pre-hybridization buffer, then hybridized with all PER probes simultaneously at 43 °C. This hybridization step was followed by a short second hybridization step at 37 °C to bind the fluorescent imagers (with ATTO-565 or ATTO-647N dyes conjugated to their 5' ends) to the probes. Slides were mounted in Fluoroshield medium (Sigma, Saint-Quentin-Fallavier, France) and observed with a Zeiss Axio Imager M2 microscope equipped with a Hamamatsu Orca-Flash 4.0LT black-and-white camera (Lordil, Lay-Saint-Christophe, France). Images were acquired with a 5x, 20x or 40x objective and with Zeiss 43 HE or AHF Cy5 ET bandpass filter sets for epifluorescence acquisition in the red or far red channels, respectively, or with brightfield or DIC settings for transmission light acquisition. Images were processed with ZEN 2.5 or Image J 1.54 software. Final figures were assembled with Microsoft PowerPoint or LibreOffice Impress.

**Relative virus quantification by one-step TaqMan RT-qPCR**

Gene-specific primer pairs and TaqMan probes for BYV and BChV were designed in

collaboration with GEVES (Angers, France) using the Primer Express 3.0.1 primer design tool (Thermo Fisher Scientific). Primer sets were designed to amplify between 75 to 200 nucleotides of the BYV and BChV genomes **(Table 1)**. The specificity of the primer pairs was controlled. Each primer pair was evaluated by a standard curve with six dilutions and three technical replicates of BChV- or BYV-infected plants. Efficiency rates (E) of 95 % (for BYV) and 94 % (for BChV) were obtained with $R^2 = 0.99$ for both viruses.

To select reference genes, we used a list of stably expressed plant genes previously established on *Arabidopsis thaliana* infected by several viruses **(G. Zhang et al., 2023)**. Orthologous genes were identified in *Beta vulgaris* using the KEGG database and primers compatible with TaqMan and SYBR Green detection were designed using the IDT Primer Request tool available online (https://eu.idtdna.com/pages/tools/primerquest). We tested the suitability and stability of the reference genes by SYBR Green PCR. Of the nine tested reference genes, five were selected with an amplification efficiency ranging from 90 % to 99 %, and with $R^2$ correlation values for the curves ranging from 0.98 to 0.99 **(S2 Table)**. The expression stability of candidate genes was then verified on five biological replicates per treatment (mono- or co-infected sugar beets) at 21 dpi. For each biological replicate, three technical replicates were analyzed. Two hundred ng RNA were used to prepare complementary DNA (cDNA) using M-MLV reverse transcriptase kit (Promega, Charbonnières-les-Bains, France) and oligo(dT) nucleotides. qPCR for reference gene evaluation was performed using Universal SYBR Green Supermix (Bio-Rad, Marnes-la-Coquette, France) with gene-specific primers (20 µM) in a CFX96 Touch Deep Well Real-Time PCR detection system (Bio-Rad). Two µl of initial cDNA were used as template in 10 µl qPCR reaction to evaluate the expression of reference genes. The cycling conditions were 98 °C for 2 min, followed by 40 cycles of 15 sec at 98 °C, 20 sec at 65 °C, with an increment ramp of 0.5 °C per sec. Data from these experiments were entered into geNorm, **(Vandesompele et al., 2002),** Bestkeeper **(Pfaffl et al., 2004)** and Normfinder **(Andersen et al., 2004)**, programs for gene stability validation **(Kozera & Rapacz, 2013; Liu et al., 2023)**.

Relative virus accumulation was measured on five biological replicates using AgPath-ID™ One-step RT-PCR Kit for TaqMan chemistry (Applied Biosystems by Thermo Fisher Scientific). The third upper systemically infected leaf from each source plant was collected at 21 dpi. The whole leaf was homogenized in liquid nitrogen. One hundred mg of ground tissue was used for total RNA extraction using NucleoSpin RNA Plant Kit (Macherey-Nagel, Hoerdt, France). After spectrometric quantification with a Nanodrop 2000, 30 ng of RNA were used in a 23 µL reaction volume. The cycling conditions were 95 °C for 10 min, followed by 40 cycles

of 15 sec at 95 °C, 1 min at 65 °C, using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). The tubulin gene was used as a reference gene and the relative accumulation was determined using the $2^{-DDCt}$ method (Livak & Schmittgen, 2001).

**Table 1: Primer and probe sequences used for virus quantification by RT-qPCR.**

| Virus/sugar beet gene | Sequence (5'---3') | Primer orientation and Probe |
|---|---|---|
| BYV | 5' - TAC TGT TCC AAA CCA GGT CCT TG - 3' | Forward |
| | 5' - ROX - TTG CTT CTT TTT CAA CTC CAC CAC CCT GT - BHQ-2 - 3' | Probe |
| | 5' - GTG CAA CGC AGT TCG AAA CTA A - 3' | Reverse |
| BChV | 5' - GGG ACC ATG GCA CCA TCT T - 3' | Forward |
| | 5' - VIC - TCC CTT ACC ACC GGA TAT TAC CCA ACT CCT - BHQ-1 - 3' | Probe |
| | 5' - GTA ATC TGA CAG CTT TTT CTG AAG AGG - 3' | Reverse |
| Tub[a] | 5' -GGCTTTCTTGCATTGGTACAC - 3' | Forward |
| | 5' - FAM - CGAGGCTGAGAGCAACATGAACGA- BHQ -1 - 3' | Probe |
| | 5' - CATCCTGGTACTGCTGGTATTC - 3' | Reverse |

[a]tubulin

## Aphid feeding behavior

We used the electrical penetration graph (EPG) system (Giga-8 DC-EPG amplifier, EPG Systems, Wageningen, Netherlands) as described by Tjallingii **(Tjallingii, 1988)** to investigate the feeding behavior of *M. persicae*. We created electrical circuits that each included one aphid and one plant by tethering a thin gold wire (12.5 μm diameter and 2 cm long) on the insect's dorsum using conductive silver glue (EPG Systems). To facilitate the tethering process, aphids were immobilized at the edge of a pipette tip with negative pressure generated with a vacuum pump (model N86KN.18, KNF Neuberger, Freiburg, Germany). Eight apterous aphids were connected to the Giga-8 DC-EPG amplifier (EPG Systems) and each one was placed on the third upper leaf of an individual plant. The circuit was closed by inserting a copper electrode into the plant substrate. The feeding behavior was recorded for 8 h at a temperature of 20±1 °C and constant light inside a Faraday cage. Each plant and aphid were used only once. Acquisition of the EPG waveforms were carried out with PROBE 3.5 software (EPG Systems). This software was also used to identify the different waveforms correlated with the phases of *M. persicae* feeding behavior, as described **(Tjallingii & Hogen Esch, 1993)**. The Excel workbook developed by Sarria et al. **(Sarria et al., 2009)** was used to calculate the parameters

of EPG data. For a given behavior, the parameters of occurrence and total duration were measured and used to analyze the feeding behavior. We decided to select the following parameters for analysis because they are relevant for the acquisition of phloem-limited viruses by aphids, *i.e.*, the total durations and occurrences of stylet penetration, pathway phases, extracellular salivation phase, phloem sap ingestion phases, and the time needed by the aphid to perform the first phloem phase.

**Statistical analyses**

Statistical analyses were made with R version 4.3.2 (https://www.r-project.org/).

Differences in virus accumulation between mono- and co-infections were determined using a Student test.

We used Generalized Linear Models (GLMER, package: "lme4") with a likelihood ratio and chi-squared ($\chi^2$) test to assess whether co-infection affected virus transmission. Data on transmission rate were analyzed following a binomial error distribution, and "session" was treated as random factor.

We used Generalized Linear Model (GLM) with the likelihood ratio and the chi-squared($\chi^2$) test to assess whether mono or co-infection affected aphid feeding behavior. As feeding duration parameters were not normally distributed, we used GLM using a gamma (link = "inverse") distribution, and parameters related to frequency of penetration were modeled using GLM with Poisson (link = "identity") distribution. The parameter "time to first phloem phase" was modeled using the Cox proportional hazards (CPH) model and we treated cases where the given event did not occur as censored. The assumption of the validity of proportional hazards was checked using the functions "coxph" and "cox.zph", respectively (R packages: "survival" and "RVAideMemoire").

The fit of all generalized linear models was controlled by inspecting residuals and QQ plots. When a significant effect was detected, to test for differences between treatments, a pairwise comparison using estimated marginal means (package R: "emmeans") (*p*-value adjustment with Tukey method) at the 0.05 significance level was used.

## RESULTS

### Virus transmission by aphids from sugar beet co-infected with BChV and BYV

We wanted to know whether virus acquisition from BYV/BChV co-infected source plants would affect the aphid transmission of each virus. For this, *Myzus persicae* aphids were allowed to acquire BChV and/or BYV from mono- or co-infected sugar beets and then transferred onto test plants for virus inoculation **(Fig. 1)**. The BYV transmission rate was not affected by sugar beet co-infection (aphid transmission rate of 75 % from mono-infected plants and 82 % from BYV/BChV co-infected plants) **(Fig. 1A)**. BChV transmission was significantly reduced when aphids fed on BYV/BChV co-infected plants compared to BChV mono-infected plants (30 % *vs* 48 %, respectively) **(Fig. 1B)**.

Aphids feeding on co-infected plants transmitted both viruses together at 19 % transmission rate, which was lower than expected, based on the virus transmission efficiency of each virus (36 % expected co-infection rate, calculated as the product of the transmission rates using mono-infected source plants). BYV alone was present in 63 % of the aphid-inoculated plants whereas BChV was detected alone in 11 % of the infected plants **(S1 Fig.)**.

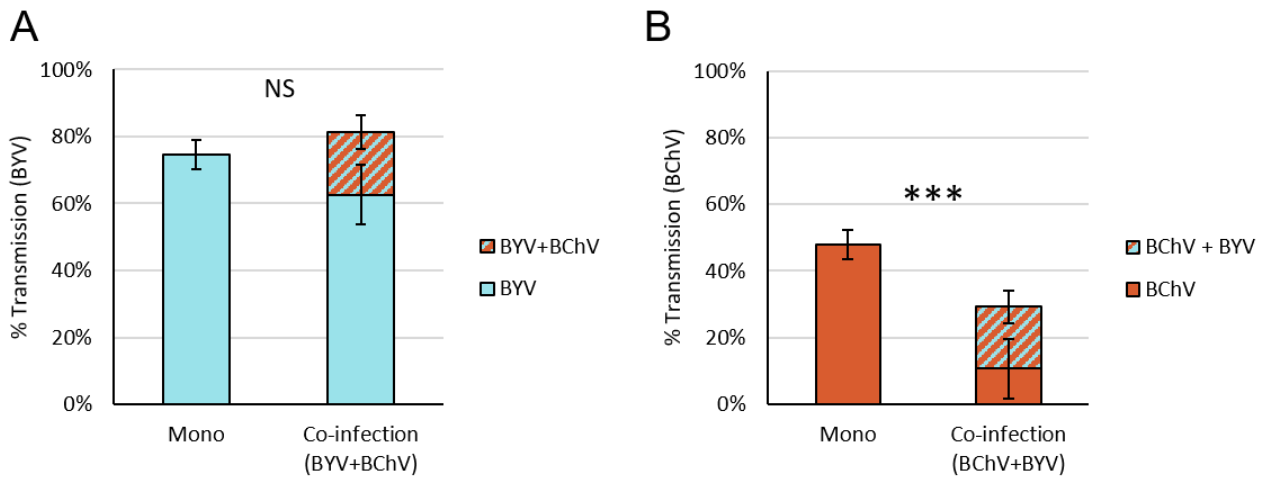



**Figure 1. Aphid transmission efficiency of (A) BYV and (B) BChV from mono or co-infected plants.** *Myzus persicae* acquired virus from mono or co-infected leaves for 24 h. Then, three aphids were transferred per test plant for 72 h inoculation. A DAS-ELISA assay was performed 3 weeks later to detect infection. The percentage of infected plants is indicated (% Transmission). The difference was statistically significant for BChV but not for BYV transmission (GLMER, BYV: p-value = 0.060; BChV: p-value< 0.001; n = 75, five independent experiments; df = 1;). NS = not significant, *** = p-value < 0.001.

### Aphid feeding behavior on mono-infected and co-infected plants or co-infected with both viruses

Aphid feeding behavior plays an important role in virus transmission. To address whether aphid feeding behavior was impacted on sugar beet co-infected with BChV and BYV, compared to mono-infected sugar beet, we used the electrical penetration graph technique (EPG). Aphid total durations of probing time, intercellular pathway, intercellular salivation and the time spent to reach the phloem were the same for all conditions **(Fig. 2A)**. However, aphids spent significantly more time (~ one extra hour) ingesting phloem sap (E2 phase) on BYV mono-infected than on healthy or BChV-infected plants. The total number of all feeding phases were not affected by the different conditions except for the number of extracellular salivations (E1e) that was significantly lower on BYV infected plants than on BChV-infected plants, their number on healthy and BYV/BChV co-infected plants was intermediate (**Fig. 2B**).

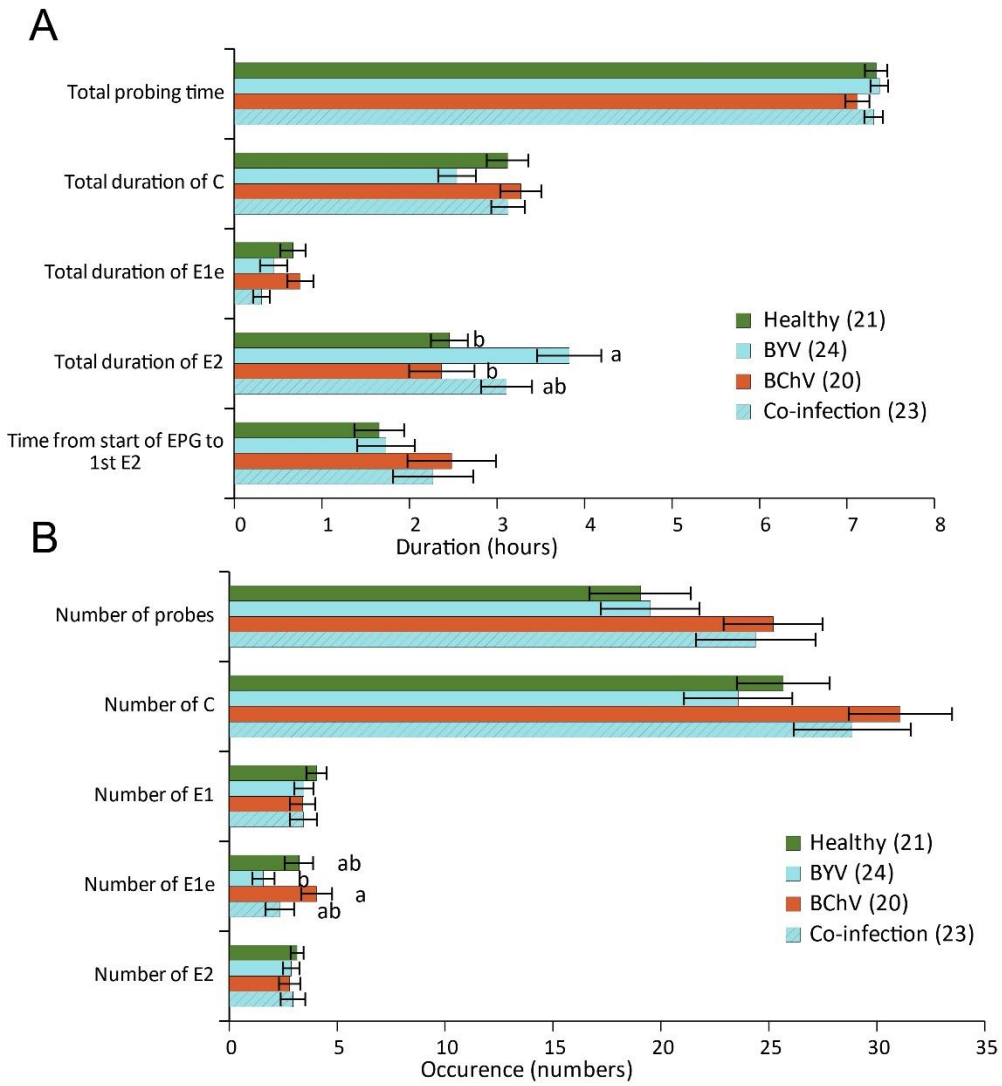



**Figure 2. Feeding behavior of *Myzus persicae* on healthy, BYV and BChV mono-infected or BYV/BChV co-infected sugar beets.** (A-B) The behavior of individual aphids was recorded by electrical penetration graph (EPG) for 8 h on the third upper leaf. Selected EPG parameters are presented according to (A) duration or (B) occurrence. The histogram bars display means and standard errors (SEM). Different letters indicate significant differences between plant infection status as tested by GLM (Generalized linear model) followed by pairwise comparisons

using package R: emmeans (P<0.05 method: Turkey). Statistical analysis of the duration of the events indicates a significant difference for the duration of phloem-sap ingestion (E2) on BYV-infected *vs* healthy or BChV-infected plants (GLM, Df = 3, $X^2$ = 15.98, P = 0.001) but no differences for the total duration of stylet penetrations (probing time) (GLM, Df = 3, $X^2$=2.585, P = 0.460), the total duration of pathway phases (C) (GLM, Df = 3, $X^2$ = 6.962, P = 0.073), the total duration of intercellular salivation (E1e) (GLM, Df = 3, $X^2$ = 5.645, P = 0.130) and the time until first sap ingestion from the phloem (Cox, Df = 3 ,p= 0.526). Statistical analysis of the occurrence of events revealed significant differences for the intercellular salivation between BYV and BChV infected plants (GLM, Df = 3, $X^2$ = 8.581 ,P = 0.035) but no differences for the number of stylet insertions (GLM, Df = 3, $X^2$ = 4.725,P = 0.193), pathway phases (C) (GLM, Df = 3, $X^2$ = 4.954, P = 0.175), salivation phase (E1) (GLM, Df = 3, $X^2$ = 1.006, P = 0.800), and phloem sap ingestion (GLM, Df = 3, $X^2$ = 0.459, P = 0.928). n = 20-24 as indicated in the graphs.

## BChV and BYV accumulation in mono- or co-infected plants

To address whether BChV and BYV accumulation could be impacted by co-infection, we quantified virus titers by RT-qPCR on sugar beet mono- or co-infected with BYV and BChV. To account for potential uneven virus distribution in the leaf, the entire leaf was ground and processed for RNA extraction and virus quantification. A reference gene, tubulin, was selected for calibration, among six tested, prior to virus quantification **(S2 Table)**. No significant differences in virus accumulation between mono-infected plants or BYV/BChV co-infected plants were observed **(Fig. 3)**.

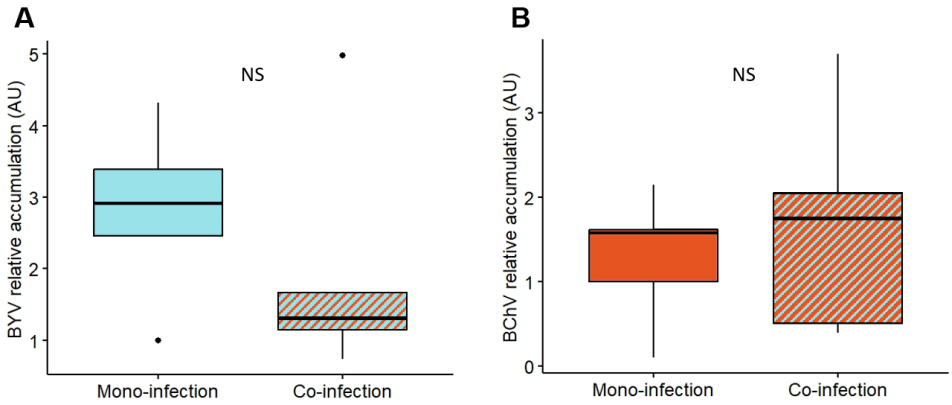


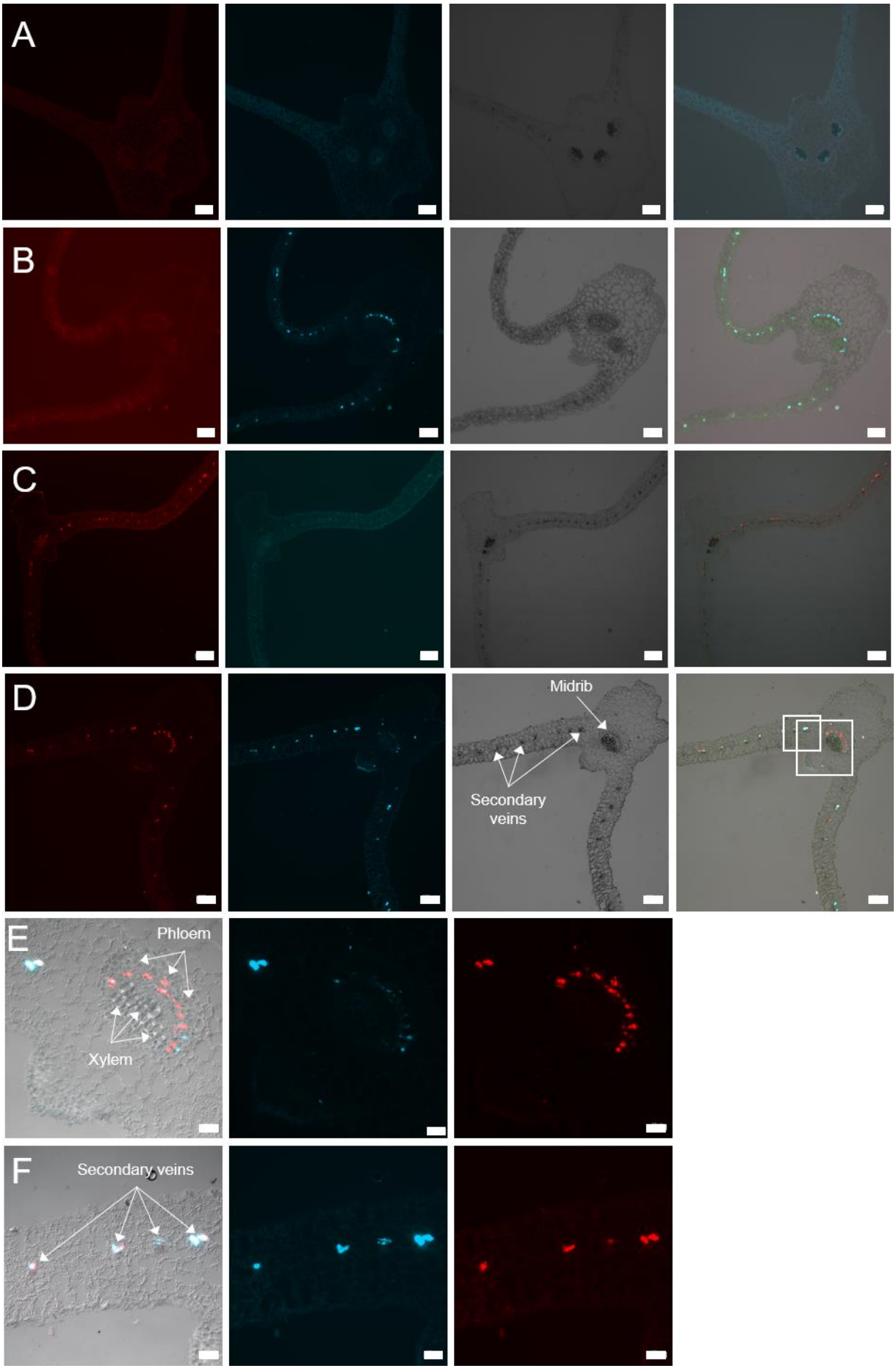

**Figure 3. Relative accumulation of BYV and BChV in mono- and co-infected plants at 21 dpi.** Virus accumulation in source plants was measured by multiplex TaqMan RT-qPCR as described in Materials and Methods. Box plots show median (black line), 25%-75% percentiles (box) and 10%-90% percentiles (whiskers), and outliers (dots). Differences were not statistically significant (Student t-test, BYV: t = -0.90817, p-value = 0.390; BChV: t = 0.59456, p-value= 0.569; n = 5; df = 8). NS = not significant.

## Tissue distribution of BChV and BYV in mono- and co-infected plants

To assess if co-infection affected tissue localization of BChV or BYV, SABER-FISH was performed on mono- and co-infected plants at 21 dpi. In cross-sections of BYV mono-infected leaves, fluorescence corresponding to BYV genomes was observed exclusively in phloem cells as expected **(Fig. 4B)**. The label was found in the phloem of primary (midribs) and secondary veins and the label intensity, estimated by visual observations, was equal in both locations

**(Fig. 4B)**. In BChV mono-infected plants, fluorescence corresponding to BChV genomes was also observed, as expected, in the phloem of primary and secondary veins **(Fig. 4C)**. However, the BChV label was less intense in the primary veins than in the secondary veins. Co-infection altered the distribution of BYV and BChV **(Fig. 4D)**. Compared to mono-infection, less cells were labeled with BYV in midribs and more cells in the secondary veins in co-infected plants. Compared to mono-infected plants, BChV was present in more phloem cells of primary veins in co-infected plants, while the BChV label in secondary veins was unchanged. Both viruses were still confined to phloem cells and no escape in non-phloem cells was noticed **(Fig. 4D-F)**.

We estimated the percentage of co-infected cells and mono-infected cells in co-infected plants. Ten to eleven sections from co-infected plants were observed in two independent experiments **(Table 2)**. We noticed a slightly lower percentage of cells infected with BChV (63.2 % of cells containing only BChV or co-infected) compared to BYV (74.3 % of cells containing only BYV or co-infected) **(Table 2**). Between 30-50 % of all infected cells were co-infected, and similar percentages of cells were mono-infected with BChV or BYV. This suggests that there is no exclusion between the two viruses at the cellular level. The label intensity of each virus seemed to be similar in mono-infected and co-infected cells, indicating that they did not interfere with each other's accumulation.


A
B
C
D
Midrib
Secondary veins
E
Phloem
Xylem
F
Secondary veins

**Legend to Figure 4 (Previous page). Tissue distribution of BChV and BYV in leaves mono-infected with BChV, BYV or co-infected with both viruses.** Leaves were processed three weeks after inoculation by SABER-FISH for detection of BChV and BYV. (A-D) Representative images showing transversal sections of (A) healthy, (B) BYV mono-infected, (C) BChV mono-infected, and (D) BChV/BYV co-infected leaves. The first column shows the BChV label (red), the second the BYV label (turquoise), the third bright field acquisitions and the last column presents merges. (E-F) Magnifications of the regions outlined in (D) showing virus distribution in (E) the midrib and (F) the leaf lamina. The first column shows image merges, the second the BYV signal (red), and the last column the BChV label (red). Scale bars = 200 µm (A-D) and 50 µm (E-F).

## Intracellular distribution of BYV and BChV in mono and co-infected plants

Cross sections of phloem tissue allow only a limited view of the phloem cell and sieve tube lumen because their elongated shapes are arranged perpendicular to the section plane. To better assess if co-infection affected intracellular distribution of BChV or BYV, SABER-FISH was performed on longitudinal sections of infected plants at 21 dpi **(Fig. 4)**. In mono-infected plants, BYV label was present in infected cells as spherical cytoplasmic inclusions that aligned like pearl chains, similarly as reported before using light microscopy **(Esau, 1960) (Fig. 4A)**. In the case of BChV mono-infected plants, the virus label was spread uniformly in the cytoplasm of infected cells **(Fig. 4B)**. Similar label patterns were also found in immunofluorescence experiments **(Fig. S2)**, suggesting that the FISH label corresponded, at least partially, to virions.

**Table 2: Number of cells infected by one or both viruses in sections of co-infected leaves.**

| | **Exp. 1** | | | **Exp. 2** | | |
|---|---|---|---|---|---|---|
| Virus(es) observed | Plant #1 | Plant #2 | Plant #3 | Plant #1 | Mean[c] | %[d] |
| **BChV** | 32[a] (33.0 %[b]) | 13 (20.6 %) | 3 (6.3 %) | 22 (34.4 %) | 17.5 ± 12.4 | 25.7 % |
| **BYV** | 33 (34.0 %) | 19 (30.2 %) | 27 (56.3 %) | 21 (32.8 %) | 25 ± 6.3 | 36.8 % |
| **BYV+BChV** | 32 (33.0 %) | 31 (49.2 %) | 18 (37.5 %) | 21 (32.8 %) | 25.5 ± 7.0 | 37.5 % |

[a] Number of cells observed on 10 to 11 sections of each plant showing a fluorescent signal for the indicated virus. [b] Percentage of cells labeled for the indicated virus/total number of labeled cells in 10 to 11 sections. [c] Mean number of cells observed on 10 to 11 sections for 4 independent plants. [d] Percentage of cells containing one or both viruses/total number of cells observed in the experiments.

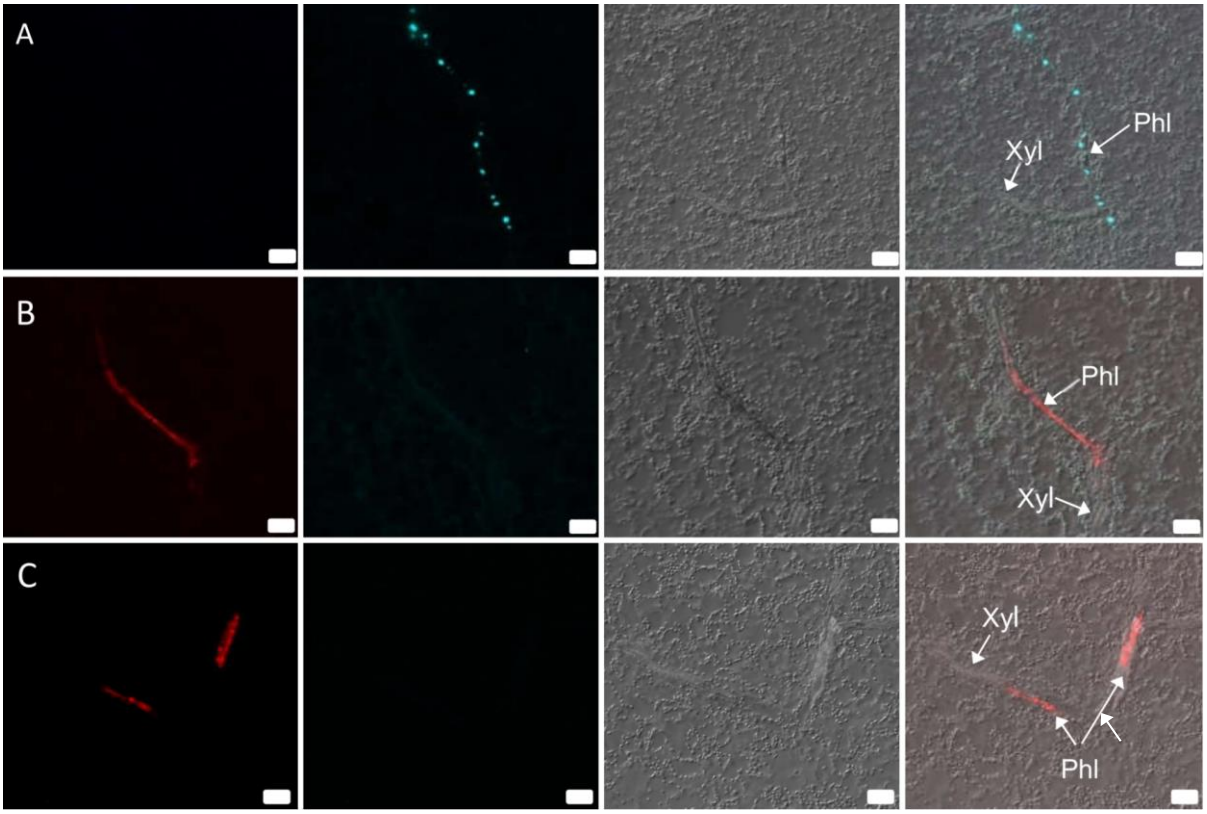



**Figure 5. Intra-cellular distribution of BYV and BChV in leaves of mono-infected plants.** Leaves were processed three weeks after inoculation by SABER-FISH for detection of BYV and BChV. The images show longitudinal sections of (A) a leaf mono-infected with BYV and (B-C) a leaf mono-infected with BChV with virus label in the phloem in (B) probably a sieve tube and (C) probably in a sieve tube and in a companion cell (left and right arrows pointing from Phl, respectively). The first column represents BChV label (red), the second BYV label (turquoise), the third differential interference contrast images, and the last column presents image merges. Xyl, xylem; Phl, phloem. Scale bars = 20 µm.

In BYV/BChV co-infected plants, both viruses showed the same intracellular distribution as in mono-infected plants when the cells were infected by one virus only, *i.e.*, pearl-like inclusions for BYV and homogenous cytoplasmic distribution for BChV **(Fig. 6A-B)**. However, in co-infected cells, the BYV label was mostly dispersed in the cytoplasm and colocalized partially with the diffuse BChV label **(Fig. 6C-D)**.

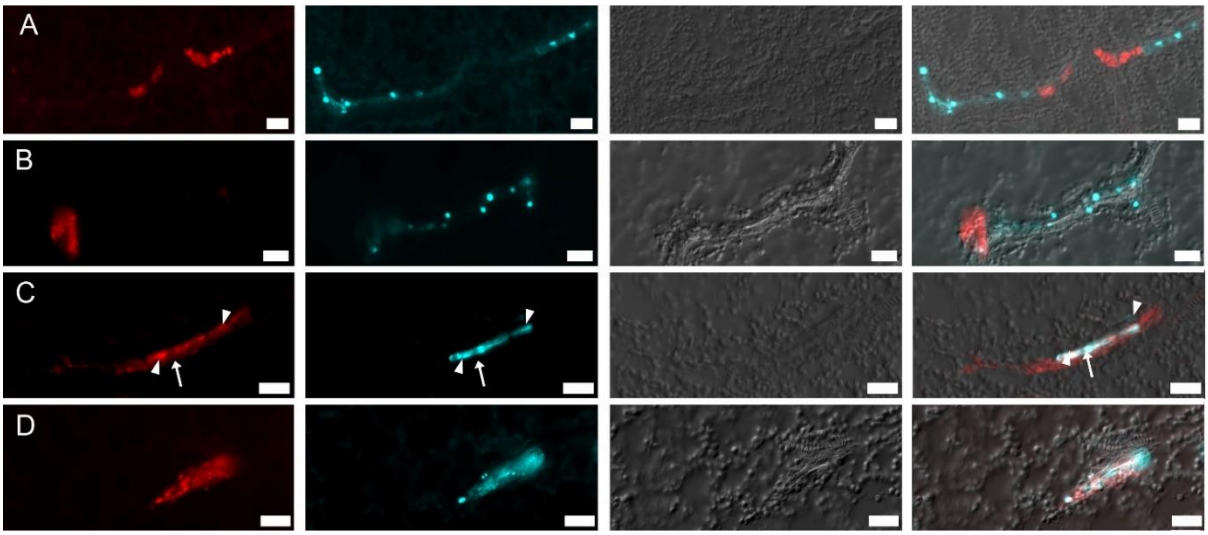



**Figure 6. Intra-cellular distribution of BYV and BChV in leaves of co-infected plants.** Leaves were processed three weeks after inoculation by SABER-FISH for detection of BChV (red) and BYV (turquoise). The images show longitudinal sections of phloem with (A-B) mono-infected cells, (C) a cell mono-infected with BChV (arrow) and an adjacent cell co-infected with both viruses (arrowheads), and (D) a co-infected phloem cell. The first column presents BChV label, the second BYV label, the third differential interference contrast images, and the last column image merges. Scale bars = 20 µm.

## DISCUSSION

Few studies have shown that viruses from various genera or families co-existing in multi-infection can modify vector transmission, and even less have addressed modifications at the cellular level. Additionally, to our knowledge, this is the first study examining the effect of co-infection by a polerovirus, (BChV) and a closterovirus (BYV), both phloem-limited and sharing the same aphid vector, on the transmission efficiency and other infection parameters. We found that co-infection of sugar beet with BYV and BChV reduced aphid transmission of BChV significantly, compared to transmission from mono-infected plants. No effect of the co-infection of sugar beet was observed on the transmission efficiency of BYV.

To find possible explanations for the reduction of BChV transmission from co-infected plants, we analyzed aphid feeding behavior, virus accumulation, and virus distribution in the leaves and the infected cells on mono- and co-infected plants.

Viral infections may impact aphid-feeding behavior, which can have a direct impact on virus acquisition and transmission **(Mauck et al., 2012; Mauck et al., 2016)**. The feeding behavior can be further modified in viral co-infections, but this has been hardly studied. One of the few published examples is that of raspberries co-infected with raspberry latent virus and raspberry leaf mottle virus. In this pathosystem, no changes in feeding behavior were observed, although aphid preferences for infected plants did change **(Lightle & Lee, 2014)**. Another example is

the co-infection of melon plants with the potyvirus watermelon mosaic virus (WMV) and the crinivirus cucurbit yellow stunting disorder virus (CYSDV). There, WMV accumulation was ten times lower in co-infected plants, but its transmission rate by aphids remained stable. EPG analysis showed that aphids on co-infected melons performed longer duration of ingestion (more specifically, pulses of the subphase II-3) during intracellular test punctures in the epidermis and mesophyll, which is the feeding behavior associated with the acquisition of non-persistently transmitted viruses like WMV. The authors argued that the changed feeding behavior might compensate for the low WMV accumulation in co-infected plants **(Domingo-Calap et al., 2020)**. Thus, aphid feeding behavior could be a determining factor that affects virus acquisition and explains the lowered transmission of BChV from co-infected plants. Since BChV should be acquired from the phloem sap**,** longer phloem sap ingestion should increase acquisition **(Gray et al., 1991; Prado & Tjallingii, 1994)**. However, our results showed that despite aphids having a tendency to ingest phloem sap for longer durations on co-infected plants than on mono-infected ones, transmission of BChV dropped. This indicates that the decreased transmission of BChV cannot be explained by aphid feeding behavior. Interestingly, for BYV, transmission from co-infected plants was not affected although aphids tended to feed less on co-infected plants than on mono-infected plants. We assume that under our experimental conditions, the 3 h sap ingestion observed for aphids feeding on co-infected plants is sufficient to charge aphids maximally with BYV and that for this reason, we did not observe a difference in transmission efficiency of BYV from co-infected or mono-infected plants. This is in line with the results by Jiménez and co-workers (2018) who demonstrated that BYV maximum acquisition was obtained after 3 to 6 h of phloem sap ingestion, with no significant increases in transmission rates observed for longer sap ingestion periods **(Jiménez et al., 2018)**. Taken together, we have no evidence that the altered aphid feeding behavior observed on co-infected sugar beets is the cause of the lower BChV transmission from these plants.

Lower or higher virus accumulation in co-infected compared to mono-infected plants may also directly affect transmission efficiency by modulating the amount of virus particles acquired by the vector. For instance, vector transmission efficiency of two criniviruses, tomato chlorosis virus (ToCV) and tomato infectious chlorosis virus (TICV), was linked to virus accumulation in mono- and co-infected tomatoes **(Wintermantel et al., 2008)**. However, an overall higher accumulation of one or both viruses in co-infected plants does not necessarily lead to increased vector transmission. Rather, the virus titer increase needs to occur in the aphid feeding sites (e.g., sieve tubes for polerovirus acquisition) **(Prado & Tjallingii, 1994)**. In this sense, a recent

study showed that co-infection of tomato with a begomovirus and a crinivirus resulted in a synergistic effect on the accumulation of both viruses, but only increased the acquisition efficiency of the begomovirus **(Li et al., 2021)**. An increase in PLRV accumulation in *Nicotiana clevelandii* was observed when the plants were co-infected with the potato virus Y and the co-infection also induced phloem escape of PLRV **(Barker, 1987)**. However, aphid transmission of PLRV and PVY was not addressed in this study. Our results showed that transmission of BChV drops when plants are co-infected with BYV, although BChV levels were not statistically different in mono- compared to co-infected plants. This indicates clearly that changes in aphid transmission rates of BChV are not linked to virus titer.

Another reason to explain altered transmission might be changes in the tissue tropism of viruses in plants that might modify virus accessibility to aphids. For instance, phloem escape of aphid-vectored PLRV in tobacco was reported in plants co-infected with pea enation mosaic virus 2 (PEMV-2, **(Ryabov et al., 2001b)** or PVY **(Barker, 1987)**. The changed tissue specificity correlated with PLRV becoming mechanically transmissible in PLRV/PEMV-2 co-infections**.** In our observations, we did not find evidence of a phloem escape of BChV or BYV in co-infected plants, ruling out altered tissue tropism as the reason for reduced BChV transmission from co-infected plants. What we did observe in co-infected plants, though, was that BChV localized equally in the phloem of primary and secondary veins, while BYV was more present in those of secondary veins. However, since both viruses are acquired preferentially from sieve tube sap, it is unlikely that the tissue relocalization impacted virus acquisition greatly.

We found that in co-infected phloem cells (representing about 40 % of all infected cells), the intracellular distribution of BYV was modified. BYV was present as spherical, cytoplasmic inclusions aligning in chains in cells of mono-infected plants. BChV was spread uniformly in the cytoplasm in cells of mono-infected plants. In co-infected plants, both viruses displayed the same intracellular localization when cells were infected by only one virus. This was different in co-infected cells. While BChV maintained its diffuse distribution, the typical BYV inclusions disappeared and BYV label was diffuse in the cytoplasm and colocalized partially with BChV. We take this as evidence that the two viruses interact directly or indirectly with each other. Since no other notable differences were detected in co-infected *vs* mono-infected plants, virus-virus interactions in co-infected cells likely caused the drop in BChV transmission. At this stage, the exact mechanism of interaction between BChV and BYV is unknown. Because BChV should be acquired predominantly from the phloem sap and only to a small extent from phloem cells **(Prado & Tjallingii, 1994)**, we propose that co-infection limits BChV release from

companion cells into the sieve tubes **(Fig. 7)**. This would result in a lower BChV accessibility to aphids, compared to BChV mono-infected plants. This could be achieved by alterations of the intracellular virus trafficking or modifications of the cell wall or plasmodesmata. Several BYV and BChV proteins are known to be involved in virus movement and interaction with plasmodesmata and are therefore good candidates for further studies **(Boissinot et al., 2020; Dolja, 2003)**.

Our results show that the percentage of co-infected cells and those mono-infected with BYV or BChV were in the same range (about 30 %) in co-infection. This suggests that co-infection is a random event and that the viruses do not exclude each other. Further, there was no alteration in the accumulation of either virus, indicating that there is no competition for plant resources and that they do not interfere with each other's replication. This is indicative of neutralism. This assumption is also reinforced by the fact that BYV/BChV co-infection, compared to infection with BYV alone, does not have a more severe impact on sugar beet root mass (https://www.itbfr.org/publications/fiches-bioagresseurs/les-jaunisses-virales-et-leurs-pucerons-vecteurs/, accessed on 4 Avril 2024). Note that the same sugar beet cultivar was used in both studies.

In conclusion, our results shed some light on the mode of interaction between two unrelated viruses, a polerovirus and a closterovirus, a combination that has never been studied before. Except for the antagonism observed for BChV transmission by aphids, the outcome of the sugar beet co-infection with BChV and BYV is fairly neutral for all other parameters analyzed. The reasons for BChV decreased aphid transmission by aphid are unknown but the subcellular localization of both viruses in co-infected plants strongly suggests interactions between them that are worth exploring. Finally, the alteration of aphid transmission of BChV from co-infected plants might have consequences at the epidemiological level. For example, it might explain partially the huge changes in incidences of the two viruses from one year to another **(Hossain et al., 2021)**.

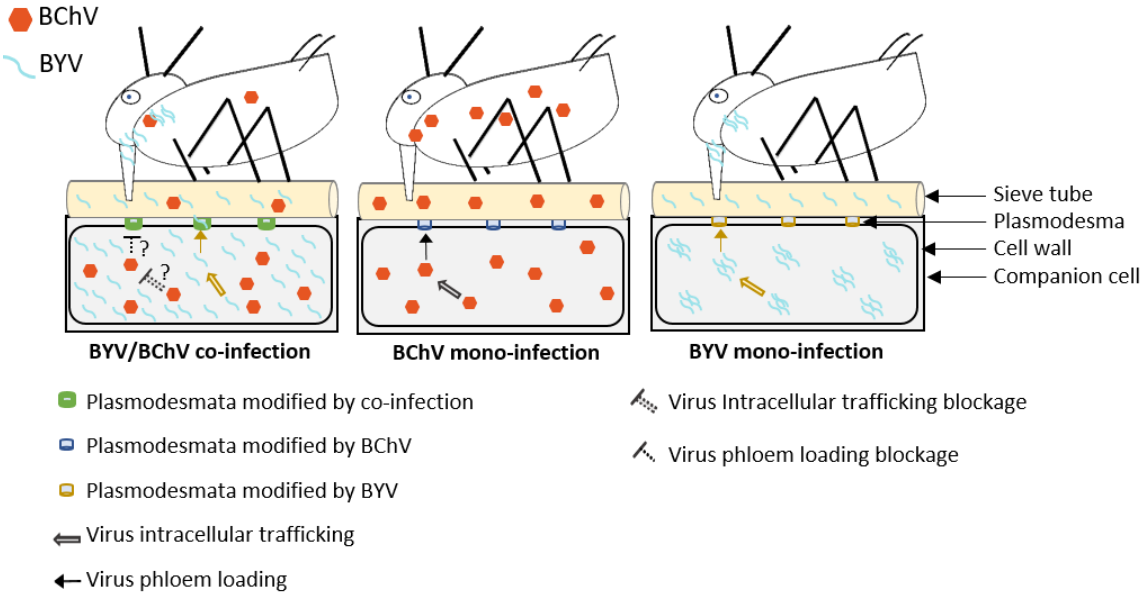



**Figure 7. Model for explaining the reduction of BChV transmission from BChV/BYV co-infected plants.** In mono-infected plants (middle and right cell), BChV and BYV particles are dispatched from infected companion cells through plasmodesmata into the sieve tube lumen and acquired by aphids ingesting phloem sap from the sieve tubes. In co-infected plants, roughly one-third of the infected phloem cells are co-infected. Co-infection does not affect virus accumulation measurably, and the two viruses might even accumulate to similar levels in mono-infected and co-infected cells, as presented in the schema (left cell). BYV and BChV interact in co-infected cells directly or indirectly. This inhibits the passage of BChV towards and/or through plasmodesmata and BChV release into the phloem sap, whereas phloem sap loading with BYV remains unchanged. Consequently, the BChV load in the sieve tube is significantly lower, while that of BYV is not changed, and aphids acquire and transmit less BChV from the sieve tubes of co-infected plants than from those of mono-infected plants. Two scenarios are thinkable that are not mutually exclusive: Co-infected phloem cells inhibit BChV release totally, and the BChV particles in the phloem sap origin from BChV mono-infected cells, or BYV infection interferes with phloem loading from all companion cells. In the former case, BYV operates only in co-infected cells; the latter case requires the movement of a sgnal or factor from BYV-infected cells to uninfected and BChV-infected cell.

## AUTHORS' CONTRIBUTIONS

**SK:** Conceptualization, methodology, validation, formal analysis, investigation, writing – original draft preparation, writing – review & editing, visualization

**QC:** Methodology, formal analysis, writing – review & editing

**CV:** Resources

**VB:** Conceptualization, methodology, investigation, writing – original draft preparation, writing – review & editing, supervision, project administration, funding acquisition

**MD:** Conceptualization, methodology, investigation, writing – original draft preparation, writing – review & editing, visualization, supervision, project administration, funding acquisition

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## DATA AVAILABILITY

All relevant data are within the manuscript and its Supporting Information files.

## COMPETING INTERESTS

The authors have declared that no competing interests exist.

## SUPPORTING INFORMATION

**S1 Fig.** Aphid transmission efficiency of BYV and BChV from co-infected plants.

**S2 Fig.** Tissue distribution of BChV and BYV in roots mono-infected with BChV or BYV.

**S1 Table.** Primers with concatemer sequences used for Primer Exchange Reaction.

**S2 Table.** Amplification efficacy (%) of reference genes, and the associated correlation coefficient ($R^2$).

**S1 Data.** Transmission.

**S2 Data.** EPG experiments.

**S3 Data.** RT-qPCR virus accumulation.

**S4 Data.** Co-infection cell count.

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# Chapter 3: New biological features of sugar beet infecting poleroviruses and impact of co-infection on virus transmission by aphids

Hélène Schlaefli, Aurélie Marmonier, Souheyla Khechmar, Catherine Reinbold, Claire Villeroy, Quentin Chesnais, Martin Drucker, Véronique Brault

INRAE, Université de Strasbourg, SVQV, Colmar, France

**Correspondence :**
Véronique Brault
veronique.brault@inrae.fr

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## ABSTRACT

Beet mild yellowing virus (BMYV) and beet chlorosis virus (BChV) are two poleroviruses infecting sugar beet that induce leaf yellowing and can cause high yield losses. Like other poleroviruses, the closely related BMYV and BChV are phloem-limited and strictly transmitted by aphids in a circulative and persistent manner. In this study, we showed that BMYV and BChV were internalized in the aphid's body at the posterior midgut. In sugar beet, we observed that each virus was distributed evenly throughout the infected plants when inoculated at an early stage of development, while old leaves escaped infection when the plants were inoculated at a later stage. Plant development was also negatively correlated with susceptibility to virus inoculation. Plant co-infection had no major impact on virus accumulation, and BMYV and BChV were shown to co-localize in about 40% of infected cells, revealing no viral exclusion at the cellular level. Co-acquisition of both viruses by aphids, from infected plants or purified viruses, resulted in an increased transmission efficiency suggesting a cooperative effect of both viruses occurring at the inoculation step. Our results shed light on fine virus-virus interactions with potential epidemiological outcome.

## INTRODUCTION

Since the ban of neonicotinoid insecticides in 2018, sugar beet yellowing is a re-emerging plant disease in all sugar beet growing countries with a major impact on sugar beet yield. Sugar beet yellowing is caused by a complex of viruses comprising beet mild yellowing virus (BMYV) and beet chlorosis virus (BChV) both belonging to the *Polerovirus* genus in the *Solemoviridae* family, beet yellows virus (BYV, *Closterovirus* genus, *Closteroviridae* family), and beet mosaic virus (BtMV, genus *Potyvirus*, *Potyviridae* family). Sugar beets can also be infected with beet western yellows virus-USA (BWYV-USA, *Polerovirus*), a virus that has not been reported so far in Europe (Beuve et al., 2008; Hossain et al., 2021b; Stevens et al., 2005). All these viruses induce leaf yellowing which decreases the photosynthetic capacity of the plant resulting in reduced root weight, sugar content and processing quality. However, extent of damage depends on the year, the sugar beet variety, the moment of infection and other undetermined factors (Stevens et al., 2004a). BMYV, BChV, BYV, BWYV and BtMV are all transmitted by aphids, mainly *Myzus persicae*, using different transmission modes, *i.e.*, persistent and circulative for BMYV, BChV and BWYV, semi-persistent and non-circulative for BYV, and non-persistent and non-circulative for BtMV (Brault et al., 2007; S. Gray & Gildow, 2003, 2003; Kozlowska-Makulska et al., 2009; Schliephake et al., 2000; Stevens et al., 2005). In nature, these viruses can be found alone in yellowing-exhibiting sugar beets, but

mixed infections have been reported with a frequency that varies depending on the year (Hossain et al., 2021b). Interactions between sugar beet viruses may have agronomical consequences like the coinfection of BYV and BtMV which reduced sugar beet biomass in greenhouse conditions and resulted in an increased accumulation of both viruses (Wintermantel, 2005b). Additive effects of BtMV when combined with BWYV or BYV on yield losses have also been reported in the field, and a higher yield loss was also observed when BYV was combined with BMYV (Russell, 1963). However, a moderate impact on yield loss was monitored in plots co-inoculated with BYV and BChV (Hossain et al., 2021b).

BMYV and BChV are closely related poleroviruses which possess a positive-sense single stranded RNA genome of about 5.6 kb encapsidated into icosahedral particles of about 30 nm composed of the major coat protein (CP) and a minor component referred to as the readthrough protein (RT*) (Mayo & Ziegler-Graff, 1996; Stevens et al., 2005). Alignment of the full-length genomic sequences of BMYV (Guilley et al., 1995), and BChV (Hauser et al., 2002c) reveals high sequence homologies within the 3' part of their genomes. This region encompasses the major coat protein (CP encoded by ORF3), the movement protein (encoded by ORF4), and the readthrough domain (encoded by ORF5) sequences. In addition, this region contains a small ORF, ORF3a, upstream of ORF3, which is involved in long-distance virus movement and which is found in both viruses (Smirnova et al., 2015b). In contrast, the 5' end of both genomes displays less than 30% sequence homology (Hauser et al., 2002c). High sequence similarity in both virus genomes is potentially responsible for the occurrence of virus recombinants observed in BMYV and BChV co-infected plants (Kozlowska-Makulska et al., 2015b), although a polymerase template switch during the cDNA synthesis that could result in chimeric genomes, cannot be completely discarded (Cocquet et al., 2006). Poleroviruses are phloem-limited and localization of BChV in phloem cells of sugar beets has been confirmed (Esau & Hoefert, 1971; Khechmar et al., 2024; Mayo & Ziegler-Graff, 1996; Mutterer et al., 1999b). Sugar beet poleroviruses have a wide host range, but BMYV and BChV display differences in host specificity (Beuve et al., 2008; Hauser et al., 2002c; Stevens et al., 1994). BChV and BMYV are mainly transmitted by *Myzus persicae* but can also be propagated from plant to plant by *Macrosiphum euphorbiae* and, in rare occasions, by *Aphis fabae* (Kozlowska-Makulska et al., 2009; Schliephake et al., 2000).

Sugar beets display mature plant resistance (MPR) to aphids, a mechanism resulting in an increased mortality rate and a decreased fecundity of aphids when plants reach an advanced developmental stage (Kift et al., 1996, 1998; Panter & Jones, 2002; Schop et al., 2022). This

phenomenon has been observed for decades in the field and impacts control measures which take into consideration the plant developmental stage. Indeed, after canopy closure, insecticide treatments decreased since the plant is considered less sensitive to the disease (Dewar, 1994). Interestingly, the negative impact of MPR on *M. persicae* survival is exacerbated on healthy sugar beets compared to plants infected with BMYV and even more when compared to plants infected with BYV (Kift et al., 1996; Zhu et al., 2024).

The present study was conducted to gain more insights into sugar beet poleroviruses (BMYV and BChV) biology by addressing sugar beet susceptibility to these viruses depending on the developmental stage and virus distribution in plants. Since both viruses can be present at the same time in sugar beets, and are both transmitted by *M. persicae*, we also addressed the effect of sugar beet coinfection on virus accumulation, virus cellular localization, and aphid transmission.

## MATERIALS & METHODS

### Virus, aphids, and plants

The beet chlorosis virus strain BChV-2a was collected in England from sugar beet (Hauser et al., 2002c). The beet mild yellowing virus strain 2itb is a French isolate also collected from sugar beet (Guilley et al., 1995). Both viruses were purified from infected spinach (*Spinacia oleracea*) collected 4 to 5 weeks after inoculation using viruliferous aphids and following the procedure described in (van den Heuvel et al., 1991). The *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) biotype WMp2 originated from the Netherlands (Reinink et al., 1989b). Aphids were reared on Chinese cabbage (*Brassica rapa L. pekinensis var. Granaat*) in a growth chamber under 20 ± 1°C, and 16 h photoperiod. The commercial variety of sugar beet (*Beta vulgaris* cv Auckland; SesVanderHave, Belgium) was kindly provided by the French Technical Institute of Sugar Beet (Paris). Sugar beet plants were grown in greenhouses until the first two true leaves developed (2 weeks after sowing). Plants were then grown in laboratories and growth chambers under 21±1°C and 14 h of light and 10 h of darkness.

### Virus inoculation

To inoculate sugar beets with one or both viruses, two techniques were used: (I) non-viruliferous *M. persicae* were fed with an artificial medium (Harrewijn, 1983) containing purified BChV or BMYV virions or a mixture of both purified viruses. After a 24 h acquisition period, aphids (1 to 3 aphids per test plant depending on the experiment) were transferred onto 3-week-old sugar beets for a 72 h inoculation period. Aphids were then killed by an insecticide

(Pirimor). Plants were grown for three weeks before being tested by DAS-ELISA first and then by IC-RT-PCR or RT-PCR to distinguish the two poleroviruses; (II) alternately, non-viruliferous *M. persicae* were fed on sugar beet infected with BChV or BMYV alone or with both viruses. For each transmission experiment, three source plants for the same condition (infected only with BMYV, BChV or coinfected with BMYV and BChV) were used. Non-viruliferous aphids were deposited either on whole infected plants, or on detached leaves laid on a 1% agarose in $H_2O$ in petri dishes. After a 24 h acquisition period, 3 aphids per test plant) were transferred onto 3-week-old sugar beet plants for 72 h that were thereafter processed as described above.

**Virus detection**

***DAS-ELISA***

Both viruses were detected in sugar beets by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with a turnip yellows virus (TuYV)-specific polyclonal antiserum at $1/200^e$ dilution (Loewe Biochemica, Sauerlach, Germany) 3 weeks post-inoculation (Clark & Adams, 1977). Because of high sequence similarities between the structural proteins of TuYV (referred to previously as BWYV) and those of BChV and BMYV viruses (Hauser et al., 2002c) the TuYV antiserum is able to detect by DAS-ELISA BChV, BMYV as well as several other closely-related poleroviruses. For each plant, 2 to 3 leaves were sampled. Absorbance values were measured at 405 nm with a spectrophotometer Infinite F50 equipped with the software Magellan reader (Tecan, Männedorf, Switzerland). Samples were considered infected when the $OD_{405\ nm}$ was twice the mean value of non-infected control plants (i.e., 3 technical replicates) plus three times the standard deviation.

***RT-PCR and IC-RT-PCR***

To discriminate between the two poleroviruses, two procedures were developed. For RT-PCR, total RNA was extracted using NucleoSpin RNA Plant Kit (Macherey-Nagel, Hoerdt, France). The RT-PCR was performed using the virus-specific primers (Table S1) by the one-step procedure (One step RT-PCR kit, Qiagen, Hilden, Germany) following instructions of the supplier. The primers final concentrations were adjusted depending on the virus (0.6 µL for each primer at 25 µM for BChV and 1.2 µL for each primer at 25 µM for BMYV). To detect BMYV and BChV by immunocapture (IC)-RT-PCR, a first step similar to DAS-ELISA was performed. Briefly, the ground leaf sample was deposited in coated ELISA plate (dilution Loewe antiserum $1/200^e$). After three washes, 10 µL of RNase free water were added to each well, and the plate was sealed before being incubated at 80°C for 20 min, followed by 5 min on

ice. RT-PCR one step (One step RT-PCR kit, Qiagen, Hilden, Germany was then performed on 4.5 µL with the aforementioned primers concentrations.

***Virus quantification by quantitative RT-PCR (RT-qPCR)***

Fragments of 2 to 3 young non-inoculated leaves (100 mg) were collected for each plant at specific times, ranging from 3 to 13 weeks post-inoculation. Total RNA was extracted using NucleoSpin RNA Plant Kit (Macherey-Nagel, Hoerdt, France). After quantification (Nanodrop 2000, ThermoFisher Scientific, Illkirch, France), 200 ng of viral RNA were converted to cDNA using a specific reverse primer (Table S1) and the M-MLV reverse transcriptase kit (Promega, Charbonnières-les-Bains, France). The forward and reverse primers (Table S1) were used to amplify the cDNA using a CFX cycler (Biorad, Schiltigheim, France) programmed as follows: 3 min 95°C, 40 amplification cycles for 10 s at 95°C, 30 s at 60°C. Melt curve analysis was performed from 60°C to 95°C with 5 s of 0.5°C increments. Dilution series of $10^8$ to $10^3$ viral cDNA copies obtained from RNA extracted from purified virions were used for calibration. The comparison between the Ct values of the calibration standard and the samples provided an absolute quantification of BMYV or BChV genomes.

**Virus observation using Transmission Electron Microscopy (TEM)**

For ultrastructural TEM observations, *M. persicae* reared on pepper seedlings (*Capsicum annuum*) were allowed to acquire purified virus (90 ng/µL for BChV and 100 ng/µL for BMYV) included in an artificial medium for 48 h to 72 h (Bruyère et al., 1997b) before being processed as described in (Alliaume et al., 2018; Reinbold et al., 2001).

**Probe synthesis and *in situ* hybridization by SABER-FISH**

For virus localization, sugar beets were infected using viruliferous aphids fed with an artificial medium containing each of the purified virus or a mixture of both. Three weeks later, we used SABER-FISH, which is a multiplexable nucleic acid-based detection method to detect the viral genomes. It includes a signal amplification step enabling rapid and highly sensitive target revelation. We used the protocol that was originally used for frozen retinal tissue as described by Kishi (Kishi et al., 2019) with some adaptations. For detection of BMYV and BChV, we designed 16 (BMYV) and 20 (BChV) specific primers with OligoMiner (Beliveau et al., 2018). A TTT and a concatemer sequence were added at the 3'-end of each primer (concatemer 27 for BChV and 28 for BMYV, see Table S2). Probes were obtained by primer extension reaction and a SABER-FISH protocol adapted to paraformaldehyde-fixed, paraffin-embedded leaf sections was carried out as described (Khechmar et al., 2024).

Processed sections were mounted in Fluoroshield medium and observed with a Zeiss Axio Imager M2 microscope equipped with a Hamamatsu Orca-Flash 4.0LT black-and white camera. Images were acquired with a 5x or 20x objective and with Zeiss 43 HE or AHF Cy5 ET bandpass filter sets for epifluorescence acquisition in the dsRED or Far Red channels, respectively, or with brightfield or DIC settings for transmission acquisition. Images were processed with ZEN 2.5 or Image J 1.54 software. Final figures were assembled with Microsoft PowerPoint.

## RESULTS

### 1. Susceptibility of sugar beet to BChV and BMYV during plant development

To analyze sugar beet susceptibility to BMYV and BChV depending on the plant developmental stages at the time of virus inoculation, series of sugar beets of different ages were inoculated with each virus. Preliminary experiments were first performed to (i) test the reactivity of the antiserum towards each virus, and (ii) to establish the conditions for efficient BChV and BMYV *in vitro* acquisition by aphids from purified virus. As shown in Figure S1, the antiserum used in the DAS-ELISA test recognizes both viruses similarly. Testing a range of different concentrations of purified virus particles (BMYV or BChV) in the artificial medium offered to aphids, we observed an increased transmission efficiency linked to the increased virus concentrations that reached a plateau when virus concentration was above 50 ng/µL or 70 ng/µL for BMYV or BChV, respectively (Figure S2). In these artificial conditions, a more efficient aphid transmission was observed for BChV when compared to BMYV, which cannot be explained by different affinities of the antiserum used in the DAS-ELISA test for BMYV or BChV detection (Figure S1).

Sugar beet plants of different age (from 3-week-old to 7-week-old) were then inoculated using viruliferous aphids fed with an artificial medium containing purified virus particles (50 ng/µL) as virus source. Five aphids were deposited at the basis of each test plant and let free to inoculate any leaf of the developed plant. The 3-week-old plants (representing plants with two true leaves and the cotyledons at the time of inoculation) were the most susceptible for both viruses (Figure 1). The percentage of infected plants dropped with the plant stage at the time of virus inoculation and at a rate comparable for both viruses. At the latest developmental stage assayed (7 weeks post-sowing), 6.30% of plants could still be infected with BChV whereas inoculation of BMYV with viruliferous aphids was unsuccessful (Figure 1).

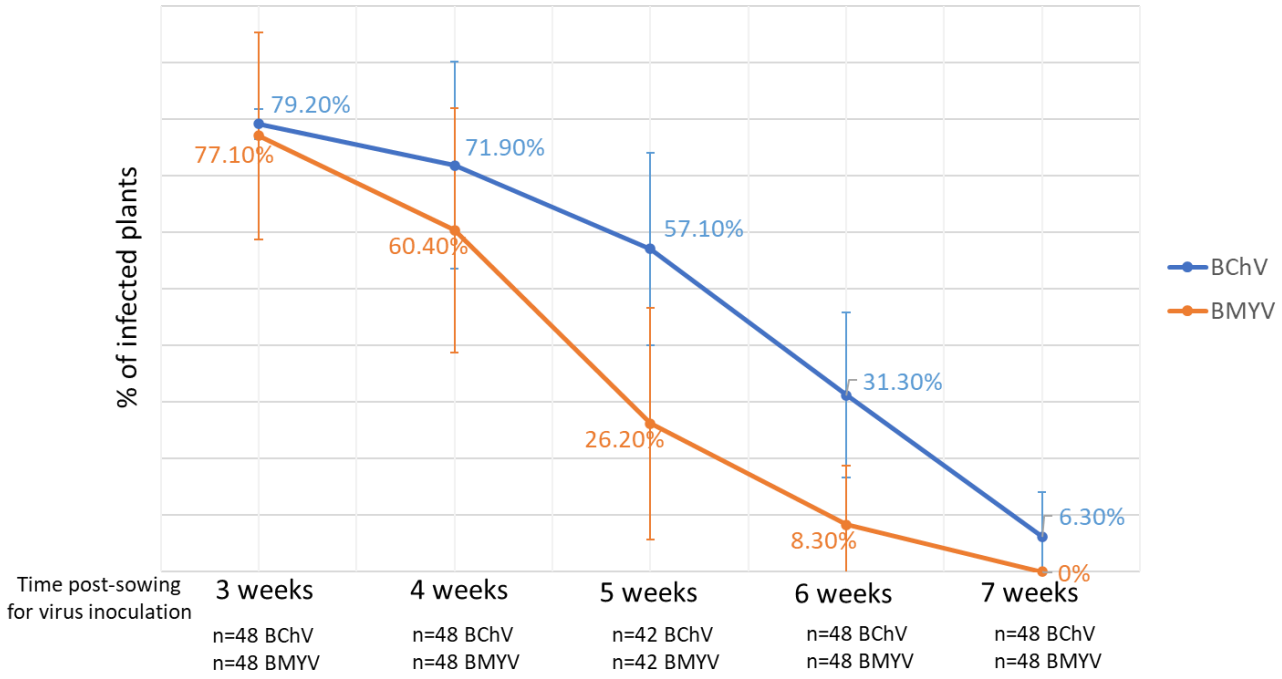


**Fig. 1. Effect of sugar beet developmental stage at the time of virus inoculation on BMYV or BChV aphid inoculation.** Sugar beets ranging from 3-week-old to 7-week-old were inoculated with 5 viruliferous aphids fed on an artificial medium containing purified virus (BMYV or BChV at 50 ng/µL). A DAS-ELISA assay was performed 3 weeks post-inoculation. The number of plants in each condition is indicated as well as the mean percentage of infected plants. Three independent experiments were performed.

## 2. Virus distribution in the plant depending on the developmental stage at the time of inoculation

Virus repartition in BChV- or BMYV-infected plants was first estimated in sugar beets inoculated at 3 weeks post-sowing, which was shown to be the most sensitive stage (Figure 1). A DAS-ELISA was performed on each leaf of infected plants. As shown in Figure 2, virus accumulation in each leaf of infected plants was in the same range for each virus and decreased in young leaves. In particular, in BMYV-infected plants, the virus was almost undetectable by DAS-ELISA in the last two youngest leaves tested, whereas BChV was still detected at a low level in corresponding leaves (Figure 2). When sugar beets were inoculated at a later stage of development (5 weeks post-sowing), a similar trend of virus accumulation in each leaf was again observed for both viruses. BChV and BMYV accumulated at a lower level in the newly developed leaves. The viruses did not systematically invade all leaves of the infected plants, in particular the oldest leaves remained uninfected (Figure S3).

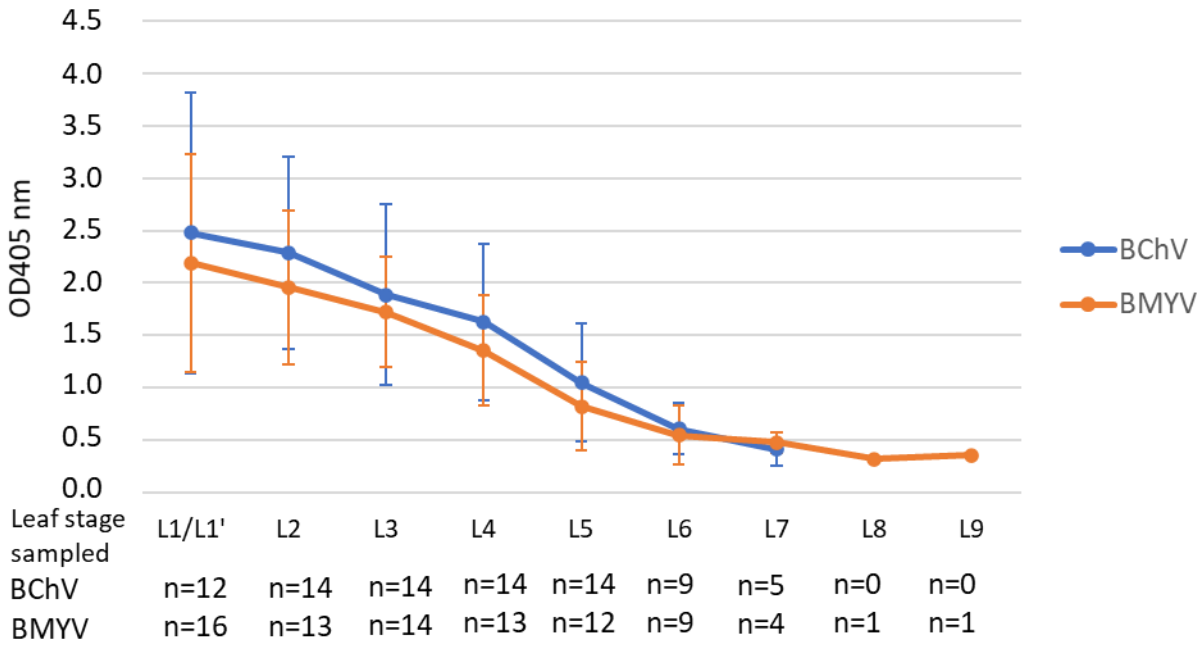


**Fig. 2. BMYV or BChV distribution in mono-infected sugar beets inoculated 3 weeks post-sowing.** Sugar beets were inoculated with BChV or BMYV after feeding aphids on an artificial medium containing purified virus for 24 h. At the time of inoculation only leaves 1 and 1' (L1/L1') were fully developed. A DAS-ELISA assay was performed 3 weeks after inoculation on each leaf of 5 infected plants (L1/L1': the oldest leaves; L7-L9: the youngest leaves at the time of sampling). The mean absorbance +/-SE (OD405 nm) of each leaf sample and the number of leaves tested is indicated. Two independent experiments were performed.

### 3. Identification of BChV and BMYV intestinal internalization sites in *M. persicae*

To identify the intestinal uptake site of BChV and BMYV in *M. persicae*, TEM analyses were conducted on aphids previously fed on purified viruses. As shown in Table 1 and Figure 3, BChV and BMYV virus particles were observed as free particles in the lumen of the digestive tube of *M. persicae*, but also in intestinal invaginations of the apical plasmalemma of posterior midgut cells, and in single or tubular vesicles in the cytoplasm of these cells. The viruliferous status of the aphids was verified by transferring part of the potentially viruliferous aphids on non-infected plants (1 to 3 aphids per plant) for virus inoculation (Table S3). No virus-like particles were observed in the posterior midgut or hindgut cells of non-viruliferous aphids.

**Table 1.** Virus particles of BChV or BMYV observed by TEM in the digestive tube of *M. persicae* after *in vitro* acquisition of viruses

| | stomach | posterior midgut | | | hindgut | |
|---|---|---|---|---|---|---|
| | lumen | lumen | invaginations | cytoplasm | lumen | cytoplasm |
| BChV | 3/7[a] | 13/14 | 9/14 | 5/14 | 3/6 | 0/6 |
| BMYV | 1/8 | 12/15 | 8/15 | 5/15 | 2/7 | 0/7 |
| controls[b] | 0/5 | 0/5 | 0/5 | 0/5 | 0/3 | 0/3 |

[a]number of *M. persicae* in which virus particles were observed/total number of aphids observed at the location

[b]controls: non-viruliferous aphids

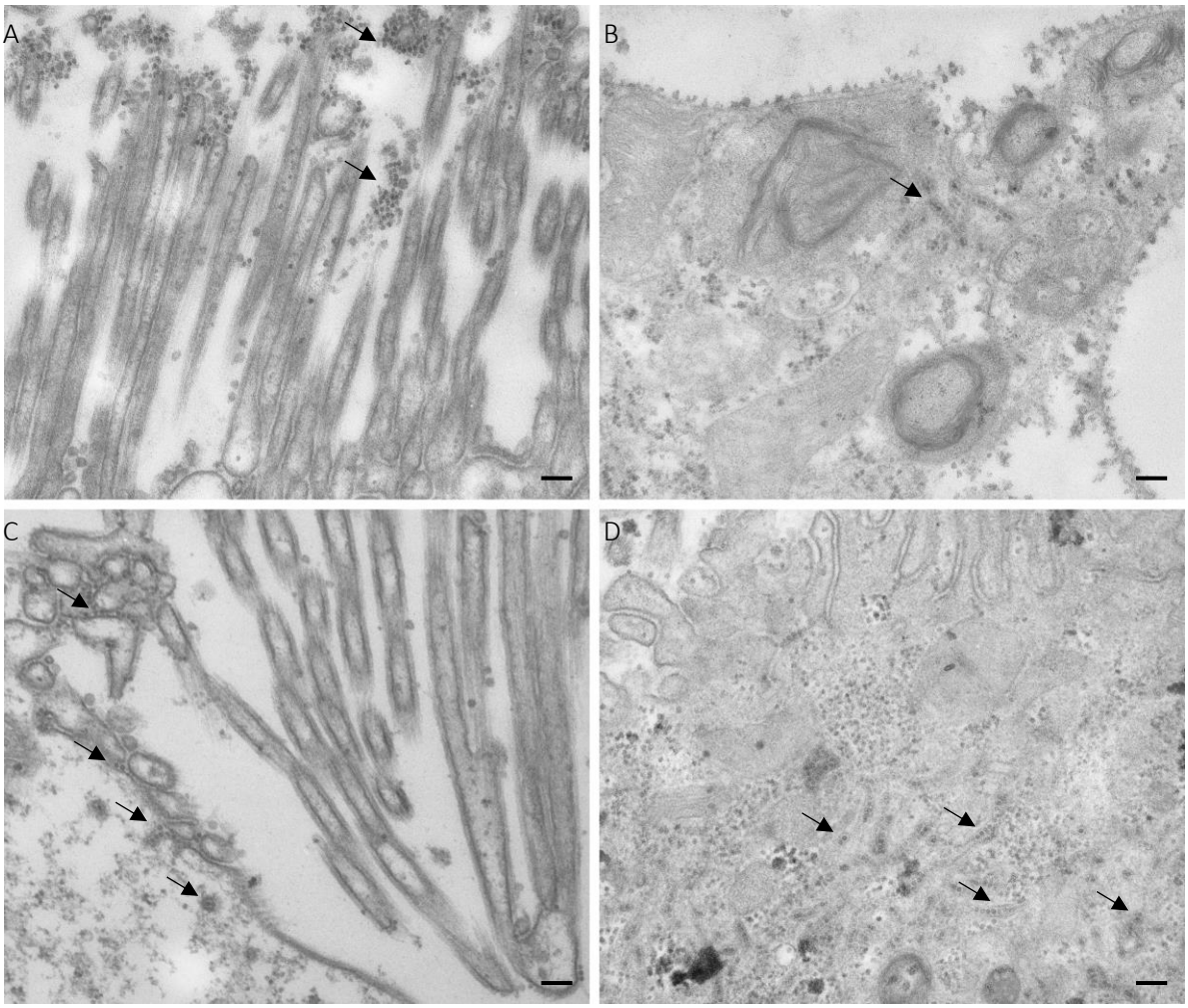


**Fig. 3. Transmission electron microscopy (TEM) observations of BChV and BMYV virus particles in the posterior midgut of *M. persicae***. BChV particles were observed in the lumen (**A**) or in the cytoplasm of the posterior midgut cells (**B**). BMYV particles were observed in the lumen (**C**) or in the cytoplasm of the posterior midgut cells (**D**). Arrows indicate the presence of virus particles. The scale bars represent 200 nm.

## 4. Kinetics of BChV and BMYV accumulation in mono- or co-infected plants

To address whether virus accumulation was impacted in co-infected plants, we performed RT-qPCR on sugar beet mono- or double-infected with BMYV and BChV. Three weeks post-inoculation and up to 13 weeks, leaf samples from the four youngest leaves were collected every

week, mixed and processed for total RNA extraction and RT-qPCR. When BMYV accumulation was compared at each time point between mono- or co-infected plants, no significant differences were observed all along the experiment, except at 9 weeks post-inoculation where a higher accumulation of BMYV was observed in mono-infected plants compared to co-infected plants (Figure 4A). For BChV, no significant differences in virus accumulation were observed in mono-infected plants when compared to co-infected plants at all time points analyzed (Figure 4B). We observed a higher accumulation of BMYV genomes (about 5-fold) in mono- or coinfected plants when compared to BChV genomes accumulation for all time points analyzed (Figures 4A & 4B). In a second independent experiment, we observed the same tendency, *i.e.* no effect of co-infection on BChV accumulation (Figure S4). BMYV levels could not be measured in the second experiment, due to insufficient numbers of plants mono-infected with BMYV. Note that in this experiment, BChV titers were 10-fold higher in all plants compared to the first experiment, highlighting variability in virus accumulation depending on the experiments.

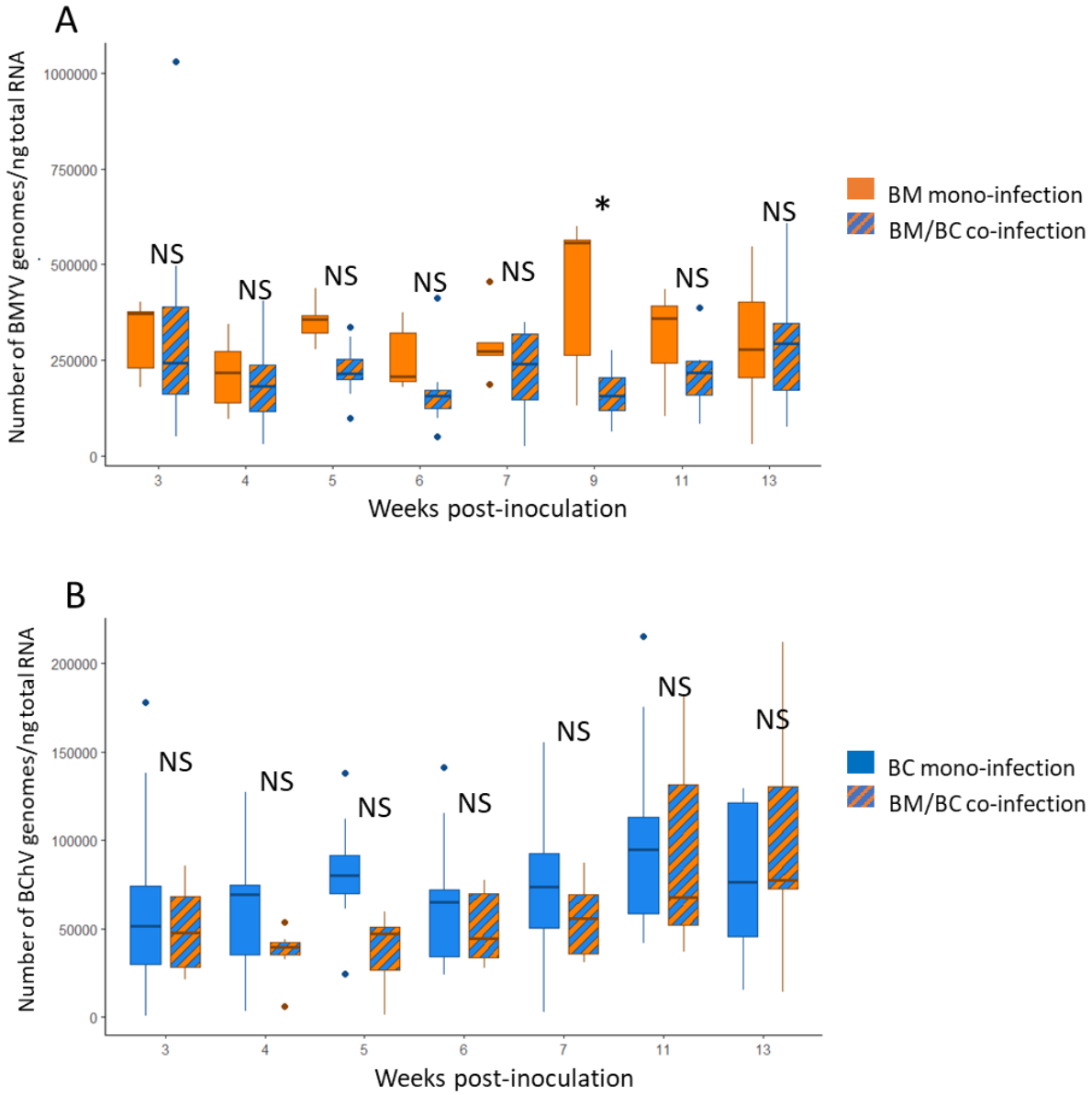


**Fig. 4. Accumulation of BChV and BMYV in mono- or coinfected sugar beets.** Leaf samples of infected plants were collected 3 to 13 weeks post-inoculation and virus accumulation was measured by RT-qPCR. The number of BMYV or BChV genome copies are normalized to ng of total plant RNA. Sugar beets were inoculated with one or the two viruses (BMYV or BChV) using aphids fed on an artificial medium containing one or both purified viruses for 24 h. **A**) BMYV accumulation in mono- or coinfected sugar beet leaves (Nonlinear Mixed-Effect Models, mono- vs. coinfection $P$ =0.030, time $P$ =0.351, infection: time $P$=0.337) ; **B**) BChV accumulation in mono- or coinfected sugar beet leaves (Nonlinear Mixed-Effect Models, mono- vs. coinfection $P$ = 0.253, time $P$ =0.004, infection:time $P$ =0.260). Box plots show median (line), 25–75% percentiles (box), 10–90% percentiles (whisker), and outliers (dots). Eight BChV-infected plants, five BMYV-infected and eight BChV and BMYV co-infected plants were analyzed. The co-infected sugar beet leaves are the same in A and B.

## 5. Localization of BChV and BMYV in mono and co-infected plants

We assessed whether co-infection affected tissue localization of BChV or BMYV. For this, SABER-FISH was performed on mono- and co-infected plants at 21 days after inoculation. In cross-sections of BMYV mono-infected leaves, fluorescence corresponding to BMYV genomes was observed exclusively in phloem cells, as expected (Figure 5A). The label was

found in the phloem of primary (midribs) and secondary veins and the label intensity, estimated by visual observation, was comparable in both locations (Figure 5A). Similarly, in BChV mono-infected plants, similar fluorescent signals corresponding to BChV genomes were observed in the phloem of primary and secondary veins (Figure 5B). Co-infection did not change the tissue distribution of BMYV and BChV (Figure 5C). Both viruses were still confined to phloem cells and no escape in non-phloem cells was observed (Figure 5C). We evaluated the percentage of co-infected cells and mono-infected cells in co-infected plants. Ten to eleven sections from co-infected plants were processed in two independent experiments (Table 2). We noticed a much lower percentage of cells mono-infected with BMYV (6.40 % of all infected cells) compared to BChV (55.40 % of infected cells containing only BChV) (Table 2). Around 40 % of the total number of infected cells were co-infected. This suggests that there is no exclusion between the two viruses at the cellular level.

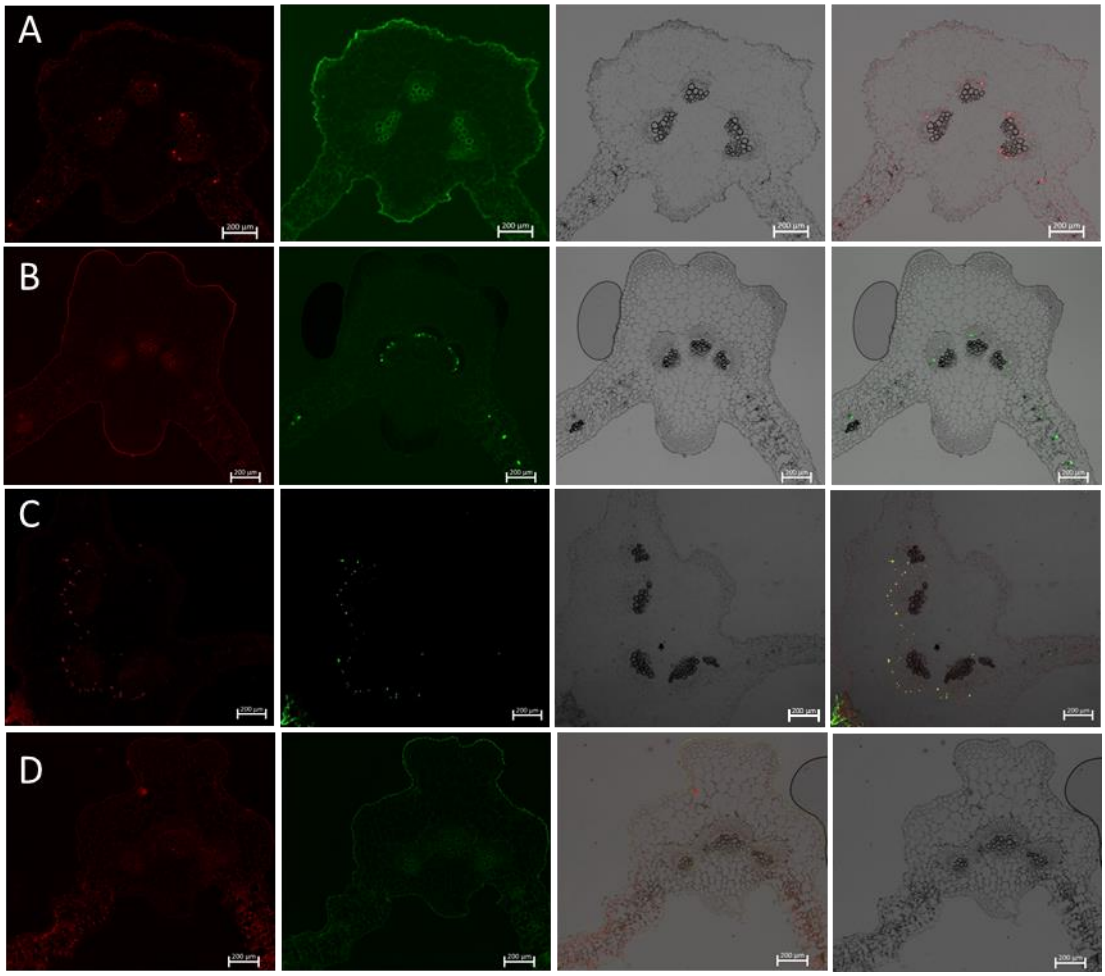


**Fig. 5. SABER-FISH detection of BChV and BMYV genomes in sugar beets mono-infected with BChV or BMYV or coinfected with both viruses**. Representative images of a cross sections of sugar beet leaves infected with BMYV (**A**), BChV (**B**), BMYV and BChV (**C**) or non-infected (**D**). The first column shows BMYV label (red), the second BChV label (green), the third bright field acquisition and the last column shows merges. Scale bar (200 µm) is indicated on each image.

**Table 2.** Number of cells infected by one or both viruses in sections of co-infected sugar beet leaves

| | Plant #1 | Plant #2 | Plant #3[b] | Plant #4 | %[c] |
|---|---|---|---|---|---|
| **BMYV** | 0[a] (0%) | 1 (4.2%) | 0 (0%) | 12 (23.1%) | **6.8%** |
| **BChV** | 24 (28.2%) | 17 (70.8%) | 43 (100%) | 29 (55.8%) | **63.7%** |
| **BMYV+BChV** | 61 (71.8%) | 6 (25.0%) | 0 (0%) | 11 (21.1%) | **29.5%** |

[a]number of cells observed on 10 to 11 sections of each plant showing a fluorescent signal for the indicated virus.
[b]co-infection of plant #3 was confirmed by IC-RT-PCR.
[c]%: percentage of labelled cells/total number of fluorescent cells. Two experiments were performed.

## 6. Effect of plant co-infection by BChV and BMYV on virus transmission by *M. persicae*

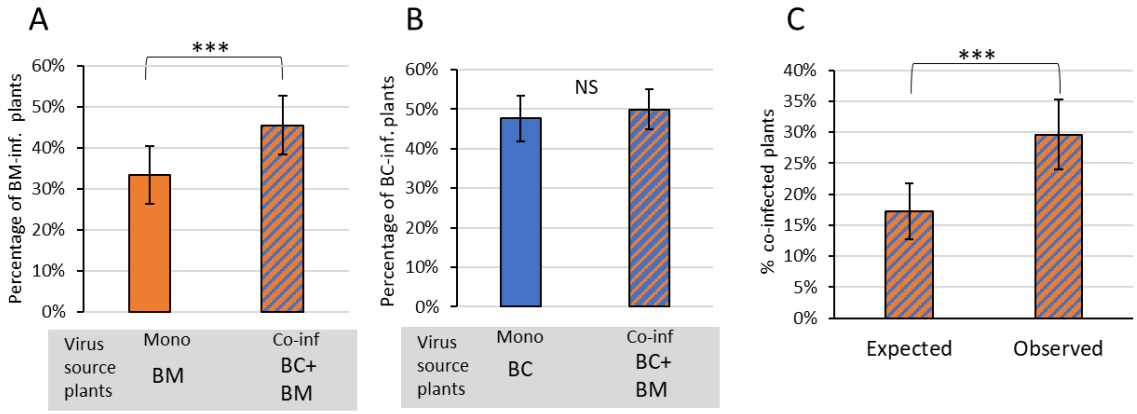


**Fig. 6. Aphid transmission efficiency of BMYV and BChV when acquired from mono- or co-infected sugar beets.** *Myzus persicae* acquired virus from mono or co-infected leaves for 24 h. Three aphids were then transferred per test plant for 72 h inoculation period. A DAS-ELISA assay was performed 3 weeks later to detect infection, and IC-RT-PCR to discriminate between BChV and BMYV. (**A**) Percentage of BMYV-infected plants when aphids acquire BMYV from mono- (orange), or co-infected (hatched) plants. (**B**) Percentage of BChV-infected plants when aphids acquire BChV from mono- (blue), or co-infected (hatched) plants. The difference was statistically significant for BMYV transmission, but not for BChV transmission (Pearson's Chi-squared test; BMYV: df = 1, p-value = 0.000398; n=228, 11 independent experiments; BChV: df = 1, p-value 0.399; n=228, 11 independent experiments; Table S4). (**C**) Percentage of observed and expected co-infected test plants calculated for each individual experiment from the transmission efficiency of each virus from mono-infected plants ([% BMYV * % BChV]/100 = expected % of co-infected plants). The difference between expected and observed was statistically significant (Chi-squared test for given probabilities; df = 1, p-value = 2.436e-08; n=228, 11 independent experiments; Table S4).

To address the effect of the co-infection of sugar beet with BChV and BMYV on the virus transmission efficiency of each virus by *M. persicae*, transmission experiments were performed using co-infected plants as virus source, and mono-infected plants as controls. BMYV transmission efficiency was statistically higher when aphids acquired BMYV from co-infected plants (Figure 6A and Table S4). In contrast, no change in BChV transmission efficiency was observed when aphids acquired the virus from mono- or co-infected plants (Figure 6A and Table S4). Interestingly, both viruses were transmitted together at a higher rate than expected

(Figure 6B and Table S4). The higher transmission efficiency of BMYV from co-infected plants did not correlate with a higher BMYV accumulation in co-infected plants (Figure 4A).

To discriminate between an indirect plant effect on the virus transmission efficiency and a direct effect of virus co-acquisition, virus transmission experiments were conducted using an artificial medium containing similar quantities of each virus as virus source. As controls, aphids were fed on an artificial medium containing each virus alone. This experiment was also conducted to avoid transmission efficiency variations due to uneven virus distribution in doubly infected source leaves. For similar virus concentrations in the medium, BChV transmission efficiency, when acquired alone, was higher compared to BMYV (~20-30% transmission for BChV and ~4 % transmission for BMYV; Table 3). Interestingly, when the two viruses were acquired simultaneously by aphids from the artificial medium, a statistically significant increase in the transmission efficiency of BChV (~50-55% of plants infected with only BChV or with BChV and BMYV) and BMYV (~16% of plants infected with only BMYV or with BChV and BMYV) was observed (Table 3). We observed that from the medium containing a mixture of both viruses, BMYV was never transmitted alone.

**Table 3.** Transmission efficiency of BMYV or BChV when acquired from an artificial medium containing purified BMYV or BChV or a mixture of both viruses

| Inoculum source | BMYV only | | Co-acquisition | | Chi-square, df | p-value |
|---|---|---|---|---|---|---|
| Exp. 1 | 2/44[a] | 4.54%[b] | 8/45[c] | 17.8%[d] | **3.906, 1** | **0.048** |
| Exp. 2 | 5/120 | 4.17% | 10/63 | 15.8% | **7.523, 1** | **0.006** |

| Inoculum source | BChV only | | Co-acquisition | | Chi-square, df | p-value |
|---|---|---|---|---|---|---|
| Exp. 1 | 8/34[e] | 23.5%[f] | 25/45[g] | 55.6%[h] | **8.167, 1** | **0.004** |
| Exp. 2 | 12/40 | 30.0% | 32/63 | 50.8% | **4.323, 1** | **0.038** |

[a]number of test plants infected with BMYV when aphids acquired the virus from a medium containing only purified BMYV (50 ng/µL; 1 aphid per test plant).
[b]percentage of BMYV-infected plants.
[c]number of test plants infected with BMYV and BChV when aphids were fed with a medium containing both viruses (50 ng/µL of each virus; 1 aphid per test plant) (no test plants only infected with BMYV have been detected in this condition).
[d]percentage of co-infected plants.
[e]number of test plants infected with BChV when aphids acquired the virus from a medium containing only purified BChV (50 ng/µL; 1 aphid per test plant).
[f]percentage of BChV-infected plants.
[g]number of test plants infected with BChV or with BMYV and BChV when aphids were fed with a medium containing both viruses (50 ng/µL of each virus; 1 aphid per test plant).
[h]percentage of co-infected plants.
Two independent transmission experiments were performed.

## DISCUSSION

BMYV and BChV are two closely related poleroviruses infecting sugar beet and are strictly transmitted by aphids. Since the European ban on neonicotinoids in 2018, insecticides used in seed coating to control aphid populations, sugar beet poleroviruses-induced disease has re-emerged causing a serious crisis in the sugar beet industry. The present study was dedicated to acquiring knowledge on the biology of these viruses and their interactions in the plants.

We showed that these two viruses invade all leaves of plants inoculated at an early stage of development with a reduced accumulation in the youngest developed leaves (Figure 2). In contrast, old leaves escaped infection when plants were inoculated at a later stage of development. An uneven distribution of sugar beet poleroviruses in the plant was already reported by van der Werf et al (van der Werf et al., 1989) who observed that the youngest leaves were systemically infected but without symptoms, while the intermediate leaves were infected and symptomatic. The older leaves, fully developed at the time of virus inoculation, remained green and healthy.

We also confirmed, in controlled conditions, the decreased sensitivity of sugar beets to poleroviruses when inoculated at a late stage of development (Figure 1). This phenomenon, mature plant resistance, has been observed and exploited in the field for decades, with a higher aphid threshold level before spraying insecticides when plants reach a certain level of maturity. This resistance of sugar beets towards BMYV and BChV has already been observed in the field and resulted in a low impact on sugar beet yield (Borgolte et al., 2024b; Smith & Hallsworth, 1990b). Stevens et al. (2004) also showed that plants inoculated with BMYV or BChV early in the season (May or June) exhibited the highest level of virus incidence and the strongest effect on yield. Sugar beet genetic factors sustaining mature plant resistance are envisaged, but environmental factors seem also to play an important role (Schop et al., 2022). The effect of the leaf stage on inoculation resistance and thus in the establishment of the mature plant resistance, as mentioned in Schop et al. (2022), was not addressed in our experiments, because here aphids were free to move and inoculate virus on any leaf of the developed plants. The molecular mechanisms sustaining mature sugar beet resistance remain to be explored.

BMYV and BChV are both transmitted by *M. persicae* in a persistent and circulative mode implying an active virus transport from the gut lumen to the hemolymph. We identified the posterior midgut cells as the intestinal uptake site of both viruses in *M. persicae* (Figure 3). Although the precise identification of the intestinal receptors of BMYV and BChV has not been

addressed here, we previously observed that the reduction of ephrin receptor expression in *M. persicae* resulted in a lower capacity of these aphids to transmit BMYV and the closely related TuYV (Mulot et al., 2018b). Therefore, ephrin receptors localized at the apical plasmalemma of posterior midgut cells could be hijacked by BMYV and BChV particles to get access to intestinal cells' cytoplasm. Whether the two closely related poleroviruses use the same intestinal, or different, receptors for intestinal uptake cannot be concluded at this time. Our transmission experiments, using a mixture of similar amount of BMYV and BChV in an artificial medium for aphid feeding, did not reveal any competition between the two viruses (Table 3). It is therefore conceivable that the two poleroviruses use different receptors at the posterior midgut, or the same receptors that could be sufficiently abundant to avoid any competition between the two viruses.

Addressing interactions between the two poleroviruses in co-infected sugar beets at the whole plant level did not reveal any synergism nor major antagonism with regard to virus accumulation of each virus (Figure 4). A lower BMYV accumulation in co-infected plants compared to plants infected only with BMYV, was however observed but only at 9 weeks post-inoculation, and not later on. This phenomenon could be assimilated to cross-protection but the transitory nature of this phenomenon has yet to be explained. Although both viruses were inoculated simultaneously in our experiments, a scenario unfavorable for cross-protection, it is still conceivable that cross-protection occurred if the two viruses invaded non-inoculated leaves with a timely delay. Cross-protection has been previously reported between strains of barley yellow dwarf virus (formerly in the *Luteoviridae* family, but now classified in the *Tombusviridae* family) (Wen et al., 1991). At the cellular level, BMYV and BChV were observed together in phloem cells which implies no cellular exclusion. No phloem escape of these two phloem-limited viruses was observed in BMYV/BChV co-infected plants, as expected (Figure 5). Indeed, phloem escape of phloem-limited viruses has only been reported in co-infection with a systemic virus. For example, co-infection of the polerovirus, potato leafroll virus, with a potyvirus infecting all tissues, potato virus A, resulted in phloem release of the polerovirus in *Nicotiana benthamiana* (Savenkov & Valkonen, 2001b). Similarly, BMYV became mechanically transmissible to *N. benthamiana* when the inoculum was a mixture of BMYV and pea enation mosaic virus 2 (*Umbravirus*), a feature linked to BMYV phloem restriction alleviation (Mayo et al., 2000). Sugar beet co-infected cells could be the site of virus recombination, and indeed Kozlowska-Makulska et al. (Kozlowska-Makulska et al., 2015b) identified recombinants between BMYV and BChV in the coat protein sequence. This

powerful mechanism may initiate the formation of new, often more virulent strains or even virus species (Aguilera & Pfeiffer, 2019; McDonald et al., 2016b; Q. Wang et al., 2019). Whether these recombinants would display specific biological features was not addressed in the study of Kozlowska-Makulska et al. (Kozlowska-Makulska et al., 2015b). It is interesting to note that recombination is probably responsible for the emergence of BChV from a poleroviral-like ancestor that brought the 5' half of the genome and BMYV, or BWYV, contributing to the 3' half (Hauser et al., 2002c). Similarly, BMYV-2itb was also supposed to arise from recombination events between a poleroviral ancestor for the 3' part of the genome and a CABYV-like ancestor for the 5' part (Guilley et al., 1995). Last, a recombination between two poleroviruses infecting pepper may have resulted in a recombinant with a vector switch, becoming transmitted by whiteflies (Ghosh et al., 2019b). Importance of recombination events in the *Solemoviridae* family is therefore highlighted by these different examples.

We addressed BMYV and BChV interactions with regard to their transmission by *M. persicae* and showed that both viruses could be simultaneously acquired by a single aphid. When BMYV was acquired from plants co-infected with BChV, its overall transmission efficiency was elevated compared to an acquisition from a plant only infected with BMYV, even if this phenomenon was not systematically observed in all the independent experiments we performed (Figure 6, Table S4). No such increase in virus transmission efficiency was noticed for BChV when the virus was acquired from co-infected plants, discarding the hypothesis that a modification of the aphid feeding behavior on co-infected plants would account for the higher BMYV acquisition from infected plants. As no increase in BMYV accumulation was monitored in co-infected plants 3 weeks after inoculation (Figure 4), the time point when source plants were used for virus acquisition, the virus titer cannot be the cause for the BMYV transmission efficiency increase. Interestingly, when virus co-acquisition was performed using artificial medium containing both viruses in similar amounts (Table 3), the stimulatory effect on the virus transmission efficiency was systematically observed for both viruses. These results suggest that there might be a cooperative effect between the two viruses either during passage through the aphid or during the initiation of infection when they are co-inoculated simultaneously in the same cells by aphids, which we forced by membrane acquisition and might be different when using co-infected plants as virus source. We believe the latter possibility to be more likely. This cooperative effect could be caused by an additive effect of the capacity of each virus to counteract plant defenses such as RNA silencing (Figure 7). Because the "stimulatory" effect on the transmission efficiency was only observed for BMYV when it was acquired from plants,

we suspect a better ability of BChV to counteract plant defense compared to BMYV. A weaker ability of BMYV to suppress plant defense could result in a lower number of cells in which BMYV infection is initiated. In co-infected cells, this deficiency in the defense suppression activity could be compensated by BChV infection. This hypothesis of cooperation between the two viruses is reinforced by our results showing that both viruses were more often than expected found to be transmitted together when acquired from co-infected plants, suggesting a beneficial effect of being inoculated together than alone (Figure 6C). RNA silencing suppressor activity of poleroviruses was attributed to the P0 protein encoded by the open reading frame located at the 5' extremity of the genome (Pazhouhandeh et al., 2006b). BMYV-P0 was shown to display silencing suppressor activity in *N. benthamiana* whereas the authors did not identify such an activity for BChV-P0 from different isolates from France and Poland (Hauser et al., 2002d). It is possible that the patch infiltration assay in *N. benthamiana* developed in this study was not adapted to reveal the silencing suppression activity of the P0-BChV, or that this function is fulfilled by another not yet identified BChV protein. However, other unrelated events, not relying on the plant defense suppression activity of P0, could also be involved in the cooperative effect of BMYV and BChV, possibly occurring at the aphid-inoculation step.

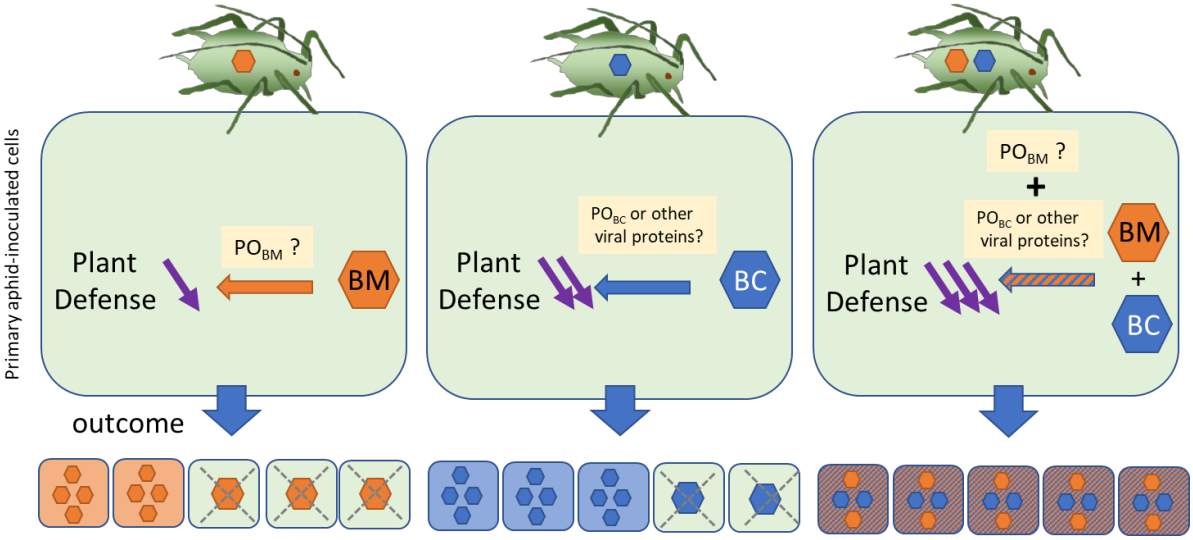


**Fig. 7.** Model for explaining the cooperative effect of BMYV and BChV co-inoculation on the success of their co-infection. We hypothesize an additive effect of the plant defense suppression by each of the viruses, BMYV or BChV, in co-inoculated cells. The dampening of plant defenses could be caused by concert action of P0 or other BMYV and BChV proteins. The purple arrows represent the strength of the plant defense suppression by either or both viruses together.


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**Author contributions**

Conceptualization: VB, MD; methodology: HS, AM, SK, MD; investigation: HS, AM, SK, CR; resources: CV; formal analysis: QC; writing original draft preparation: VB, HS, AM, SK, CR; writing-review and editing: VB, MD, QC; supervision: VB, MD, AM; funding acquisition: VB.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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# Chapter 4: Synergistic interactions between the beet mosaic potyvirus and the beet yellows closterovirus decrease transmission of the closterovirus

Souheyla Khechmar [1,2], Quentin Chesnais [1,2], Véronique Brault [1,2]*, Martin Drucker [1,2]*

[1] SVQV, UMR 1131, INRAE Centre Grand Est, Colmar, France

[2] Université Strasbourg, Strasbourg, France

* martin.drucker@inrae.fr, veronique.brault@inrae.fr

Short title: Co-infection decreases aphid transmission

Keywords: plant virus, virus co-infection, virus localization, aphid transmission, virus accumulation, aphid behavior, plant-virus-virus interactions.

## ABSTRACT

Infection of a host by more than one virus can impact the outcome of infection including transmission. Here we studied the effect of co-infection of sugar beet on aphid transmission of two unrelated viruses, the non-circulative semi-persistent beet yellows closterovirus (BYV), and the non-circulative non-persistent beet mosaic potyvirus (BtMV). Co-infection exacerbated plant growth reduction and mosaic symptoms compared to mono-infections. Aphid transmission of BYV from co-infected sugar beet was reduced by 50 %, while BtMV transmission was not impacted. Because both viruses accumulated significantly more in co-infected plants than in mono-infected ones, BYV titers cannot account for the reduced transmission of the virus. Virus localization by SABER-FISH showed that co-infection did not relieve phloem restriction of BYV. BtMV tissue distribution was unchanged in co-infected leaves compared to mono-infected ones. BtMV accumulated in periplasmic aggregates in mono-infected and co-infected cells, while BYV formed spherical inclusions in mono-infected cells and displayed a granular and more diffuse distribution in co-infected cells. This indicates BYV-BtMV interactions in co-infected plants, and hypotheses to explain inhibition of BYV transmission are presented.

## INTRODUCTION

Virus yellows (VY) in sugar beet is an emerging disease affecting this crop, caused by a complex of up to four aphid-transmitted virus species, with the green peach aphid (*Myzus persicae*) being the most important vector **(Wintermantel et al., 2008)**. The viruses can be found alone or in different combinations that all cause beet leaves to yellow prematurely and contribute to yield losses to various degrees. This disease has become increasingly problematic in many sugar beet cultivation areas, especially in USA and Europe. The viruses present in Europe were named according to the type of chlorosis they cause i.e. beet yellows virus (BYV; family *Closteroviridae*, genus *Closterovirus*), beet mild yellowing virus (BMYV; family *Solemoviridae*, genus *Polerovirus*), and beet chlorosis virus (BChV; family *Solemoviridae*, genus *Polerovirus*). While BYV was the predominant virus in many European countries, more recent surveys showed a shift with BMYV becoming more prevalent in Germany and Belgium, and BChV in France **(Hossain et al., 2021)**. A third polerovirus, beet western yellows virus (BWYV), in contrast, is up to now restricted to the USA and Asia and has not been detected in Europe **(Stevens et al., 2004; Wintermantel, 2005; Xiang et al., 2008)**. The forth virus, beet mosaic virus (BtMV; family *Potyviridae*, genus *Potyvirus*) is often associated with VY due to

its transmission by the same vectors, but it generates in sugar beet leaves mosaic symptoms instead of chlorosis. This highly prevalent virus is not considered by itself as a virus causing yellowing although there is evidence that it can exacerbate symptoms and accelerate their appearance when present together with BYV or BWYV-USA in greenhouse conditions **(Wintermantel, 2005)**. A field study using different virus isolates and sugar beet varieties reported a significant effect of BtMV-BMYV co-infection but not of BtMV-BYV co-infection on symptom expression and sugar yield **(Borgolte et al., 2024)**. Consequently, it is thought that BtMV can exert synergistic effects on symptom development in co-infections with the other members of the VY disease.

Interactions between BtMV and other yellowing viruses have not been studied in depth. However, considering that BYV, BChV and BMYV are phloem-limited viruses **(Dolja, 2003; Dolja & Koonin, 2013; Esau et al., 1967)** and BtMV is a systemic virus that it is able to infect and replicate in all plant cells **(Fujisawa et al., 1967)**, it is conceivable that multi-infection with BtMV could alleviate the phloem-restriction of BYV, BChV and BMYV. The broader tissue specificity of these viruses could potentially enhance virus titers, by increasing the number of infected cells, which could ultimately have an impact on the virus transmission efficiency and disease severity. Additionally, since tissue tropism is tightly linked to insect transmission mode, this new virus localization could also modify the virus transmission parameters. Indeed, studies on co-infections with other viruses identified synergisms between potyviruses and members of both the *Solemoviridae* and *Closteroviridae* families. One example of potyvirus/polerovirus synergism is that of the polerovirus potato leaf roll virus (PLRV) and the potyvirus potato virus Y (PVY), where the phloem-restricted PLRV accumulated more and could infect non-phloem tissue in co-infection with PVY in *Nicotiana clevelandii* but not in potato **(Barker, 1987)**. Barker obtained similar results with BWYV and another potyvirus, potato virus V **(Barker, 1989)**. Savenkov and Valkonen **(2001)** found evidence for suppression of RNA silencing by a potyvirus protein (HC-Pro of potato virus A) as the cause for the increased titer of PLRV in co-infection, but not for PLRV phloem escape that was observed in co-infected plants **(Savenkov & Valkonen, 2001)**. Srinivasan and Alvarez **(Srinivasan & Alvarez, 2007)** showed that co-infection with PVY and PLRV caused more severe symptoms in potato than single infections and that it had an effect on the fecundity and the preference of two aphid species vectoring these viruses.

The knowledge on synergistic effects in multiple infections of potyviruses with members of the *Closteroviridae* family is limited. One of the few examples is co-infection of the lettuce

infectious yellows virus (LIYV, genus *Crinivirus*, family *Closteroviridae*), and the potyvirus turnip mosaic virus (TuMV). The crinivirus benefits from the interaction and replicates better in co-infected Nicotiana plants, with some evidence of potyvirus-mediated RNA-silencing suppression being one of the molecular causes **(Wang et al., 2009)**. Another example is sweet potato virus disease (SPVD) resulting from co-infection with sweet potato feathery mottle virus (SPFMV, genus *Potyvirus*) and sweet potato chlorotic stunt virus (SPCSV, genus *Crinivirus*, family *Closteroviridae*). In contrast to other potyvirus co-infections, the potyvirus benefits in this case from the co-infection with a 600-fold increase of SPFMV titer in co-infected plants, compared to mono-infected plants **(Karyeija et al., 2000)**. It was also demonstrated that the insertion of leader-proteinase (L-Pro) encoded by the closterovirus BYV into the genome of another potyvirus, tobacco etch virus (TEV), suppresses infection and interferes with accumulation and systemic movement of TEV **(Dolja et al., 1997)**. Therefore, interactions between BtMV and BYV are perfectly conceivable.

In this work, we focused on the co-infection of sugar beet with BYV and BtMV. Both viruses are mainly transmitted by the two aphid species *Myzus persicae* and *Aphis fabae* **(Dusi & Peters, 1999; German-Retana et al., 1999)**. BtMV and BYV use different modes of transmission: non-circulative and non-persistent for BtMV, and non-circulative and semi-persistent for BYV. Both transmission modes have in common virus retention outside aphid cells, either on the stylets or esophagus of the vectors. The non-persistent viruses are characterized by short acquisition and inoculation times in the range of seconds to minutes and by short retention times (minutes to hours). Semi-persistent transmission mode is characterized by similar acquisition times as non-persistent transmission, but a longer virus retention in the aphid and consequently transmissibility, ranging from hours to a few days **(Bragard et al., 2013; Brault et al., 2010; Jiménez et al., 2018)**.

Here, we studied mixed viral infection of sugar beet and examined its effect on vector transmission. Our results show that co-infection of sugar beet with BtMV and BYV had no impact on BtMV transmission, but did decrease transmission of BYV by 50 %.

## MATERIALS AND METHODS

### Plants, viruses and aphids

The virus-susceptible sugar beet variety used in this study was “Auckland”. Seeds were kindly provided by the seed company SESVanderHave (Tienen, Belgium) via the Technical Institute

of Sugar Beet (ITB, Paris, France). One week after germination, plantlets were transplanted into individual pots and grown in a climate chamber at 22 to 25 °C with a day/night cycle of 16 h of light and 8 h of darkness.

One aphid clone was used throughout the experiments: The *Myzus persicae* biotype WMp2 **(Reinink et al., 1989)**. Aphids were maintained on Chinese cabbage (*Brassica rapa* subspc. *Pekinensis* var. Granaat) in a culture chamber under controlled conditions at 20±1 °C with a day/night cycle of 16 h of light and 8 h of darkness.

The BYV_BBRO_UK isolate used in this study was kindly supplied by Prof Mark Varrelmann of the Institut für Zuckerrübenforschung (IfZ, Göttingen, Germany) but was originally sampled in the UK **(Stevens et al., 1997)**. The isolate was maintained on sugar beet plants by continuous transmission using aphids.

We used the BtMV-Adon isolate in this study, which was collected by ITB from a field in Adon region, France. It was propagated on sugar beet plants by mechanical inoculation.

**Virus detection by DAS-ELISA**

BtMV and BYV detection in plant samples was conducted at 21 dpi using double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) as reported **Adams & Clark, 1977)** using beet mosaic virus and beet yellows virus-specific antibodies, respectively (Loewe Biochemica, Sauerlach, Germany). Samples were considered infected when the $OD_{405\ nm}$ was twice the mean value of non-infected control plants (three technical replicates) plus three times the standard deviation.

**Establishing co-infection in source plants**

To produce virus-infected source plants, two different inoculation methods were used: mechanical inoculation using infected frozen leaves freshly ground in water and carborundum for BtMV and inoculation using viruliferous aphids for BYV. To obtain the co-infected plants, non-viruliferous aphids were allowed to feed on BYV-infected plants for 24 h, before being transferred on sugar beet plants that had been mechanically inoculated with BtMV 24 h before. The inoculation access period was 3 days. Aphids were then carefully removed using a fine paint brush and plants were checked weekly to remove any remaining living nymphs. After aphid inoculation, the plants were maintained in a growth chamber under controlled conditions (22-25 °C and 16/8 h light/dark photoperiod) for three weeks before being analyzed by DAS-ELISA to verify infection.

**Aphid transmission experiments**

After feeding on detached source leaves for 24 h (three leaves from three independent infected source plants that were placed on 1 % agarose in petri dishes), three aphids were placed on each non-infected sugar beet plant for 72 h. We inoculated 15 test plants for each condition (mono- or co-infection). Three days after aphid inoculation, aphids were killed with pirimicarb insecticide. The transmission rates were determined at 21 dpi by testing for the presence of the two viruses by DAS-ELISA.

**Localization of viruses by SABER-FISH**

Virus localization by SABER-FISH was done as described **(Khechmar et al., 2024)**. Briefly, signal amplification by exchange reaction fluorescent *in situ* hybridization (SABER-FISH) uses a strand-displacing polymerase and catalytic DNA hairpins to generate FISH probes that consist of a target-specific sequence and a concatemer part binding complementary fluorescent imager oligonucleotides. This enables adjustable signal amplification, allowing highly sensitive detection of nucleic acids and reducing the number of required probes. For the detection of BYV and BtMV, we designed 25 primers for each virus with OligoMiner **(Beliveau et al., 2018)**. A TTT and a concatemer sequence were added to the 3'-end of each primer (concatemer 30 for BtMV and 26 for BYV, see **Table S1**). Leaf pieces were fixed with 4 % paraformaldehyde, embedded in paraffin, and 10 µm sections processed as described **(Khechmar et al., 2024)**. Epifluorescence, bright field and DIC images were acquired with a Zeiss Axio Imager M2 microscope equipped with a Hamamatsu Orca-Flash 4.0LT black-and-white camera, 5x, 20x or 40x objectives and Zeiss 43 HE and AHF Cy5 ET bandpass filter sets (all from Lordil, Lay-Saint-Christophe, France). Images were processed with ZEN 2.5 or Image J 1.54 software. Final figures were created with Microsoft PowerPoint or LibreOffice Impress.

**Relative virus quantification by one-step TaqMan RT-qPCR**

Multiplex TaqMan real time RT-qPCR was used to quantify relative titers of the two viruses. Primers and probes for BYV and BtMV were designed using the Primer Express 3.0.1 primer design tool (Thermo Fisher Scientific, Illkirch, France). The amplicons comprised between 75 to 200 base pairs of the BYV and BtMV genomes **(Table 1)**. Each primer pair was controlled for its specificity and was evaluated by a standard curve with six dilutions and three technical replicates of BtMV- or BYV-infected plants. Efficiency rates (E) of 95 % (for BYV) and 104 % (for BtMV) were obtained with $R^2 = 0.99$ for both viruses.

Selection of the reference genes were performed as described **(Khechmar et al., 2024)** except that the expression stability of the five selected reference genes was performed on five biological replicates corresponding to 5 plants infected only with BYV, only with BtMV or co-infected with BYV and BtMV. Titers of BtMV and BYV were quantified from five biological replicates of source plants using AgPath-ID™ One-step RT-PCR Kit for TaqMan chemistry (Applied Biosystems by Thermo Fisher Scientific). The third upper systemically infected leaf (the third leaf starting from the youngest leaf) from each source plant was collected at 21 dpi. The whole leaf was homogenized in liquid nitrogen. One hundred mg of ground tissue was used for total RNA extraction using NucleoSpin RNA Plant Kit (Macherey-Nagel, Hoerdt, France). After spectrometric quantification with a Nanodrop 2000 (Thermo Fisher Scientific), 30 ng of RNA were used in a 23 µl reaction volume. The cycling conditions were 95 °C for 10 min, followed by 40 cycles of 15 sec at 95 °C, 1 min at 65 °C, using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Marnes-la-Coquette). The ubiquitin conjugating enzyme E2 gene was used as an internal control (Table S1) and the relative viral RNA accumulation was determined using the $2^{-DDCt}$ method **(Livak & Schmittgen, 2001)**.

**Supplementary Table 1:** Primer and probe sequences used for virus quantification by RT-qPCR.

| Virus | Sequence (5'---3') | Primer orientation and Probe |
|---|---|---|
| BYV | 5' - TAC TGT TCC AAA CCA GGT CCT TG - 3' | Forward |
| | 5' - ROX - TTG CTT CTT TTT CAA CTC CAC CAC CCT GT - BHQ-2 - 3' | Probe |
| | 5' - GTG CAA CGC AGT TCG AAA CTA A - 3' | Reverse |
| BtMV | 5' - CTC GCT ATG CAT TCG ATT TCT ATG - 3' | Forward |
| | 5' – ATTO647N- TGA TCG TGC ACG TGA AGC TCA TTT CC - BHQ-3 - 3' | Probe |
| | 5' - GCT TGT TGG ACA CGC TTG C - 3' | Reverse |
| UCE2[a] | 5' -GGCTTTCTTGCATTGGTACAC - 3' | Forward |
| | 5' – ROX™ - CGAGGCTGAGAGCAACATGAACGA- BHQ -2 - 3' | Probe |
| | 5' - CATCCTGGTACTGCTGGTATTC - 3' | Reverse |

[a] ubiquitin-conjugating enzyme E2

## Aphid feeding behavior

Aphid probing and feeding was monitored by the electrical penetration graph (EPG) system (Giga-8 DC-EPG amplifier, EPG Systems, Wageningen, Netherlands) as described by Tjallingii **(Tjallingii, 1988)**. We created electrical circuits that each included one aphid and one plant. Adult apterae aphids were tethered to a thin gold wire of an insect electrode (12.5 µm

diameter and 2 cm long) using conductive silver glue (EPG Systems). For wiring, aphids were immobilized at the edge of a pipette tip connected to a vacuum pump (model N86KN.18, KNF Neuberger, Freiburg, Germany). Silver conductive glue was used to attach the opposite end of the gold wire to a 3 cm long copper wire that was soldered to a brass nail using flux enhancement. This electrode nail was inserted into the input connector. The circuit was closed by inserting a copper electrode into the potting soil of the plant. Eight apterous aphids were connected to the EPG amplifier and each one was placed on the third upper leaf of an individual plant. EPG recording was performed inside a Faraday cage during 24 h at a temperature of 20±1 °C and constant light. Each plant and aphid were used only once. Acquisition of the EPG waveforms were carried out with PROBE 3.5 software (EPG Systems). This software was also used to identify the different waveforms correlated with the phases of *M. persicae* feeding behavior, as described **(Tjallingii & Hogen Esch, 1993)**. The Excel workbook developed by Sarria et al. **(Sarria et al., 2009)** was used to calculate the parameters of EPG data. For a given behavior, the parameters of occurrence and total duration were measured and used to analyze the feeding behavior. We decided to select the following parameters for analysis because they are relevant for the acquisition of phloem-limited viruses by aphids, *i.e.*, the total durations and occurrences of stylet penetration, pathway phases, extracellular salivation phase, phloem sap ingestion phases, and the time needed by the aphid to perform the first phloem phase.

**Statistical analyses**

All statistical analyses were made with R version 4.3.2 (https://www.r-project.org/).

For viral load measurements, statistical analysis were performed using a student test when normality and homoscedasticity was confirmed.

The comparison of transmission rates was performed by applying Generalized Linear Models (GLMER, package: "lme4") with a likelihood ratio and chi-squared ($\chi^2$) test. Data on transmission rate were analyzed following a binomial error distribution, and "session" was treated as random factor.

Behavioral variables between mono and co-infection were processed using Generalized Linear Model (GLM) with the likelihood ratio and the chi-squared ($\chi^2$) test. As feeding duration parameters were not normally distributed, we used GLM using a gamma (link = "inverse") distribution, and parameters related to frequency of penetration were modeled using GLM with Poisson (link = "identity") distribution. The parameter "time to first phloem phase" was modeled using the Cox proportional hazards (CPH) model and we treated cases where the given

event did not occur as censored. The assumption of the validity of proportional hazards was checked using the functions “coxph” and “cox.zph”, respectively (R packages: “survival” and “RVAideMemoire”).

The fit of all generalized linear models was controlled by inspecting residuals and QQ plots. When a significant effect was detected, to test for differences between treatments, a pairwise comparison using estimated marginal means (package R: “emmeans”) (*p*-value adjustment with Tukey method) at the 0.05 significance level was used.

## RESULTS

### Symptoms of BYV and BtMV mono-infected and co-infected plants

BtMV and BYV symptoms were compared in single- and co-infected plants. Plants infected with BtMV alone showed reduced growth and exhibited typical mosaic symptoms on upper leaves 4 weeks after virus inoculation. BVY-infected plants showed interveinal yellowing symptoms on old leaves 4 weeks after inoculation, but hardly any growth reduction. The BYV and BtMV co-infections resulted in more severe symptoms, including severe mosaic on the upper leaves, interveinal yellowing of older leaves and a noticeable reduction of plant height. It also resulted in faster appearance of BYV symptoms in co-infected plants (first symptoms were observed 2 weeks after virus inoculation), compared with plants infected with BYV alone **(Fig. 1)**.

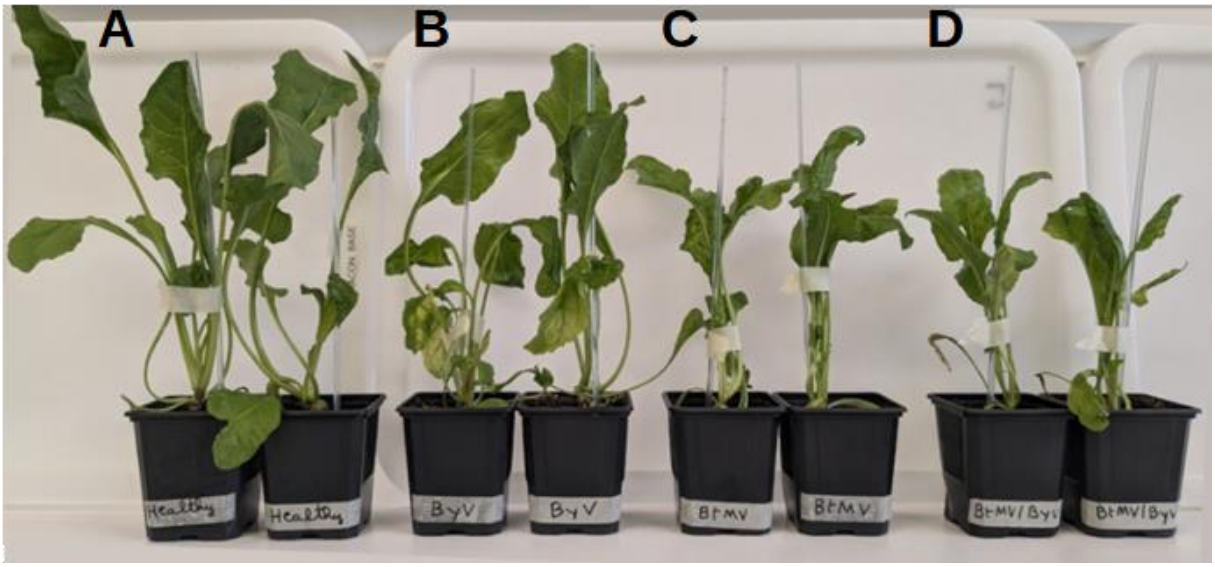



**Fig. 1. Symptoms induced by mono- or co-infection with BYV and BtMV at 28 days post-inoculation.** (A) healthy control plant, (B) BYV-infected plant, C) BtMV-infected plant, and D) plant co-infected by BYV and BtMV.

### Virus transmission by aphids from sugar beet co-infected with BtMV and BYV

To determine whether the co-infection affected virus transmission by *M. persicae*, we performed transmission experiments using, as virus source, single-infected of co-infected sugar beet plants **(Fig. 2)**. In five independent experiments, we observed a significant reduction in BYV transmission when aphids acquired BYV from co-infected plants compared to mono-infected plants (71 % *vs* 36 % transmission rate, respectively) **(Fig. 2A)**. In contrast, co-infection did not have any effect on BtMV transmission rate (aphid transmission rate of 45 % from mono-infected plants and 48 % from BYV/BtMV co-infected plants, respectively, **Fig. 2B)**.

Interestingly, aphids feeding on co-infected plants transmitted both viruses together with 19 % transmission rate, which was lower than expected, based on the virus transmission efficiency of each virus from mono-infected plants (36 % expected co-infection rate, calculated as the product of the proportion of infected over inoculated test plants using mono-infected source plants, **Fig. S1).**

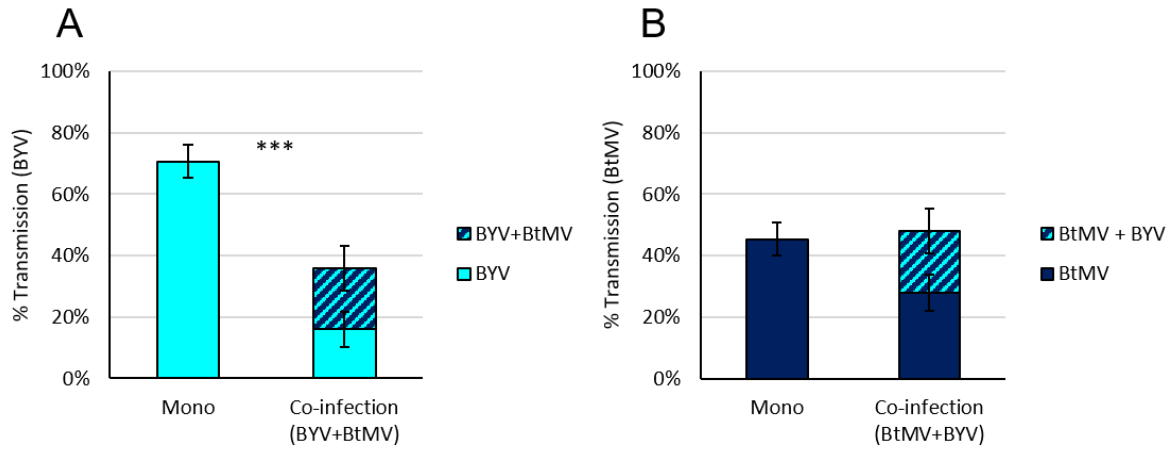
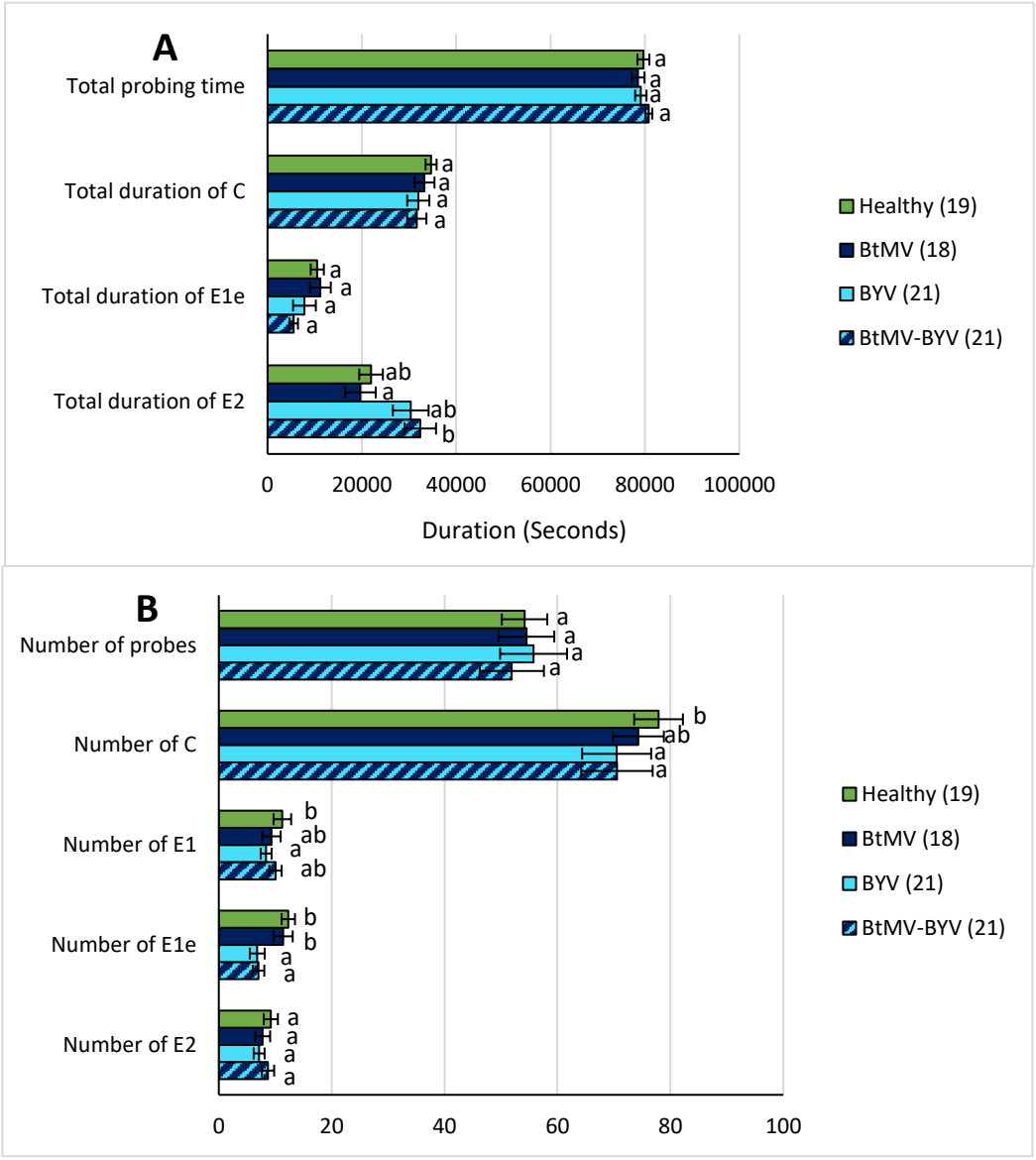



**Figure 2. Aphid transmission efficiency of (A) BYV and (B) BtMV from mono or co-infected plants.** *Myzus persicae* acquired virus from mono or co-infected leaves for 24 h. Then three aphids were transferred per test plant for 72 h inoculation. A DAS-ELISA assay was performed 3 weeks later to detect infection. The percentage of infected plants is indicated (% Transmission). The difference was statistically significant for BYV but not for BtMV transmission (GLMER, BYV: p-value = 1 ; BtMV: p-value< 0.001; n = 75, five independent experiments; df = 1;). NS = not significant, *** = p-value < 0.001.

### Aphid feeding behavior on mono-infected and co-infected plants

The feeding behavior of aphids was monitored using EPG to determine whether it was influenced by single or co-infections of the source plants. We let wired aphids feed on (i) healthy plants, (ii) plants mono-infected with BYV, (iii) plants mono-infected with BtMV, and (iv) plants co-infected with BYV/BtMV for 24 hours. Parameters studied were total durations of probing time (stylet inserted in the plant), intercellular pathway and extracellular salivation

were the same for all conditions **(Fig. 2A)**. The results indicated that aphids spent significantly more time ingesting phloem sap (E2 phase) on BYV/BtMV co-infected than on BtMV infected plants **(Fig. 2A)**. The total number of the probes and phloem sap ingestion were not affected by the different conditions. However, the number of extracellular salivations (E1e) that was significantly lower on BYV mono-infected and co-infected plants with BtMV than on healthy plants, the number of pathway phase (C) was lower on BYV and BYV/BtMV infected plants compared to BtMV and healthy ones, and the number of salivation phase (E1) was lower on BYV infected sugarbeets than healthy plants (**Fig. 2B**).



**Legend to Figure 2 (previous page). Feeding behavior of *Myzus persicae* on healthy, BYV and BtMV mono-infected or BYV/BtMV co-infected sugar beets (n18-21).** (A-B) The behavior of individual aphids was recorded by electrical penetration graph (EPG) for 24 h on the third upper leaf. Selected EPG parameters are presented according to (A) duration or (B) occurrence. The histogram bars display means and standard errors (SEM). Different letters indicate significant differences between plant infection status as tested by GLM (Generalized linear model) followed by pairwise comparisons using package R: emmeans ($P<0.05$ method: Turkey). Statistical analysis of the duration of the events indicates a significant difference for the duration of phloem-sap ingestion (E2) on BYV mono- and BYV/BtMV co-infected plants *vs* BtMV mono-infected plants (GLM, Df = 3, $X^2 = 10.10$, P = 0.0017) but no differences for the total duration of stylet penetrations (probing time) (GLM, Df = 3, $X^2=2.113$, P = 0.549), the total duration of pathway phases (C) (GLM, Df = 3, $X^2 = 1.388$, P = 0.708), the total duration of extracellular salivation (E1e) (GLM, Df = 3, $X^2 = 5.421$, P = 0.143). Statistical analysis of the occurrence of events revealed significant differences between healthy and BtMV mono-infected *vs* BYV mono-infected and BYV/BtMV co-infected plants for the extracellular salivations (GLM, Df = 3, $X^2 = 52.66$ ,P = ), pathway phases (C) (GLM, Df = 3, $X^2 = 10.15$, P = 0.0173) and phloem salivation phases (E1) (GLM, Df = 3, $X^2 = 8.99$, P = 0.029), but no differences for the number of stylet insertions (GLM, Df = 3, $X^2 = 3.01$,P = 0.390) and phloem sap ingestions (GLM, Df = 3, $X^2 = 6.329$, P = 0.096). n = 18-22 as indicated in the graphs.

## BtMV and BYV accumulation in mono- or co-infected plants

The accumulation of BtMV and BYV was quantified in infected plants to see whether virus titers were impacted by co-infection. For this, relative accumulation was measured in sets of infected plants 21 dpi. In co-infected plants, we observed a significant (4 to 10-fold, depending on the experiment) increase of BtMV and BYV titers, compared to plants only infected with BtMV or BYV **(Fig. 3)**. These results suggest a synergistic interaction between the two viruses.

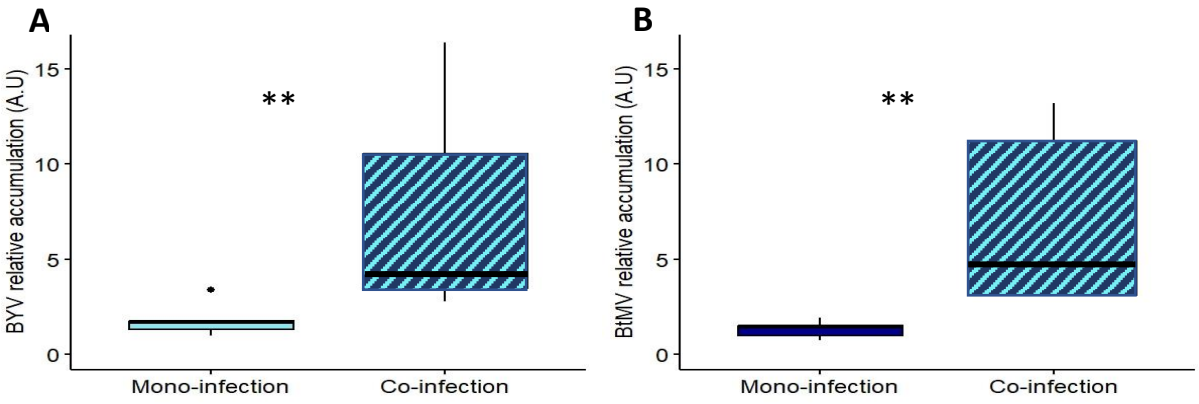



**Figure 3. Relative amounts of BYV and BtMV RNA in mono- and co-infected plants at 21 dpi.** Virus accumulation in source plants was measured by multiplex TaqMan RT-qPCR as described in Materials and Methods. The box plots show medians (black line), 25-75 % percentiles (box) and 10-90 % percentiles. Differences were statistically significant (Student t-test, BYV: t = -0.90817, p-value = 0.00341 BtMV: t = 0.59456 p-value=0.0005761 n = 5; df = 8). **= significant.

## Tissue distribution of BtMV and BYV in mono- and co-infected plants

SABER-FISH was used to see the tissue distribution of BtMV and BYV in mono and co-infected plants at 21 dpi. In cross-sections of BtMV mono-infected leaves, BtMV label was observed in phloem, epidermal and mesophyll, as expected for systemic viruses **(Fig. 4A)**. In BYV mono-infected leaves, BYV fluorescence was observed exclusively in the phloem, as expected for phloem-limited viruses **(Fig. 4B)**. Co-infection did not affect tissue localization of

either virus. BYV label was still restricted to the phloem, and no phloem escape was noticed, and BtMV was detected in different tissues as in mono-infected plants. The number of infected cells varied considerably from one section to another, reflecting an uneven, patchy distribution of both viruses within a leaf at this stage of infection.

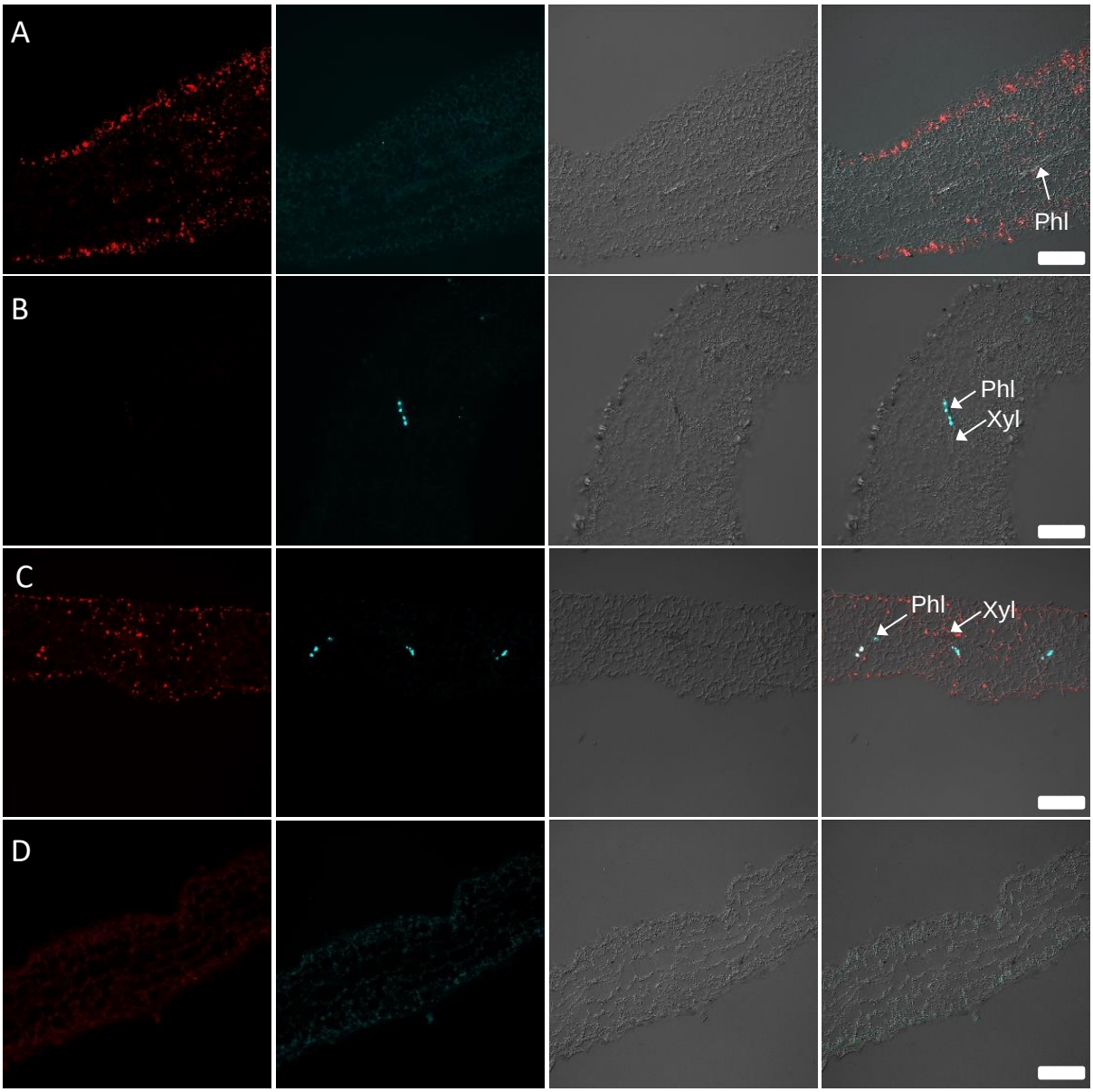



**Figure 4. Tissue localization of BtMV and BYV RNA in leaves mono-infected with BtMV or BYV or co-infected with both viruses.** Leaves were processed three weeks after inoculation by SABER-FISH for detection of BtMV and BYV. (A-D) show transversal sections of (A) BtMV mono-infected, (B) BYV mono-infected, (C) BtMV/BYV co-infected leaves, and (D) healthy leaves. The first column shows the BtMV label (red), the second the BYV label (turquoise), the third bright field acquisitions and the last column presents merges. Scale bars = 100 µm; Phl, phloem; Xyl, xylem.

**Intracellular distribution of BYV and BtMV in mono and co-infected plants**

To see whether co-infection affected intracellular distribution of BtMV or BYV, we performed SABER-FISH on longitudinal sections of infected plants at 21 dpi **(Fig. 5)**. In mono-infected plants, BtMV label was detected throughout the cytoplasm of infected phloem cells **(Fig. 5A)**. In epidermis and mesophyll cells the BtMV label was concentrated in aggregates that seemed to be pressed against the cell periphery **(Fig. 5B)**. In the case of BYV, label was present as spherical cytoplasmic inclusions as reported before **(Khechmar et al., 2024)** in Khechmar et al.,2024 **(Fig. 5B)**. In co-infected cells of BYV/BtMV co-infected plants, BtMV localized to periplasmic aggregates as in mono-infected cells. The BYV label was observed throughout the cytoplasm in a diffuse form, but small cytoplasmic inclusions, similar to the ones observed in mono-infected plants, were also seen in co-infected cells. BYV co-localized partially with BtMV in phloem cells **(Fig. 5C)**.

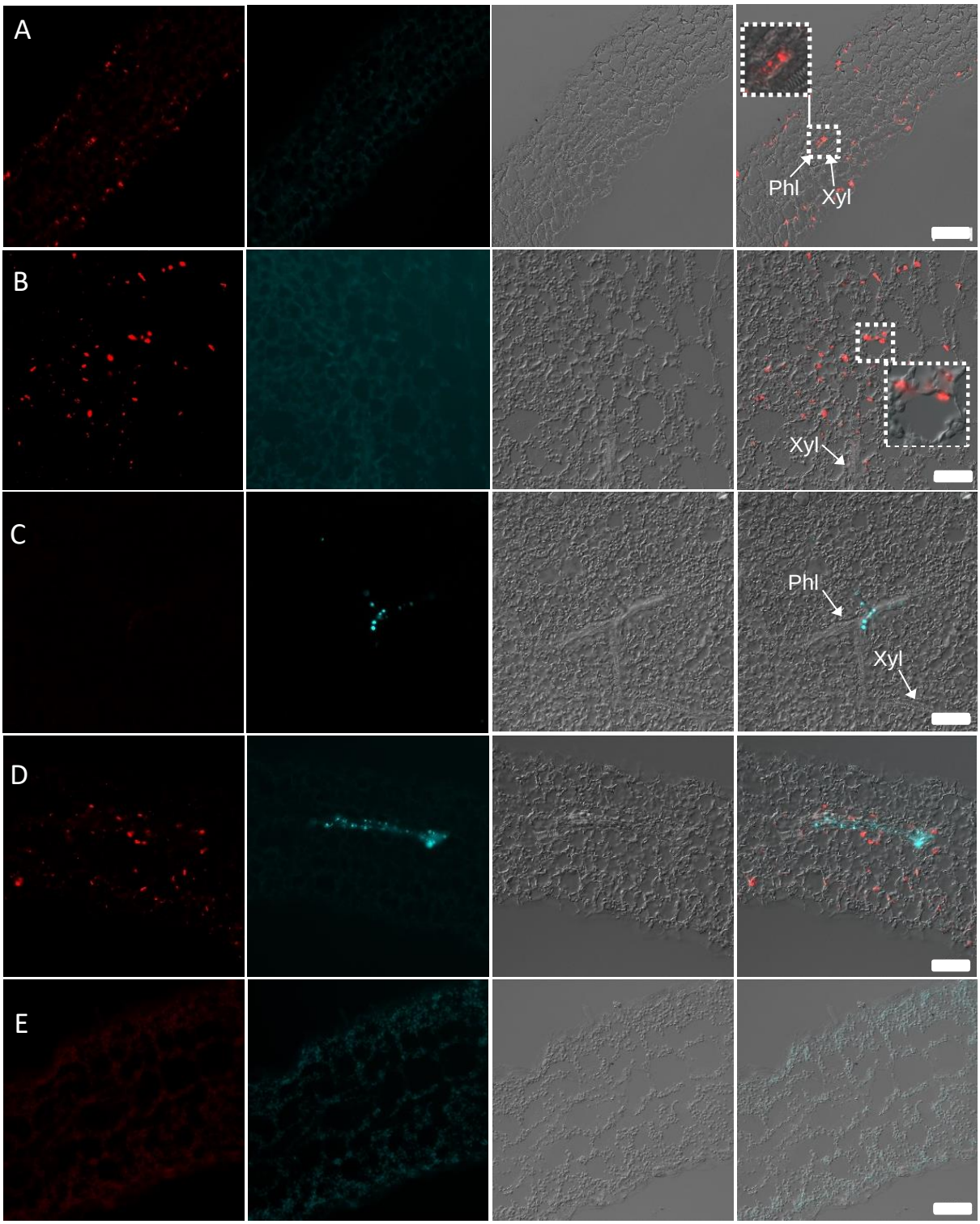



**Figure 5. Intracellular distribution of BYV and BtMV in mono and co-infected leaves.** Leaves were processed three weeks after inoculation by SABER-FISH for detection of BtMV and BYV. The images show longitudinal sections of (A-B) leaves mono-infected with BtMV, (C) a leaf mono-infected with BYV, (D) a leaf co-infected with BtMV and BYV, and (E) a healthy leaf. The first column represents BtMV label (red), the second BYV label (turquoise), the third differential interference contrast images, and the last column presents image merges. Xyl, xylem. Scale bars = 50 µm.

## DISCUSSION

Our data demonstrated that interactions between BYV and BtMV resulted in enhanced titers of both viruses, and a co-incident increase in symptom severity in doubly-infected plants. Similar results were reported by Wintermantel who observed in greenhouse experiments symptom aggravation and more severe stunting of BtMV/BYV co-infected plants, compared to single infections by these viruses **(Wintermantel, 2005)**. In addition, we observed like Wintermantel only a marginal effect of BYV and a more pronounced effect of BtMV on plant growth. In contrast, a recent study did not observe a measurable effect of BtMV/BYV co-infection on symptoms under field conditions **(Borgolte et al., 2024)**. Therefore, experimental conditions and in particular environmental factors might be important for symptom expression. Wintermantel (2005) also observed increased virus titers of BtMV and BYV in co-infected plants, compared to mono-infected plants. A higher potyvirus accumulation was also observed in plants co-infected with the SPFMV potyvirus and the phloem-limited crinivirus SPCSV **(Karyeija et al., 2000)**. However, in most of the reported studies of co-infections involving a potyvirus, the stimulating effect on virus accumulation was observed for the other co-infected virus **(Savenkov & Valkonen, 2001; Wang et al., 2009)** Interestingly, all these reports including ours, showed that co-infection involving a potyvirus resulted in more severe symptoms than single-infected plants.

The increase in BYV and BtMV titers did not correlate with a modification of the virus cellular tropism in doubly-infected plants. This excludes BYV tissue escape and expansion of infection area as the reason for higher accumulation. Consequently, increased titers of BYV and of BtMV could be due to a higher number of infected cells or to higher virus accumulation in infected cells of doubly infected plants. Our SABER-FISH data **(Fig. 4-5)** do not allow to distinguish between the two possibilities because of the uneven, patchy distribution of the viruses that we observed in infected leaves. Another work showed that mixed infection with a potyvirus increased the number of cells infected with potato leaf roll virus **(Savenkov & Valkonen, 2001)** while co-infection by turnip mosaic potyvirus and lettuce infectious yellows crinivirus enhanced crinivirus titers in infected cells **(Wang et al., 2009)**. Thus, the outcome of co-infection depends on the co-infecting viruses, but also the host plant cultivars **(Tatineni et al., 2010)**.

The synergic interaction between BtMV and BYV in co-infected plants could be due to additive effects on viral anti-plant defenses. The BtMV RNA silencing suppressor P1/HC-Pro could be involved in such a mechanism as shown for two other potyviruses in co-infection with a

polerovirus or a crinivirus, respectively **(Domingo-Calap et al., 2021; Savenkov & Valkonen, 2001)**. Concerning BYV, the p21 silencing suppressor might be implicated **(Reed et al., 2003)**. Because P1/HC-Pro and p21 have different modes of action in the RNA silencing pathway, they should not interfere with another and could have additive effects on suppression of host defenses resulting in higher virus accumulation **(Hýsková et al., 2024; Ye & Patel, 2005)**.

Intracellular distribution of BYV was affected in co-infected cells. The typical spherical BYV inclusions were less present in co-infected cells and a diffuse label throughout the cytoplasm was observed that colocalized partially with the BtMV label. Cellular co-infection is therefore possible excluding cellular exclusion between the two viruses. The modified subcellular localization of BYV in co-infected cells is also indicative of direct or indirect interactions between the two viruses. Similar modifications were observed in sugar beet co-infected by BYV and BChV polerovirus where likewise dissolution of BYV inclusions was observed in co-infected cells **(Khechmar et al., 2024)**. The significance of this cellular alteration remains unclear, but atypical intracellular virus inclusions containing both viruses have been described before in other co-infections involving a potyvirus **(Martin et al., 2004)**.

A modified aphid feeding behavior on co-infected plants *vs* mono-infected plants and in particular a reduced phloem sap ingestion could explain a lower BYV uptake and transmission, because this virus is principally acquired from phloem sap **(Jiménez et al., 2018)**. However, our results showed that despite aphids having a tendency to ingest phloem sap for longer durations on co-infected plants than on mono-infected ones, transmission of BYV dropped. This indicates the drop in BYV transmission in co-infection with BtMV does not seem to be linked to a reduction in phloem ingestion time. Interestingly, for BtMV, transmission from co-infected plants was not affected although aphids tended to feed phloem sap longer and the number of pathway phases was lower on co-infected plants compared to BtMV mono-infected plants, behavior changes that are thought to be disadvantageous for transmission of non-circulative viruses. Taken together, we have no evidence that the altered aphid feeding behavior observed on co-infected sugar beets is the cause of the lower BYV transmission from these plants.

We did not observe increased BtMV or BYV transmission by aphids despite a considerable higher virus titer in co-infected plants. This indicates that virus quantity alone is not crucial for virus acquisition and that other factors such as virus tissue and cellular localization or virus morphs define virus accessibility to vectors and subsequent transmission. Co-infection lowered BYV transmission while BtMV transmission was unchanged. The fact that BtMV transmission

was not affected by BYV discards a potential competition for aphid receptors binding and suggests that the two stylet-borne viruses occupy different binding sites in the aphid. This is comforted by microscopic observations, where another potyvirus, potato virus Y, was localized in the stylet tips of *M. persicae* , while the closterovirus citrus tristeza virus was detected in the cibarium of *Toxoptera citricida* aphids **(Killiny et al., 2016; Mondal et al., 2021)**. So the two viruses occupy different regions in the stylets and suggests that the reduction of BYV transmission was due to another cause. Another explication is that, since HC-Pro and BtMV particles can be acquired from epidermis, mesophyll and phloem cells while BYV is essentially acquired from sieve tube sap, and because aphids do first punctures in the tissue before reaching the phloem, HC-Pro and BtMV should be acquired before BYV. Its binding to the stylet tips might hinder passage of BYV and subsequent binding of BYV to the cibarium.

Yet another possibility would be that BtMV blocks phloem loading of BYV and that a decreased BYV concentration in the phloem sap reduces BYV acquisition from co-infected plants. This hypothesis was already evoked to explain BChV inhibition of transmission from BYV and BChV co-infected plants **(Khechmar et al., 2024)**. The potyviral CI and P3N-PIPO proteins that localize to plasmodesmata, might be involved in impeding BYV phloem loading **(Wei et al., 2010)**.

Taken together, we present here that co-infection by BYV and BtMV is synergistic in terms of symptom severity and virus accumulation, but antagonistic for BYV aphid transmission. Intracellular redistribution of BYV in co-infected cells strongly suggests cellular interactions between the two viruses that could lead to the observed phenotypes, but the nature of BYV-BtMV interactions remains to be elucidated.

**Authors' contributions**

**SK:** Conceptualization, methodology, validation, formal analysis, investigation, writing – original draft preparation, writing – review & editing, visualization

**QC:** Methodology, formal analysis, writing – review & editing

**VB:** Conceptualization, methodology, investigation, writing – original draft preparation, writing – review & editing, supervision, project administration, funding acquisition

**MD:** Conceptualization, methodology, investigation, writing – original draft preparation, writing – review & editing, visualization, supervision, project administration, funding acquisition

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**Data Availability**

All relevant data are within the manuscript and its Supporting Information files.

**Competing interests**

The authors have declared that no competing interests exist.

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# Conclusion & Discussion

In this PhD thesis, I described the impact of sugar beet viral multi-infection on the biology of four different viruses – BtMV, BYV, BMYV and BChV – with a focus on transmission. Preliminary experiments were carried out to test virus transmission by aphids from plants infected with the 15 possible combinations of co-infection. Five combinations were identified as having a reproducible effect on the rate of aphid virus transmission, and three of them (BChV/BYV, BtMV/BYV and BChV/BMYV) were subsequently studied in greater depth. The different virus combinations had different outcomes; BYV/BChV co-infection decreased transmission of BChV by 50 %, but had no impact on BYV transmission, BYV/BtMV co-infection correlated with lower transmission of BYV, and interestingly, co-infection of the two poleroviruses BMYV/BChV resulted in mutual transmission synergy. Analysis of aphid feeding behavior, virus accumulation, virus distribution in tissues and virus intracellular localization allowed us to rule out all parameters except virus localization as possible causes. For BMYV/BChV co-infection, about 30 % of infected cells were co-infected, without impacting cell phenotype. Whether and how this could increase transmission, is discussed in **Chapter 2**. For BYV/BChV and BYV/BtMV co-infections, we observed a different intracellular distribution of BYV in mono- *vs* co-infected cells. The form of BYV accumulation shifted from distinct intracellular spherical inclusions in mono-infected cells to a rather diffuse distribution in BtMV/BYV and in BChV/BYV co-infected cells. The reasons for the decrease in transmission by aphids of BChV in BChV/BYV co-infected plants or BYV in BtMV/BYV co-infected plants are currently unknown, but the subcellular localization of both viruses in co-infected plants strongly suggests the existence of interactions between the two viruses that decrease acquisition by aphids. One of the hypotheses put forward is that the concentration of viruses is reduced in phloem sap from which both BChV and BYV are acquired by aphids. How could this be achieved? We propose that alterations in intracellular targeting to plasmodesmata (PD) or intercellular trafficking or modifications of PD could reduce the release of BChV or BYV particles from nucleated phloem cells to the sieve tubes. Several viral proteins and host factors can be implicated in this modification. The alternative suggestion to explain the lower virus acquisition by aphids would be linked to a partial obstruction of the food canal in the sylets due to virus particles binding to their external cuticular receptors which would limit accessibility to receptors or movement of the other virus.

**Hypotheses and factors involved in aphid transmission modification in the context of multi-infection:**

- **Viral proteins interactions**

Good candidates are viral movement proteins (MP) that affect PD size exclusion limits (SEL) and allow cell-to-cell movement of large molecules which transfer would not normally be possible. MP proteins are expressed by most viruses. How could MPs of BtMV and BYV interfere with virus translocation into sieve tube sap? Potyviruses and closteroviruses are filamentous viruses that require multiple proteins for cell-cell movement **(Otulak & Garbaczewska, 2011)**. Modification of plasmodesmata SEL and cell-to-cell movement of potyviruses is monitored by collaboration of CP, HC-Pro, CI, 6K2, P3N-PIPO and VPg. CP and HC-Pro have been shown to induce an increase in PD SEL, traffic through PD and facilitate cell-to-cell movement of viral RNA, while CI in concert with CP was shown to have a role in cell-to-cell movement by forming continuous channels through the center of the CI inclusions and the PD. These channels contain viral RNA and CP in the form of fibrous material resembling potyvirus particles **(Otulak & Garbaczewska, 2011)**. The mechanism of VPg in cell-to-cell transport is not defined. Viral MPs association with viral replication complexes (VRCs) is crucial for intracellular movement as it was demonstrated in the case of the potyvirus TuMV. Its 6K2 protein induces, together with other viral proteins, vesicular VRCs that are used for movement to PD and intercellular movement **(A. Wang, 2021; Xue et al., 2023)**.

BYV encodes besides a non-structural movement protein, the hydrophobic membrane protein p6 **(Peremyslov et al., 2004)**, four structural MPs that binds to the 5’ of end of the virus particles, the so-called tail, CPm, Hsp70h, p64, and p20 **(Prokhnevsky et al., 2002)** that are required for virion assembly and subsequent cell-to-cell movement (CPm, Hsp70h, p64) or phloem-loading (p20). This suggests that the formation of the “tailed” virions is a prerequisite for intercellular trafficking. Hsp70h was shown to localize at the PD, this suggests a specific role played by the thin tails in the entry of virions into the narrow channels of PD **(Alzhanova et al., 2000; Prokhnevsky et al., 2002)**.

Relocalization of movement proteins or alteration of their expression or virion binding capacity caused by mixed infection could hinder viral movement within and between cells. In the case of mixed infection, heterologous interactions between viral proteins of different virus species

can also occur **(Leastro et al., 2020)** that can potentially affect virus movement within the cell or from cell-to-cell.

In the case of BtMV/BYV co-infection, interaction between movement proteins of the two viruses could lead to inhibition of BYV movement and release from companion cells to phloem lumen. We speculate that a potential direct or indirect interaction between MPs of the two viruses inhibits the formation of the BYV "tailed" virions that is a prerequisite for intercellular trafficking. Another possible scenario is that BtMV forms a physical barrier in plasmodesmata that can inhibit movement of BYV: CI structures on PD (see above) could potentially affect the PD binding of the Hsp70h of BYV and thereby the movement of the virus through the PD in mixed infection. Since there is also some assumption that 6K2 replication vesicles are within the PD-located conical structures in potyvirus-infected cells, we can also suggest a possible steric inhibition of BYV movement through PD by these vesicles.

Modification of PD SEL and cell-to-cell movement in poleroviruses is also mediated by several viral proteins. Four of the seven ORFs of the well characterized turnip yellows virus (which is closely related to BChV and BMYV) encode proteins involved in viral movement, underlining the complexity of this essential step in the viral cycle. These are the ORFs encoding major (CP) and minor (RT) capsid proteins, the movement proteins P4 (also referred to as P17) and P3a. The latter is a well conserved small 5 kDa protein in poleroviruses which is essential for virus systemic movement within the plant (Smirnova et al., 2015a). A number of small hydrophobic viral proteins (4 to 9 kDa) have been characterized for other plant viruses and very often, they are associated with a movement protein capable of binding RNA. These proteins are essential for cell-to-cell movement and long-distance transport of the virus **(Morozov & Solovyev, 2020; Olmedo-Velarde et al., 2023)**. The TuYV P3a protein belongs to this class of small hydrophobic proteins and is localized to the Golgi apparatus and to plasmodesmata. Poleroviral P4 is non-structural MP with properties similar to those of the TMV 30 kDa MP, as ssRNA binding, protein dimerization, phosphorylation by a membrane-bound protein kinase, plasmodesmal localization in both virus-infected and transgenic plants and ability to increase plasmodesmal SELs.

For the co-infection of the polerovirus BChV with the closterovirus BYV we hypothesize, as for BYV/BtMV, that an alteration in intracellular or intercellular trafficking or modifications of PD could reduce the release of BChV into the sieve tubes. Again, direct or indirect interactions between BYV and BChV MPs could reduce BChV phloem charge. Since both viruses are phloem-limited, we assume that they require specialized movement proteins to enter,

move through, and exit the phloem tissue. These virus proteins might compete for binding sites on host proteins or cellular structures involved in phloem transport and lead to antagonistic effect on the movement of one of the two viruses, in this case BChV.

In spite of the striking diversity of virus transport systems and lack of similarity between MPs of different virus groups, complementation of plant virus movement is a very common phenomenon. It is known as transcomplementation or heterologous complementation, allowing one virus to assist the movement of another. For instance, TMV MP complements a potato spindle tuber viroid RNA mutant impaired for mesophyll entry but not mutants unable to enter the phloem **(J. Wu & Bisaro, 2022)**. TMV MP also complements movement defective mutants of cucumber mosaic virus (CMV) **(Cooper et al., 1996)**. In our case, complementation for virus phloem loading does not seem to occur since an inhibition of the phloem acquisition of viruses is observed rather than an increase. However, it is still conceivable that in the specific combination between BtMV and BYV, the combined action of MPs enhances virus intercellular transport increasing the number of infected cells and/or the virus titers, as observed in our experiments.

For the BChV/BMYV combination, aphid transmission synergy was observed. While we discussed in **Chapter 2** that the most likely explanation is BChV/BMYV synergy during virus inoculation in a new host, by suppression of plant anti-viral defenses through the combined action of BChV and BMYV anti-defense proteins (potentially RNA silencing suppressors), an alternative scenario is that the movement proteins of the two poleroviruses interact with each other to facilitate phloem entry. This could result in higher virus charge in the phloem and higher virus loading in the vector.

- **Host proteins involvement**

Host factors might be involved. One candidate is cell wall pectin methylesterase (PME). For example, MPs of TMV, turnip vein-clearing virus (TVCV), and cauliflower mosaic virus (CaMV) were both found to interact with PME **(Chen et al., 2000)**, but the mode of action of PME in virus movement still remains unclear **(Lionetti et al., 2014)**. PME could potentially be involved in phloem sap release of BtMV, because potyvirus infection modifies PME activity **(Otulak-Kozieł et al., 2024)**.

Viruses rely on host cellular components, including the cytoskeleton, to facilitate their movement. Host factors associated with microfilaments (actin filaments) and microtubules are important cytoskeletal components that can be co-opted by viruses to facilitate intracellular and

cell-to-cell movement, but also replication. Actin filaments are primarily involved in intracellular transport and plasmodesmata modification, and microtubules play roles in the cortical anchorage of replication complexes and the formation of virus factories **(Pitzalis & Heinlein, 2018)**. Actin filaments and microtubules often work in concert **(Farhadi et al., 2020)**. For instance, the actin cytoskeleton can influence the organization and dynamics of microtubules, creating a synergistic effect that enhances transport and replication **(Yuan et al., 2023)**. Both cytoskeletal components can recruit various host factors that can also be essential for viral movement. These include motor proteins, cytoskeletal-associated proteins and other cellular machinery that viruses hijack to support their life cycle. Plant viruses do not all rely on the same cytoskeletal resources for movement and spread within host plants, some rely on actin microfilaments, and others use microtubules for intracellular movement, or both. There is evidence that P4 of PLRV requires actin filaments for its transport to PD in source leaves and microtubules for its degradation by proteasomes in sink tissues **(Vogel et al., 2007)**. It is likely that P4 of the related BChV and BMYV could utilize actin for their movement to PD as well. The closterovirus BYV likely interacts with both actin filaments and microtubules: it was shown that the BYV 6-kDa movement protein (p6) associates with the endoplasmic reticulum (ER) and is implicated in actin-mediated transport. BYV MP Hsp70h has been shown to interact with microtubules, suggesting a potential role of these cytoskeletal elements in BYV movement. In co-infection scenario, implication of actin-related transport for both viruses (BChV and BYV) could affect intracellular movement and distribution of both viruses. Similarly, BtMV is speculated to rely on both cytoskeletal components. As mentioned above, the 6K2 protein of potyviruses, so probably also of BtMV, interacts with the host cytoskeleton to induce the formation of membranous vesicles involved in viral replication and movement, and HCPro can also interact with cytoskeleton and is involved in replication, encapsidation and movement. Again, similar transport routes between BtMV and BYV may have repercussion on virus intracellular distribution.

Presently, we do not know how co-infection modifies intracellular transport of BYV, BtMV and BChV. It was shown for other pathosystems that even viruses from the same genera do not necessarily rely on the same cytoskeletal resources, as for the two tobamoviruses TMV and turnip vein-clearing virus (TVCV), where disruption of microfilaments inhibited TMV but not TVCV movement **(Harries et al., 2009)**. In co-infection scenarios where viruses use different cytoskeletal elements, (e.g. one primarily uses actin and the other microtubules), the viruses might co-exist simply without direct competition but one can also imagine complex interactions

between the viruses and the host cytoskeleton that recondition the cytoskeleton as each virus attempts to optimize its replication and movement. For example, the cauliflower mosaic virus protein P6 forms motile inclusions that traffic along actin microfilaments and stabilize microtubules **(Naghavi & Walsh, 2017)**. In general, cytoskeleton-viral interactions depend on the viruses involved, the mechanism used in these interactions and also the host plant species. So, there is a body of evidence that the cytoskeleton is important also for movement of BYV, BtMV, BChV and BMYV, but whether and how co-infection impacts movement of one or the other virus and possibly virus acquistion, remains to be determined.

- **Hindrance in vector**

The last hypothesis is that interaction between viruses in the vector changes transmission. This could occur for the non-circulative viruses BYV and BtMV in the aphid mouthparts. The mouthparts include the acrostyle which is located at the tip of the stylets and contains proteins that serve as binding sites for various plant viruses, such as the cauliflower mosaic virus (CaMV) and potentially BtMV as the related PVY seems to dock to it **(Mondal, Ghanim, et al., 2021)**. The acrostyle is considered as a transit point for other viruses including closteroviruses. The closterovirus citrus tristeza virus (CTV) was shown to be retained in the cibarium of the aphid vector, and the criniviruses lettuce infectious yellows virus (LIYV) and cucurbit chlorotic yellows virus (CCYV) are retained in the whitefly cibarium. **(A. Y. S. Chen et al., 2011; Killiny, Harper, Alfaress, Mohtar, et al., 2016; Li et al., 2016)**. Thus, BYV might also bind to the cibarium. The cibarium is a specialized structure in the aphid's foregut that controls fluxes to the intestine. If we assume that BYV is also retained in the cibarium of its aphid vector we can speculate that any obstruction of the acrostyle by BtMV could potentially hinder the binding of BYV further up in the aphid's stylets. The same hypothesis can be applied in the case of BYV/BChV. Here, reduction in BChV transmission efficiency could be due to interference between BYV and BChV in the aphid vector mouthparts: BYV, being retained in the cibarium, may create physical barriers that impedes BChV from effectively passing through the cibarium and invade the intestine.

**How to continue? What are the next steps?**

We have proposed a model for both BtMV/BYV and BChV/BYV co-infections to explain the dropin transmission of BYV and BChV, respectively. It postulates that decreased virus concentration in the phloem sap may cause the lower transmission of the virus. To verify this, we could measure virus accumulation in the phloem sap collected from co-infected plants using

techniques like aphid stylet severing or EDTA-facilitated phloem sap exudation. By comparative RNAseq analyses on co- or mono-infected plants, it could be possible to identify candidate genes involved in intracellular transport, PD function, phloem loading and so on. Using reverse genetics to inhibit their expression could provide confirmation of their involvement in virus intra- or inter-cellular transport. Analyzing protein-protein interactions (viral and host proteins) could also reinforce implication of candidate proteins in virus transport. In our pathosystem, technical obstacles need to be overcome to conduct functional validation tests. First, a full-length infectious clone of BYV is not any more available and new ones may be difficult to obtain due to its genome length, prone to rearrangements in bacteria. Full-length infectious clones of BMYV **(Klein et al., 2013)**, BChV **(Wetzel et al., 2018)** and BtMV **(Rollwage et al., 2023)** have been developed and allow functional assays at least for these three viruses. Second, sugar beet is not a model plant and plant transformation is lengthy. Nonetheless, recently, CRISPR/Cas9 was applied to sugar beet to mutate the translation initiation factor eIFiso4E which resulted in resistance to BChV **(Rollwage et al., 2024)**. *Nicotiana benthamiana* could be used as an alternative host species to conduct functional validation experiments.

To conclude, we show that virus transmission efficiency from co-infected plants is not always fully explained by differences in virus accumulation, virus localization, or aphid feeding behavior alone. While each single of these factors may be important, their combined effects on co-infecting viruses in the host plant or/and in the vector are probably more important and may alter transmission dynamics. Understanding these complexities requires considering a wider range of molecular, physiological, and ecological aspects beyond the three mentioned here.

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# Supplementary data

This part contains the supplementary data accompanying the **Chapters 1**, **3** & **4**.

## Supplementary data for Chapter 1

**Table.1:** ***Transmission experiments from mono-infected source plants.*** Indicated are the percentages of transmission. (Exp x) refers to the same experiment throughout Tables 1-4. (-) indicates that this condition was not run in the experiment.

| Mono-infection | Experiment | BYV | BChV | BtMV | BMYV |
|---|---|---|---|---|---|
| BYV | Exp 1' | 73% | | | |
| | Exp 2' | 73% | | | |
| | Exp 3' | 86% | | | |
| | Exp 4' | 40% | | | |
| | Exp 5' | 60% | | | |
| | Exp 1 | - | | | |
| | Exp 2 | 80 % | | | |
| | Exp 4 | 87 % | | | |
| | Exp5 | 60 % | | | |
| | Exp6 | 60 % | | | |
| | Exp7 | 80 % | | | |
| BChV | Exp 1' | | 53% | | |
| | Exp 2' | | 46% | | |
| | Exp 3' | | 53% | | |
| | Exp 1 | | - | | |
| | Exp 2 | | 20 % | | |
| | Exp 4 | | 47 % | | |
| | ExP5 | | 27 % | | |
| | Exp6 | | 47 % | | |
| | Exp7 | | 40 % | | |
| BtMV | Exp1' | | | 7% | |
| | Exp 4' | | | 7% | |
| | Exp 5' | | | 26% | |
| | Exp 1 | | | 60 % | |
| | Exp 2 | | | 60 % | |
| | Exp 4 | | | 53 % | |
| | Exp5 | | | 66 % | |
| | Exp6 | | | 66 % | |
| | Exp7 | | | 40 % | |
| BMYV | Exp 1 | | | | - |
| | Exp 2 | | | | 40 % |
| | Exp 4 | | | | 40 % |
| | Exp5 | | | | 27 % |
| | Exp6 | | | | 33 % |
| | Exp7 | | | | 15 % |

**Table.2:** ***Transmission experiments from double-infected source plants***

| Double infection | | BYV | BChV | BtMV | BMYV | BYV+BChV | BYV+BtMV | BChV+BtMV | BMYV+BtMV | BMYV+BYV | BMYV+BChV |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BYV+BtMV | Exp1' | 33 % | | 7% | | | 31% | | | | |
| | Exp 4' | 26 % | | 33% | | | 0% | | | | |
| | Exp 5' | 7 % | | 33% | | | 0% | | | | |
| | Exp 1 | - | | - | | | - | | | | |
| | Exp 2 | - | | - | | | - | | | | |
| | Exp 4 | 20 % | | 33 % | | | 40 % | | | | |
| | Exp5 | - | | - | | | - | | | | |
| | Exp6 | 20 % | | 26 % | | | 33 % | | | | |
| | Exp7 | 0 % | | 46 % | | | 14 % | | | | |
| BMYV+BtMV | Exp 1 | | | - | - | | | | - | | |
| | Exp 2 | | | - | - | | | | - | | |
| | Exp 4 | | | 33 % | 13 % | | | | 40 % | | |
| | Exp5 | | | 86 % | 0 % | | | | 0 % | | |
| | Exp6 | | | 14 % | 14 % | | | | 60 % | | |
| | Exp7 | | | 0 % | 20 % | | | | 0 % | | |
| BChV+BtMV | Exp1 | | - | - | | | | - | | | |
| | Exp2 | | - | - | | | | - | | | |
| | Exp4 | | - | - | | | | - | | | |
| | Exp5 | | 0 % | 14 % | | | | 7% | | | |
| | Exp6 | | 13 % | 33 % | | | | 33 % | | | |
| | Exp7 | | 0 % | 60 % | | | | 0 % | | | |
| BYV/BMYV | Exp 1 | - | | | - | | | | | - | |
| | Exp 2 | - | | | - | | | | | - | |
| | Exp 4 | 47 % | | | 7 % | | | | | 13 % | |
| | Exp5 | 73 % | | | 0 % | | | | | 0 % | |
| | Exp8 | 87% | | | 0% | | | | | 7% | |
| | Exp9 | 73% | | | 0% | | | | | 21% | |
| BMYV/BChV | Exp 1 | | - | | - | | | | | | - |
| | Exp 2 | | - | | - | | | | | | - |
| | Exp 4 | | 33 % | | 7 % | | | | | | 7 % |
| | Exp5 | | 14 % | | 0 % | | | | | | 20 % |
| | Exp6 | | 20 % | | 13 % | | | | | | 40 % |
| | Exp7 | | - | | - | | | | | | - |
| BChV/BYV | Exp 1' | 80% | 0% | | | 7% | | | | | |
| | Exp 2' | 80% | 13% | | | 7% | | | | | |
| | Exp 3' | 66% | 0% | | | 26% | | | | | |
| | Exp1 | - | - | | | - | | | | | |
| | Exp2 | - | - | | | - | | | | | |
| | Exp4 | - | - | | | - | | | | | |
| | Exp5 | - | - | | | - | | | | | |
| | Exp6 | 53 % | 20 % | | | 26 % | | | | | |
| | Exp7 | 40 % | 0 % | | | 7 % | | | | | |

**Table.3:** ***Transmission experiments from triple-infected source plants***

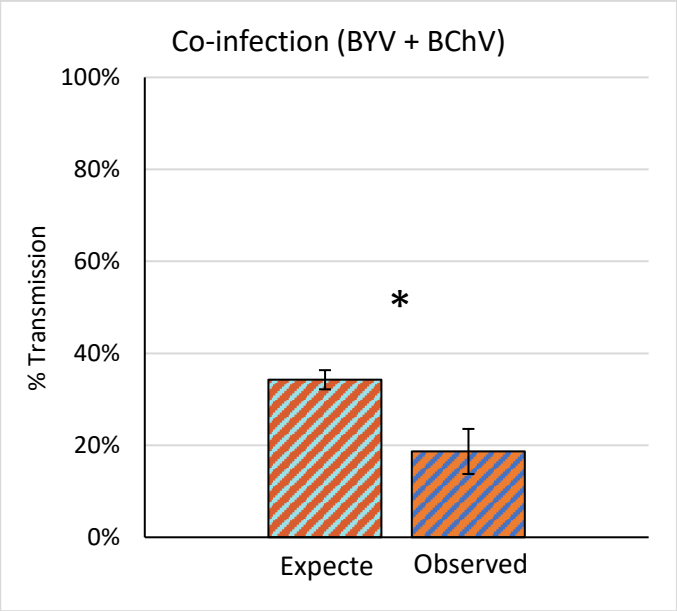
| Triple Infection | | BYV | BtMV | BChV | BMYV | BYV+ BChV | BYV+ BtMV | BChV+ BtMV | BMYV+ BtMV | BMYV+ BYV | BMYV+ BChV | BChV+BYV +BMYV | BMYV+BChV +BtMV | BtMV+BYV +BMYV | BChV+BYV +BtMV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BMYV/BChV/BYV | Exp 1 | 53 % | | | 13% | 0 % | | | | % | 0 % | 7 % | | | |
| | Exp 2 | 66 % | | 0% | 0 % | 0 % | | | | % | 0 % | 27 % | | | |
| | Exp 4 | 67 % | | 14% | 0 % | 0 % | | | | 7 % | 0 % | 0 % | | | |
| | Exp5 | 40 % | | 0% | 0 % | 7 % | | | | 7 % | 7 % | 0 % | | | |
| | Exp6 | 53 % | | 0% | 0 % | 7 % | | | | 0 % | 0 % | 40 % | | | |
| | Exp7 | 53 % | | 0% | 0 % | 0 % | | | | 0 % | 0 % | 0 % | | | |
| BChV/BMYV/BtMV | Exp1 | | % | % | % | | | % | % | | % | | % | | |
| | ExP2 | | 0 % | 27 % | 7 % | | | 13 % | 7 % | | 7 % | | 33 % | | |
| | Exp4 | | % | % | % | | | % | % | | % | | % | | |
| | Exp5 | | 10% | 40% | 0% | | | 0 | 0 | | 20% | | 0 | | |
| | Exp6 | | 0 % | 40 % | 0 % | | | 7 % | 20 % | | 0 % | | 20 % | | |
| | Exp7 | | | | | | | | | | | | | | |
| BYV/BMYV/BtMV | Exp1 | % | | % | % | | % | | % | | % | | | % | |
| | Exp2 | % | | % | % | | % | | % | | % | | | % | |
| | Exp4 | % | | % | % | | % | | % | | | | | % | |
| | Exp5 | 0 % | | 0 % | 20 % | | 0 % | | 0 % | | | | | 0 % | |
| | Exp6 | 14 % | | 7 % | 7 % | | 20 % | | 14 % | | | | | 53 % | |
| | Exp7 | % | | % | % | | % | | % | | | | | % | |
| BChV/BYV/BtMV | | 20% | 7% | 7% | | 0% | 0% | 0% | | | | | | | 0% |

**Table.4:** ***Transmission experiments from infected source plants with four viruses***

| | | BYV | BtMV | BChV | BMYV | BYV+ BChV | BYV+ BtMV | BChV+ BtMV | BtMV +BMYV | BMYV +BYV | BChV +BMYV | BMYV+BYV +BChV | BMYV+BChV +BtMV | BMYV+BChV +BtMV | BtMV+BYV +BMYV | 4 Viruses |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4 viruses | ExP1 | 33 % | 0 % | 7 % | 0 % | 0 % | 20 % | 7 % | 0 % | 0 % | 0 % | 0 % | 0 % | 7 % | 7 % | 13 % |
| | Exp2 | 0 % | 0 % | 33 % | 0 % | 0 % | 0 % | 7 % | 0 % | 0 % | 13 % | 0 % | 13 % | 0 % | 0 % | 0 % |
| | ExP4 | 0 % | 14 % | 7 % | 0 % | 20 % | 7 % | 0 % | 0 % | 0 % | 0 % | 7 % | 0 % | 0 % | 0 % | 0 % |
| | Exp5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | Exp6 | 46 % | 0 % | 0 % | 0 % | 0 % | 20 % | 0 % | 0 % | 0 % | 0 % | 7 % | 7 % | 0 % | 0 % | 7 % |
| | Exp7 | 27 % | 0 % | 27 % | 0 % | 27 % | 0 % | 0 % | 0 % | 0 % | 0 % | 0 % | 0 % | 0 % | 0 % | 0 % |

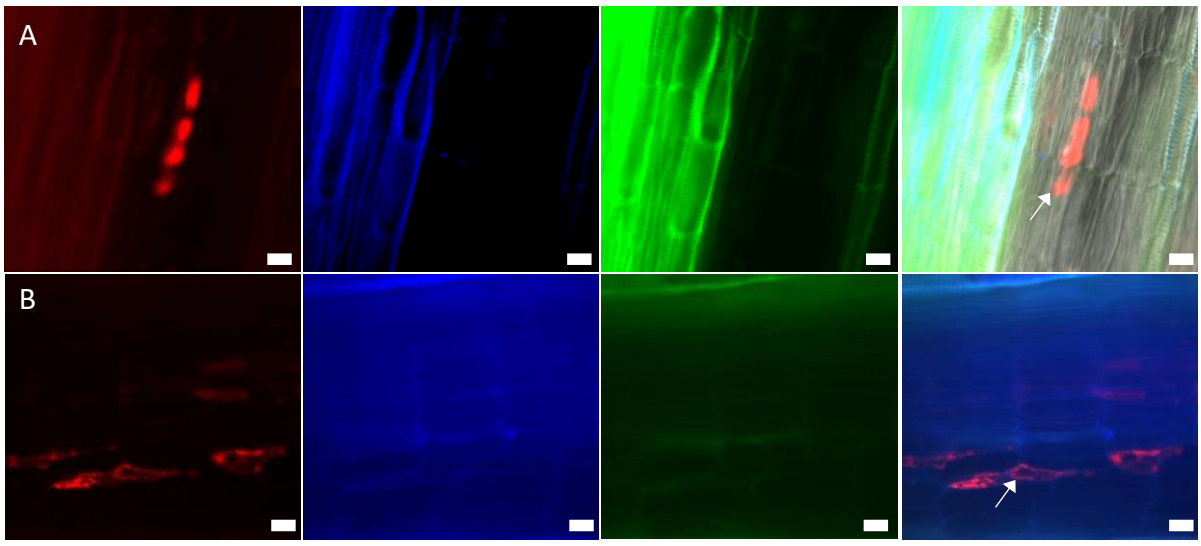
## Supplementary data for Chapter 2 (Article 1)

## S1 Figure




**S1 Figure. Aphid transmission efficiency of BYV and BChV from co-infected plants.** *Myzus persicae* acquired virus from co-infected leaves for 24 h. Then, three aphids were transferred per test plant for 72 h inoculation. A DAS-ELISA assay was performed 3 weeks later to detect infection. The percentage of expected and observed co-infected plants is indicated. Difference was statistically significant (p-value= 0.02865; n=75, five independent experiments; df=1; chi-squared($\chi$2)).

## S2 Figure




**S2 Figure. Tissue distribution of BChV and BYV in roots mono-infected with BChV or BYV**. Sugar beet roots were processed three weeks after inoculation for immunofluorescence detection of BChV and BYV. (A-B) show longitudinal sections of (A) BYV mono-infected, (B) BChV mono-infected roots. The first column shows the BYV, BChV label, the second and the third columns showed autofluorescence and the last column presents merges. The white arrows indicate the label. Scale bars = 200 µm (A-B).

## S1 Table

**S1 Table. Primers with concatemer sequences (red) used for Primer Exchange Reaction.**

| BChV | |
|---|---|
| Primer 1 | GTGCCATGGTCCCAAGAGCTCCAAATAAGATTAGGGTTTCATCATCAT |
| Primer 2 | TGTAGCCTCTTTGCAAGACGTGTATTACAAGTTCTTTGTAGCTGTTTCATCATCAT |
| Primer 3 | AGGCCATCAATGAGGCCACTATTATAGCCAGGCTTTCATCATCAT |
| Primer 4 | GGGTTTCATTGGTACAGCCCATGTAGTGAACCCCTTTCATCATCAT |
| Primer 5 | CGCGCTCATCACCATCCTTGCCGTTTCATCATCAT |
| Primer 6 | GGTCTCTCTATGCAAACTCTCTTCATCGTGGTGAACGCTTTCATCATCAT |
| Primer 7 | TCGTTTTGAAGCAAATCTCAAAGTTCATTCACTGTCCTCCTGCTTTTCATCATCAT |
| Primer 8 | CAAGTGTCTTTCGCTCGTCACTAGTAGTTCGCTGTTTTCATCATCAT |
| Primer 9 | GGTATCAACTTGGTGGACCAGTTATTGAAAACTTCTTGTGGTTCCTCACTTTCATCATCAT |
| Primer 10 | GGACTTCCATGTCATCTTCGAGCATCCAGTCCGCTACTTTCATCATCAT |
| Primer 11 | CGTAATCGTTTTGTTGTGTGGTTGTTGTTTCTGGTCAAGCGATTGCTTTCATCATCAT |
| Primer 12 | GCTTGATCCTCCTGAATTAGTTTTGTGGTTGGACTGGAATGACCTTTCATCATCAT |
| Primer 13 | GGTCTCCACGATTCAACCAATTCCGGCGTTGGATTAAAATTTCCTTTCATCATCAT |
| Primer 14 | GGAGTTTTAAGGTCTTGTCTTTGACCGGTTTTGAGTTTCTCGGAGTCTTTCATCATCAT |
| Primer 15 | CCAGTGACGGTTGAGAAAACACTCCCATTGATGGGTTTCATCATCAT |
| Primer 16 | TGCGTGTGAACCGTCGTCATGACTTATGAGCTTTCATCATCAT |
| Hairpin | Hairpin.27 : ACATCATCATGGGCCTTTTGGCCCATGATGATGTATGATGATGTTTTTTT |
| Imager | Fluor 27 /5ATTO550N/TTATGATGATGTATGATGATGT |

| BYV | |
|---|---|
| Primer1 | CTGTCGGGCACAATGGGGCTTGCACTTTATAAACCTA |
| Primer2 | CATGTCTCGGCGGGTGTCCACGACTTTATAAACCTA |
| Primer4 | GGGTTGTCGGTAGAAGCACTCAAGTGCACACGTTTATAAACCTA |
| Primer5 | GCACGACCTCGGTGCAGGCTCGTTTATAAACCTA |
| Primer6 | GGGAACTGACAGAGAGGACTTCAGTTGGGAACACGTTTATAAACCTA |
| Primer7 | CTGTAAGAGCCGACCAGCTGGAAGAGTCTTCGTTTATAAACCTA |
| Primer8 | GGGCGCGAGTTAGGTTTCGATGTAACACCTAACGTTTATAAACCTA |
| Primer9 | CGCGAGGAGTTTGTTGCCTCCTGCGTTTATAAACCTA |
| Primer10 | CGCGCGCCTGAGCTTCAACAAGTTGTTCGTTTATAAACCTA |
| Primer11 | GTTCGCCCGGAGTCACCATGGTGAGGTTTATAAACCTA |
| Primer12 | GCCATGTCGATAGCGAGAGTTAAACGTTAAAGGCAGGGTTTATAAACCTA |
| Primer13 | CACTCGAGTGTAAGAGACCGAACTGGGTTTCAACCTATTCATACTTTATAAACCTA |
| Primer14 | CCATTTCGGCGTGCGTGGGAATTTGTGCATTTATAAACCTA |
| Primer15 | CTACGAAAGGAGCCGCCACTTCAGCCAGTTCTTTATAAACCTATTTATAAACCTA |
| Primer16 | CTTCCACTCCACTCATGTTTATTGTGAATTCACCGAGAGCCGTTTATAAACCTA |
| Primer17 | GGAACAGTTTGGGCAACGAACGAGCTATCACTATGTAAGCTTTATAAACCTA |

| | |
|---|---|
| Primer18 | CGGCGCGAACACTGGGTCCGAGTTTATAAACCTA |
| Primer19 | GCGGCGTGGCCGCAGTGACTACTTTATAAACCTA |
| Primer 20 | GAGCGGCGTGGGCGAGGTAACATAACCTTTATAAACCTA |
| Primer21 | CGAGAGACTCGTCTTCCGCGCACAGGTTTATAAACCTA |
| Primer 22 | GCTGACACGTTCGTAGACTGATTCGATCGCCTTGTTTATAAACCTA |
| Primer23 | CCACTCTGCACGGCCTCCCCCATATAGAAGTTTATAAACCTA |
| Primer 24 | CCAGCAGACGGGAACCGGCCGTTTATAAACCTA |
| Primer25 | CGGTCTGCACACGTGGTAATCTGTGCTCCTTTATAAACCTA |
| Hairpin | Hairpin.26 : AATAAACCTAGGGCCTTTTGGCCCTAGGTTTATTTAGGTTTATTTTTTT |
| Imager | Fluor26 /ATTO647N/TTTAGGTTTATT TAGGTTTATT |

## Supplementary data for Chapter 3 (Article 2)

**Table S1: Primer sequences used for virus detection**

| Virus | Sequence (5'---3') | Sense | Expected size | Use |
|---|---|---|---|---|
| BMYV | CTA-ACA-GCT-ACA-GAG-CGA-GT | forward | 390 bp | RT-PCR IC-RT-PCR |
| | GCT-TTC-AGC-ACA-CCA-TAC-TG | reverse | | |
| BMYV | CCT-TCT-ACA-CCG-CGG-GAA-CC | forward | 96 bp | RT-qPCR |
| | GTC-GTG-GCC-TGC-AAT-TTG-GC | reverse | | |
| BChV | CAA-CCG-CCG-GCA-ATT-CCC-TA | forward | 115 bp | RT-PCR IC-RT-PCR RT-qPCR |
| | ACG-AGT-CCC-GTA-GGC-GAT-GT | reverse | | |

**Table S2: Primers with concatemer sequences (in red) used for Primer Exchange Reaction**

| BChV | |
|---|---|
| Primer 1 | GTGCCATGGTCCCAAGAGCTCCAAATAAGATTAGGGTTTCATCATCAT |
| Primer 2 | TGTAGCCTCTTTGCAAGACGTGTATTACAAGTTCTTTGTAGCTGTTTCATCATCAT |
| Primer 3 | AGGCCATCAATGAGGCCACTATTATAGCCAGGCTTTCATCATCAT |
| Primer 4 | GGGTTTCATTGGTACAGCCCATGTAGTGAACCCCTTTCATCATCAT |
| Primer 5 | CGCGCTCATCACCATCCTTGCCGTTTCATCATCAT |
| Primer 6 | GGTCTCTCTATGCAAACTCTCTTCATCGTGGTGAACGCTTTCATCATCAT |
| Primer 7 | TCGTTTTGAAGCAAATCTCAAAGTTCATTCACTGTCCTCCTGCTTTTCATCATCAT |
| Primer 8 | CAAGTGTCTTTCGCTCGTCACTAGTAGTTCGCTGTTTTCATCATCAT |
| Primer 9 | GGTATCAACTTGGTGGACCAGTTATTGAAAACTTCTTGTGGTTCCTCACTTTCATCATCAT |
| Primer 10 | GGACTTCCATGTCATCTTCGAGCATCCAGTCCGCTACTTTCATCATCAT |
| Primer 11 | CGTAATCGTTTTGTTGTGTGGTTGTTGTTTCTGGTCAAGCGATTGCTTTCATCATCAT |
| Primer 12 | GCTTGATCCTCCTGAATTAGTTTTGTGGTTGGACTGGAATGACCTTTCATCATCAT |
| Primer 13 | GGTCTCCACGATTCAACCAATTCCGGCGTTGGATTAAAATTTCCTTTCATCATCAT |
| Primer 14 | GGAGTTTTAAGGTCTTGTCTTTGACCGGTTTTGAGTTTCTCGGAGTCTTTCATCATCAT |
| Primer 15 | CCAGTGACGGTTGAGAAAACACTCCCATTGATGGGTTTCATCATCAT |
| Primer 16 | TGCGTGTGAACCGTCGTCATGACTTATGAGCTTTCATCATCAT |
| Hairpin | Hairpin.27 : ACATCATCATGGGCCTTTTGGCCCATGATGATGTATGATGATGTTTTTTT |
| Imager ATTO550 | Fluor 27 /5ATTO550N/TTATGATGATGTATGATGATGT |

| BMYV | |
|---|---|
| Primer1 | ATGGGACCTCGTGTTTGAGTGGGCGAGAATGTTTCAACTTAAC |
| Primer2 | TACGCGGCGGGCTTTGTTTGATGGTAAAGGTGTTTCAACTTAAC |
| Primer4 | CTCCCGTAGCTTTCTCTCGCTTTCAGCACACCTTTCAACTTAAC |
| Primer5 | CAGCAAAGGCAGAGCAGCGAGAAGAGAACGAATACAGTTTCAACTTAAC |
| Primer6 | GTTTGTTTTAAGCTGAAATTGCATAGACTGCTAGATCCTCGCTGGTTTTCAACTTAAC |
| Primer7 | GTAGCTGTTAGCGGTCGGTTCAACGAACAAGTGAAACTTTCAACTTAAC |
| Primer8 | GGAATAAGGTGGGATTTCCAATTTTGGAGTAATTCAGCAGTGTTCACTCCCTTTCAACTTAAC |
| Primer9 | CGGACTTCCATTTCATCCTCTAGAAGCCAGTCCGAAACGTTTCAACTTAAC |

| | |
|---|---|
| Primer10 | CGCCTGGTTAGATCATTTATGTCCAACGTGAGCCTGTTCTTTCAACTTAAC |
| Primer11 | CCCTCGTTATATCTTTTGTGGCTGCACTGGAAGGACCTTTCAACTTAAC |
| Primer12 | CCGGTTGTTCCGGCGGTCGGAGTTTCAACTTAAC |
| Primer13 | GGTGTAGAAGGAGCAGCACGAGGGCTTTCAACTTAAC |
| Primer14 | CTCCTCCAATTCTGGGTGGAGGCTGGTGTTTCAACTTAAC |
| Primer15 | GCCATCTTGCGGCTTGTAGCCGCTTTCAACTTAAC |
| Primer16 | CGACCGTAAGCAACATACGGGACGCCTTTCAACTTAAC |
| Primer17 | CCCATGGCGATGGCCCAGGAGGTTTCAACTTAAC |
| Primer18 | GGCTGAGAGCGCTGAGCGCGTTTCAACTTAAC |
| Primer19 | GCCAGTCCGCCCCGTTGATGTAAGACTTTCAACTTAAC |
| Primer20 | GGGTTGTGGAGAGGGAGAAGGCCCTGTTTCAACTTAAC |
| Hairpin | Hairpin.28 : ACAACTTAACGGGCCTTTTGGCCCGTTAAGTTGTGTTAAGTTGTTTTTTT |
| Imager | Fluor28 /5ATTO647N/TTGTTAAGTTGTGTTAAGTTGT |

**Table S1: Virus transmission efficiency of M.persicae fed on artificial medium containing purified BChV or BMYV and processed for TEM**

| | 1 aphid/plant | 2 aphids/plant | 3 aphids/plant |
|---|---|---|---|
| **BChV [90 ng/µL][a]** | 7/10[c] | 8/10 | 10/10 |
| **BMYV [100 ng/µL][b]** | 1/6 | 3/6 | 6/6 |

[a]virus concentration in the artificial medium fed to aphids for 72 h acquisition period

[b]virus concentration in the artificial medium fed to aphids for 48 h acquisition period

[c]number of sugar beets infected/number of plants inoculated with potentially viruliferous aphids

**Table S1: Transmission efficiency of BMYV or BChV when acquired from mono or co-infected plants.** The transmission rate of a) BMYV and b) BChV by aphids is shown for eleven independent experiments using 15 to 30 plants each, with the number of infected plants/number of plants tested for each independent experiment, and the pooled total followed by the value of a two-tailed Pearson Chi-Square test (df=1, $p < 0.05$*). c) Observed co-infected plants compared to the expected coinfection percentage calculated based on the BMYV and BChV mono-infection rates (e.g., for experiment 1, expected rate of co-infection = 0.133 [BMYV mono-infection rate]*0.60 [BChV mono-infection rate] = 8.00%) (Chi-squared test for given probabilities, df = 1, $p < 0.05$*).

**a)**

| source | BMYV | | BMYV+BChV | | Chi-square, df | p-value |
|---|---|---|---|---|---|---|
| Exp. 1 | 4/30[a] | 13,3%[b] | 24/30[c] | 80,0%[d] | **26.786, 1** | **2.273e-07** |
| Exp. 2 | 4/30 | 13,3% | 12/30 | 40,0% | **5.454, 1** | **0.019** |
| Exp. 3 | 4/30 | 13,3% | 8/30 | 26,7% | 1.667, 1 | 0.197 |
| Exp. 4 | 10/23 | 43,5% | 21/23 | 91,3% | **11.97, 1** | **0.0005407** |
| Exp. 5 | 15/20 | 75,0% | 8/20 | 40,0% | **5.013, 1** | **0.025** |
| Exp. 6 | 15/20 | 75,0% | 10/20 | 50,0% | 2.667, 1 | 0.103 |
| Exp. 7 | 6/15 | 40,0% | 2/15 | 13,3% | 2.727, 1 | 0.098 |
| Exp. 8 | 4/15 | 26,7% | 3/15 | 20,0% | 0.186, 1 | 0.666 |
| Exp. 9 | 5/15 | 33,3% | 8/15 | 53,3% | 1.222, 1 | 0.269 |
| Exp. 10 | 2/15 | 13,3% | 5/15 | 33,3% | 1.677, 1 | 0.195 |
| Exp. 11 | 3/15 | 20,0% | 8/15 | 53,3% | 3.589, 1 | 0.058 |
| **Total** | 72/228 | 31,6% | 109/228 | 47,8% | **12.542, 1** | **0.000398** |

[a]number of test plants infected with BMYV when aphids acquired the virus from plants mono-infected with BMYV; [b]percentage of infected plants; [c]number of test plants infected with BMYV alone or with both viruses when aphids acquired from plants co-

infected with BMYV and BChV; [d]percentage of infected plants. Eleven independent transmission experiments were performed.

**b)**

| source | BChV | | BChV + BMYV | | Chi-square, df | p-value |
|---|---|---|---|---|---|---|
| Exp. 1 | 18/30[a] | 60,0%[b] | 27/30[c] | 90,0%[d] | **7.2, 1** | **0.00729** |
| Exp. 2 | 7/30 | 23,3% | 13/30 | 43,3% | 2.7, 1 | 0.1003 |
| Exp. 3 | 8/30 | 26,7% | 12/30 | 40,0% | 1.2, 1 | 0.2733 |
| Exp. 4 | 19/23 | 82,6% | 9/23 | 39,1% | **9.127, 1** | **0.002519** |
| Exp. 5 | 8/20 | 40,0% | 10/20 | 50,0% | 0.404, 1 | 0.525 |
| Exp. 6 | 13/20 | 65,0% | 12/20 | 60,0% | 0.107, 1 | 0.744 |
| Exp. 7 | 7/15 | 46,7% | 6/15 | 40,0% | 0.136, 1 | 0.7125 |
| Exp. 8 | 4/15 | 26,7% | 5/15 | 33,3% | 0.159, 1 | 0.690 |
| Exp. 9 | 7/15 | 46,7% | 9/15 | 60,0% | 0.536, 1 | 0.464 |
| Exp. 10 | 6/15 | 40,0% | 5/15 | 33,3% | 0.143, 1 | 0.705 |
| Exp. 11 | 10/15 | 66,7% | 9/15 | 60,0% | 0.143, 1 | 0.705 |
| **Total** | 108/228 | 47,4% | 117/228 | 51,3% | 0.711, 1 | 0.399 |

[a]number of test plants infected with BChV when aphids acquired the virus from plants mono-infected with BChV; [b]percentage of infected plants; [c]number of test plants infected with BChV alone or with both viruses when aphids acquired from plants co-infected with BMYV and BChV; [d]percentage of infected plants. Eleven independent transmission experiments were performed.

**c)**

| | Observed | | Expected | Chi-square, df | p-value |
|---|---|---|---|---|---|
| Exp. 1 | 22/30[a] | 73,3%[b] | 8,0%[c] | 173.99, 1 | **2.2e-16** |
| Exp. 2 | 3/30 | 10,0% | 3,1% | 4.726, 1 | **0.030** |
| Exp. 3 | 7/30 | 23,3% | 3,6% | 34.164, 1 | **5.065e-09** |
| Exp. 4 | 9/23 | 39,1% | 35,9% | 0.103, 1 | 0.748 |
| Exp. 5 | 4/20 | 20,0% | 30,0% | 0.952, 1 | 0.329 |
| Exp. 6 | 8/20 | 40,0% | 48,8% | 0.613, 1 | 0.434 |
| Exp. 7 | 1/15 | 6,7% | 18,7% | 1.423, 1 | 0.233 |
| Exp. 8 | 3/15 | 20,0% | 7,1% | 3.774, 1 | 0.052 |
| Exp. 9 | 6/15 | 40,0% | 15,6% | 6.819, 1 | **0.009** |
| Exp. 10 | 5/15 | 33,3% | 5,3% | 23.312, 1 | **1.378e-06** |
| Exp. 11 | 3/15 | 20,0% | 13,3% | 0.578, 1 | 0.447 |
| **Total** | **71/228** | **31,1%** | **17,2%** | **31.112, 1** | **2.436e-08** |

[a]number of observed test plants infected with BMYV and BChV when aphids acquired the virus from co- infected plants; [b]percentage of observed co-infected plants; [c]percentage of expected co-infected plants. Eleven independent transmission experiments were performed.

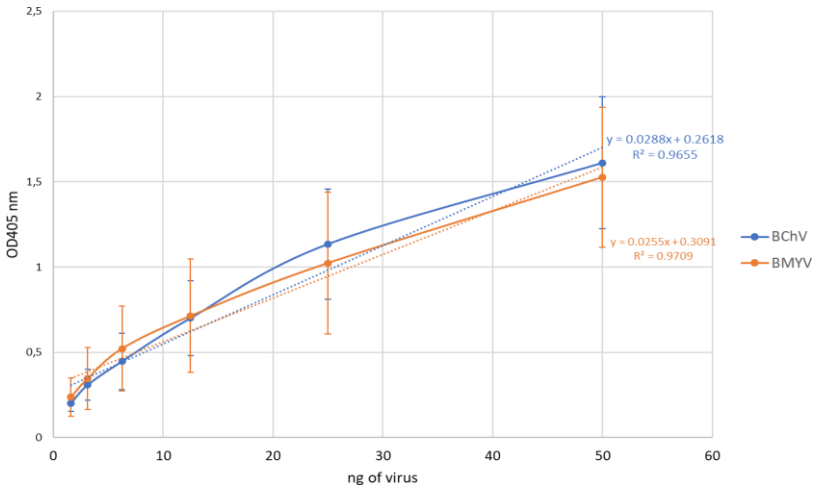



**Figure S1: Reactivity of anti-TuYV antiserum towards purified particles of BChV and BMYV.** A range of purified virus particles (BChV or BMYV) from 1.6 ng to 50 ng was tested by DAS-ELISA for each virus. OD values at 405 nm 1 h after substrate incubation. Each dot of the curves represents the mean of 4 replicates from 4 independent virus purifications performed at different dates.

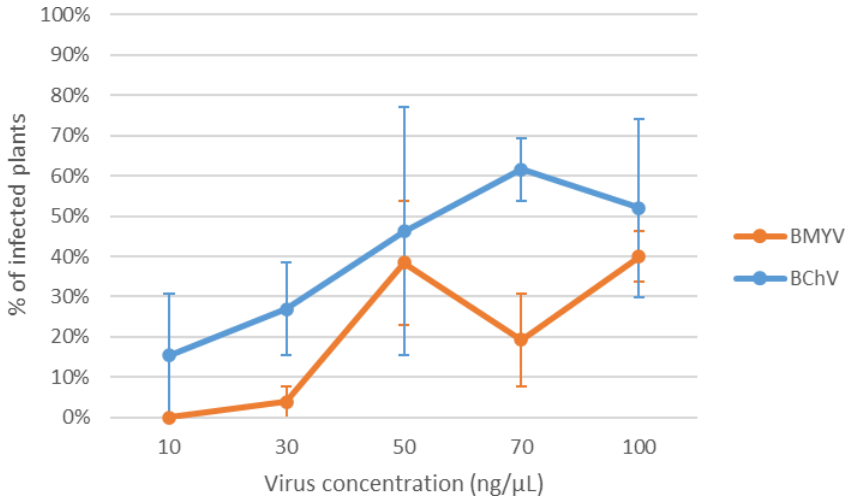



**Figure S2: BMYV and BChV aphid transmission efficiency using different virus concentrations (from 10 to 100 ng/µL) in an artificial medium to feed aphids.** After a 24 h acquisition access period, one potentially viruliferous aphid was transferred onto 3-week-old sugar beet plants. A DAS-ELISA assay was performed 3 weeks post-inoculation. The experiment was reproduced twice with n=26 plants for each virus concentration.

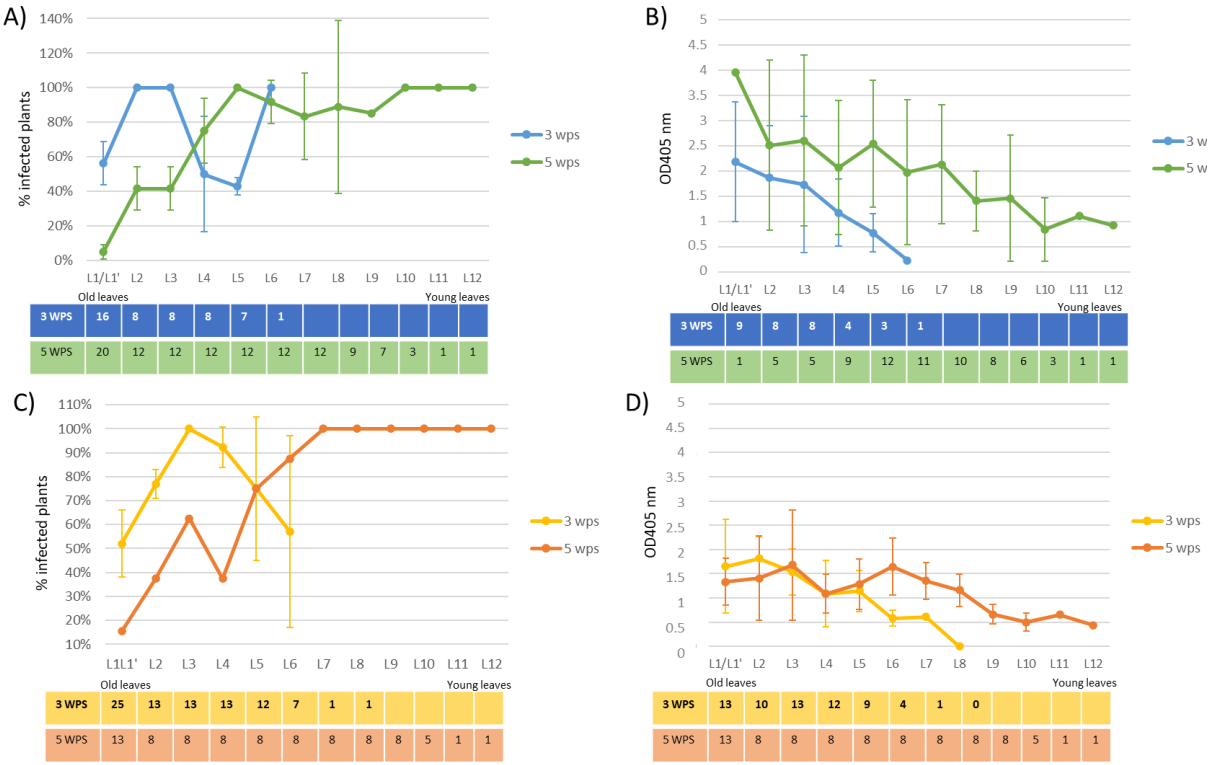



**Figure S3: Comparison of BMYV or BChV distribution in mono-infected sugar beets inoculated 3 or 5 weeks post-sowing.** Sugar beets were inoculated with BChV (**A & B**) or BMYV (**C & D**) after feeding aphids on an artificial medium containing purified virus for 24 h. A DAS-ELISA assay was performed 3 weeks after virus inoculation on each individual leaf of infected plants (the oldest leaves: L1/L1'). The absorbance value of each leaf sample at 405 nm after 1 h substrate incubation is indicated. Percentage of infected leaves for each leaf level and when the plants were inoculated at 3 or 5 weeks post-sowing with BChV (**A**) or BMYV (**C**). The number of leaves at each level present at the time of inoculation is mentioned in the tables below the graphs. Virus accumulation (OD405 nm) in each leaf tested positive by DAS-ELISA in plants inoculated 3 or 5 weeks post-sowing with BChV (**B**) or BMYV (**D**). The number of total leaves and leaves tested positive by DAS-ELISA is mentioned in the tables below the graphs. All experiments have been performed twice except the inoculation of BMYV on 5-week-old sugar beet that has been done once. wps: weeks post-sowing.

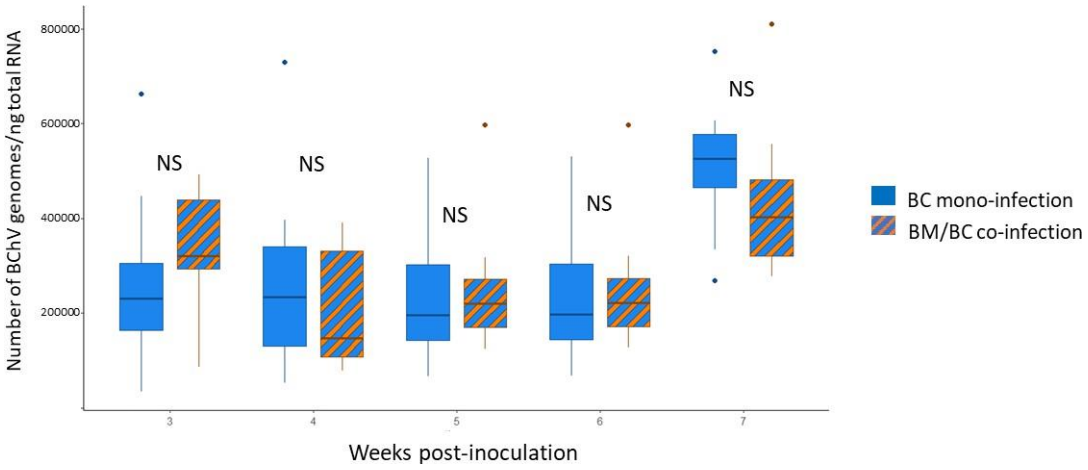
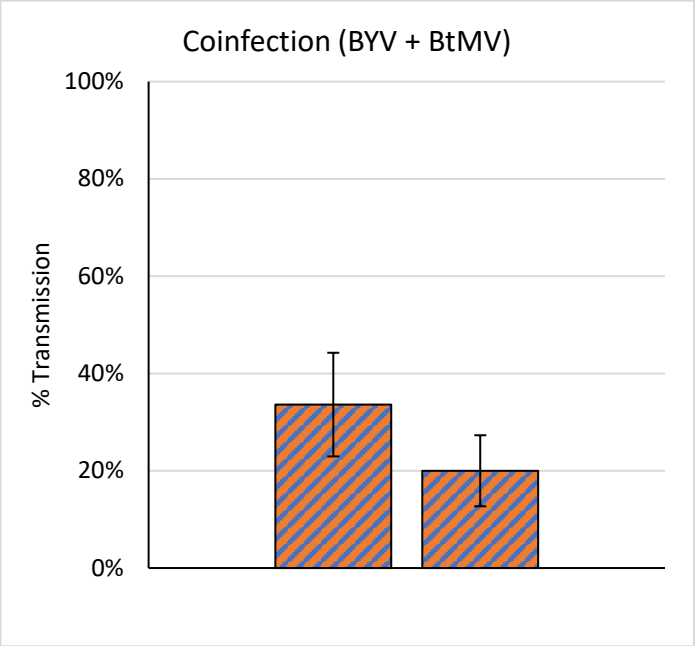



**Figure S4: Accumulation of BChV in mono- and co-infected sugar beets.** Leaf samples of infected plants were collected 3 to 7 weeks post-inoculation and virus accumulation was measured by RT-qPCR. The number of BChV genome copies are normalized to ng of total plant RNA. Sugar beets were inoculated with one or the two viruses (BMYV or BChV) using aphids fed on an artificial medium containing one or both purified viruses for 24 h. (NS: Non significant, Nonlinear Mixed-Effect Models, mono- vs. coinfection $P$=0.743, time $P$<0.001, infection:time $P$=0.761). Box plots show median (line), 25–75% percentiles (box), 10–90% percentiles (whisker), and outliers (dots). Nine BChV-infected plants, and nine BChV and BMYV co-infected plants were analyzed.

## Supplementary data for Chapter 4 (Article 3)

### S1 Figure




**S1 Figure. Aphid transmission efficiency of BYV and BtMV from co-infected plants.** *Myzus persicae* acquired virus from co-infected leaves for 24 h. Then, three aphids were transferred per test plant for 72 h inoculation. A DAS-ELISA assay was performed 3 weeks later to detect infection. The percentage of expected and observed co-infected plants is indicated. Difference was not significant (p-value= 0.1089; n=75, five independent experiments; df=1; chi-squared($\chi 2$)).

## S1 Table

**S1 Table. Primers with concatemer sequences (red) used for Primer Exchange Reaction.**

| BtMV | |
|---|---|
| Primer1 | GCCAACGAACACGTTGTGGATGTAGCTCTCTGTTTAATACTCTC |
| Primer2 | TGATGGGTCGAGCAACAGAACTGCTCTCAGTTTAATACTCTC |
| Primer4 | TGCCCTTCGCAATTGCTTGGGGACCTGTTTAATACTCTC |
| Primer5 | TGGTTGAGGTGTTGACTCTTCTGTATCCACATCTGAGTTTAATACTCTC |
| Primer6 | CCCTCTTACTATCACCTCGTCATAGCGTCGTCCTTTAATACTCTC |
| Primer7 | AGTGCATAGTTCTACCAACATCGCCTGCGGTTTAATACTCTC |
| Primer8 | CTTGCAGATATGCTCCTTCTGGGCTGGAACTTTAATACTCTC |
| Primer9 | AGCAATTTGTACGATCGACGCCGCCATTTAATACTCTC |
| Primer10 | GCTCAGAATACGCCTCTGGGGTCATCTCATTGTTTAATACTCTC |
| Primer11 | TGCAAGCTTCCTTTGGCCCTGTGGTCCTTTAATACTCTC |
| Primer12 | CAATGGGGTTCCGTCATCTAGAGTGACACAACAGCTTTAATACTCTC |
| Primer13 | CGGATCTCCAGTTGTTCCAACAACCAAGTGTCTCTTTAATACTCTC |
| Primer14 | GGCCATGTTCCAAGCATTGGGATAATAATGTCACGAACCTTTAATACTCTC |
| Primer15 | GTGATCAACTAGTATCCTCGGCAGTTCCGCACTACTTTAATACTCTC |
| Primer16 | GCTGTTCCTGCCTTGAGTACGTGGTACCCTTTAATACTCTC |
| Primer17 | CCGCCAACTCTGTACCACTTCATCTCTCCTTCAAGCTTTAATACTCTC |
| Primer18 | CTTGTGATCAATGCGCTAATCATCTTCTCTTTCACGGGTTTAATACTCTC |
| Primer19 | GGATCCTCCTCTATCAAGTACACCAATTTCTTGGGCCTGTTTAATACTCTC |
| Primer 20 | CCCCTTGAACTTCTTCACTATTGTTCCATCCGGTGTGAGTTTAATACTCTC |
| Primer21 | CTGAACACTGCCCCGCTCTTCTCTGTATCGTTTAATACTCTC |
| Primer 22 | GGAACAACTCTGTTCTGGCTCAATTGCTTGTCCCACTTTAATACTCTC |
| Primer23 | GTGCCCTGTTTCACTCGTCCTACTCTGCCTTTAATACTCTC |
| Primer 24 | CATATTGCATCATGGTTCTGGCTTGTCTGTTCGTGCTTTAATACTCTC |
| Primer25 | GTACTCCATAACCCATAATGTCACCAACTCTCTCTGGGTCTTTAATACTCTC |
| Primer26 | GAGCTTCTGCATCTGGCGCCTCTTCCTTTAATACTCTC |
| Hairpin | Hairpin.30 : AAATACTCTCGGGCCTTTTGGCCCGAGAGTATTTGAGAGTATTTTTTTTT |
| Imager | Fluor30 /565 /5ATTO565/tt GAGAGTATTT GAGAGTATTT |

| BYV | |
|---|---|
| Primer1 | CTGTCGGGCACAATGGGGCTTGCACTTTATAAACCTA |
| Primer2 | CATGTCTCGGCGGGTGTCCACGACTTTATAAACCTA |
| Primer4 | GGGTTGTCGGTAGAAGCACTCAAGTGCACACGTTTATAAACCTA |
| Primer5 | GCACGACCTCGGTGCAGGCTCGTTTATAAACCTA |
| Primer6 | GGGAACTGACAGAGAGGACTTCAGTTGGGAACACGTTTATAAACCTA |
| Primer7 | CTGTAAGAGCCGACCAGCTGGAAGAGTCTTCGTTTATAAACCTA |
| Primer8 | GGGCGCGAGTTAGGTTTCGATGTAACACCTAACGTTTATAAACCTA |
| Primer9 | CGCGAGGAGTTTGTTGCCTCCTGCGTTTATAAACCTA |
| Primer10 | CGCGCGCCTGAGCTTCAACAAGTTGTTCGTTTATAAACCTA |
| Primer11 | GTTCGCCCGGAGTCACCATGGTGAGGTTTATAAACCTA |
| Primer12 | GCCATGTCGATAGCGAGAGTTAAACGTTAAAGGCAGGGTTTATAAACCTA |
| Primer13 | CACTCGAGTGTAAGAGACCGAACTGGGTTTCAACCTATTCATACTTTATAAACCTA |
| Primer14 | CCATTTCGGCGTGCGTGGGAATTTGTGCATTTATAAACCTA |
| Primer15 | CTACGAAAGGAGCCGCCACTTCAGCCAGTTCTTTATAAACCTATTTATAAACCTA |
| Primer16 | CTTCCACTCCACTCATGTTTATTGTGAATTCACCGAGAGCCGTTTATAAACCTA |
| Primer17 | GGAACAGTTTGGGCAACGAACGAGCTATCACTATGTAAGCTTTATAAACCTA |
| Primer18 | CGGCGCGAACACTGGGTCCGAGTTTATAAACCTA |
| Primer19 | GCGGCGTGGCCGCAGTGACTACTTTATAAACCTA |
| Primer 20 | GAGCGGCGTGGGCGAGGTAACATAACCTTTATAAACCTA |
| Primer21 | CGAGAGACTCGTCTTCCGCGCACAGGTTTATAAACCTA |
| Primer 22 | GCTGACACGTTCGTAGACTGATTCGATCGCCTTGTTTATAAACCTA |
| Primer23 | CCACTCTGCACGGCCTCCCCCATATAGAAGTTTATAAACCTA |
| Primer 24 | CCAGCAGACGGGAACCGGCCGTTTATAAACCTA |
| Primer25 | CGGTCTGCACACGTGGTAATCTGTGCTCCTTTATAAACCTA |
| Hairpin | Hairpin.26 : AATAAACCTAGGGCCTTTTGGCCCTAGGTTTATTTAGGTTTATTTTTTT |
| Imager | Fluor26 /ATTO647N/TTTAGGTTTATT TAGGTTTATT |

## S2 Table

**Tables S2: Number of cells infected by one or both viruses in sections of co-infected leaves.**

Exp1

| **BtBY1** | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 | S11 | S12 | S13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BtMV | N | N | N | N | N | 4 | N | 0 | N | N | N | N | N |
| BYV | 0 | 0 | 4 | 9 | 2 | 7 | 4 | 0 | 0 | 1 | 8 | 0 | 0 |
| BtMV/BYV | 3 | 5 | 3 | 3 | 0 | 2 | 0 | 9 | 3 | 1 | 2 | 15 | 5 |

Exp2

| **BtBY2** | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 | S11 | S12 | S13 | S14 | S5 | S16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BtMV | N | N | N | N | N | N | N | 20 | 13 | N | 0 | N | N | N | N | 0 |
| BYV | 2 | 2 | 2 | 9 | 1 | 0 | 0 | 6 | 0 | 0 | 0 | 1 | 4 | 0 | 3 | 2 |
| BtMV/BYV | 0 | 1 | 2 | 1 | 2 | 4 | 3 | 0 | 1 | 2 | 4 | 0 | 1 | 3 | 1 | 1 |

Exp3

| **BtBY3** | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 | S11 | S12 | S13 | S14 | S15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BtMV | N | N | N | N | N | N | N | 20 | 13 | N | 0 | N | N | N | 0 |
| BYV | 1 | 4 | 1 | 2 | 1 | 4 | 2 | 12 | 9 | 0 | 2 | 0 | 2 | 2 | 2 |
| BtMV/BYV | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

# RESUME DE LA THESE EN FRANCAIS

## Introduction générale

L'infection mixte par deux pathogènes, ou plus, est la règle plutôt que l'exception, mais elle reste peu étudiée. Pourtant, les infections mixtes peuvent avoir un impact considérable sur l'issue de l'infection, par exemple par des effets synergiques ou antagonistes qui exacerbent ou atténuent les symptômes chez l'hôte, modifient la distribution et l'accumulation des agents pathogènes, ou altèrent leur propagation. La multi-infection virale est un phénomène très courant chez les procaryotes et les eucaryotes y compris les humains, les animaux et les plantes. C'est sur la multi-infection virale des plantes que se concentre cette thèse, puisque, comme évoqué ci-dessus, son impact sur la plante, la biologie des virus et leur transmission par vecteur reste encore très peu exploré. La betterave à sucre est un modèle très approprié pour acquérir des données sur les multi-infections virales des plantes car cette culture est fréquemment infectée par plusieurs virus appartenant à différentes familles et qui possèdent des caractéristiques biologiques distinctes. Parmi les quatre virus infectant la betterave et responsables de l'apparition de jaunisses figurent deux polérovirus (famille des *Solemoviridae*), le beet mild yellowing virus (BMYV) et le beet chlorosis virus (BChV), un closterovirus (famille des *Closteroviridae*), le beet yellows virus (BYV), et un potyvirus (famille des *Potyviridae*), le beet mosaic virus (BtMV). Un troisième polérovirus, le beet western yellows virus (BWYV) est jusqu'à présent limité aux États-Unis et à l'Asie et n'a pas été détecté en Europe **(Stevens et al., 2004 ; Wintermantel, 2005 ; Xiang et al., 2008)**. Les polérovirus et clostérovirus sont restreints au phloème alors que le potyvirus envahit toutes les cellules de la plante. Ces quatre virus sont également tous transmis par puceron mais selon des modes très différents (persistant pour les polérovirus, semi-persistant pour le clostérovirus et non-persistant pour le potyvirus) **(Kozłowska-Makulska et al., 2009; Limburg et al., 1997)**. Les mono- ainsi que les co-infections virales de la betterave peuvent causer d'importants dommages dus à un jaunissement intense des feuilles réduisant l'activité photosynthétique ce qui interfère avec le transport des assimilats et exerce des dérégulations métaboliques massives **(Clover et al., 1999)**. Les jaunisses de la betterave induisent des pertes de rendement considérables et une réduction de la teneur en sucre des pivots. Ces dégâts étaient jusqu'à présent contrôlés par l'emploi de néonicotinoïdes, insecticides aujourd'hui interdits. Ce projet de thèse, dont l'enjeu majeur est l'acquisition de connaissances fondamentales sur la multi-infection virale de la betterave, répond donc aussi à un besoin d'intensifier les recherches sur ce pathosystème, qui reste très peu étudié en France et à l'étranger, afin d'identifier et d'éprouver les nouvelles

méthodes de lutte contre ces virus, des alternatives qui devront se substituer à l'emploi des néonicotinoïdes.

## Objectifs principaux de la thèse

Notre étude s'est focalisée sur l'impact de la multi-infection de la betterave sucrière par les quatre virus (BMYV, BChV, BYV et BtMV) sur les interactions hôte-virus-vecteur avec une priorité sur les conséquences de la multi-infection sur la transmission des virus par puceron. Nous avons d'abord réalisé toute une batterie de tests de transmission afin de comparer l'efficacité de transmission des virus à partir de plantes mono-infectées versus plantes co- voir multi-infectées. Lorsque des différences de transmission ont été observées, nous avons cherché à en identifier les causes. Pour cela, l'impact de la multi-infection sur les paramètres suivants, qui pourraient tous être en relation avec l'acquisition des virus, a été étudié :

1) La localisation cellulaire du virus (tropisme) dans les plantes infectées. Nous avons cherché à savoir si la multi-infection ou, dans un premier temps, la co-infection pouvait modifier (i) la localisation intracellulaire des virus ou (ii) affecter le tropisme tissulaire, en particulier en levant la limitation au phloème du BMYV, du BChV et du BYV, puisque cette modification de localisation pourrait affecter l'accessibilité du/des virus pour le vecteur.
2) L'accumulation des virus. La question était de savoir si les interactions entre les virus entraînaient une synergie, un antagonisme ou des effets neutres au niveau du titre viral, ce qui pourrait également expliquer une transmission augmentée ou diminuée du virus.
3) Le comportement alimentaire des pucerons sur les plantes multi-infectées. Nous avons émis l'hypothèse que la mono-infection et les multi-infections virales auraient des effets différents sur le phénotype de la plante, comme par exemple sur son métabolisme, avec des conséquences sur le comportement alimentaire des pucerons. Puisque le comportement alimentaire est important pour l'acquisition des virus, sa modification par l'infection simple ou multiple des plantes pourrait avoir des conséquences sur l'acquisition et la transmission.

Les résultats de ces études sont présentés ci-dessous sous la forme de trois articles de recherche, dont l'un est soumis (article 1) à Microbiology Spectrum, un soumis à Journal of General Virology (article2) et une version provisoire d'un article encore à soumettre (article3).

## Chapitre 1: L'interaction entre un polérovirus et un clostérovirus diminue la transmission du polérovirus

Des expériences de transmission ont été réalisées pour tester les 15 combinaisons différentes des 4 virus. Nous avons ainsi identifié trois combinaisons qui ont un effet sur le taux de transmission des virus par puceron, et qui ont été étudiées plus en profondeur par la suite.

La première combinaison étudiée était la co-infection du closterovirus beet yellows virus (BYV) et du polérovirus beet chlorosis virus (BChV). Une réduction significative de la transmission du BChV à partir de plantes co-infectées BYV/BChV a été observée par rapport aux plantes infectées seulement avec le BChV. Aucun effet de la co-infection n'a été observé sur l'efficacité de transmission du BYV à partir des plantes co-infectées comparé aux plantes infectées uniquement par le BYV **Fig.1**. Pour tenter d'expliquer la réduction de la transmission du BChV à partir de plantes co-infectées, nous avons analysé le comportement alimentaire des pucerons, l'accumulation du virus, la distribution du virus dans les feuilles et dans les cellules infectées (localisation tissulaire et cellulaire) sur des plantes mono-infectées et co-infectées par le BChV et le BYV. La réduction drastique de la transmission du BChV n'était pas due à une moindre accumulation du BChV dans les plantes co-infectées **Fig.2**, ni à une réduction de l'ingestion de sève du phloème, à partir de laquelle le BChV est acquis par les pucerons **Fig.3**. En utilisant la technique d'hybridation in situ SABER-FISH sur des plantes co-infectées, nous avons observé une localisation préférentielle du BChV dans les nervures centrales des feuilles et du BYV dans les veines d'ordre inférieur, ce qui suggère une redistribution des virus dans le système vasculaire des plantes co-infectées **Fig.4**. Cependant, cette redistribution ne peut pas expliquer la réduction de la transmission du BChV car le virus est acquis à partir de la sève du phloème plutôt qu'à partir du contenu cellulaire des cellules du phloème. Le SABER-FISH a également montré que 40 % des cellules phloémiennes infectées étaient co-infectées et que la co-infection entraînait une redistribution du BYV dans ces cellules. La forme d'accumulation du BYV est passée d'inclusions sphériques intracellulaires distinctes dans les cellules mono-infectées à une distribution diffuse dans les cellules co-infectées **Fig.5 & 6**. Les raisons de la diminution de la transmission du BChV par les pucerons sont actuellement inconnues, mais la localisation subcellulaire des deux virus dans les plantes co-infectées suggère fortement l'existence d'interactions entre les deux virus qui diminuent l'acquisition du BChV par les pucerons. Parmi les hypothèses avancées, nous proposons des altérations du trafic intracellulaire ou des modifications de la paroi cellulaire ou des plasmodesmes qui pourraient

réduire la libération des particules du BChV des cellules nucléées du phloème vers les tubes criblés **Fig.7**. Ces résultats constituent la première mise en évidence de l'impact de la multi-infection sur la transmission des virus par des pucerons en utilisant la betterave comme pathosystème. C'est aussi la première fois que les interactions cellulaires et tissulaires entre un polérovirus et un clostérovirus sont étudiées.

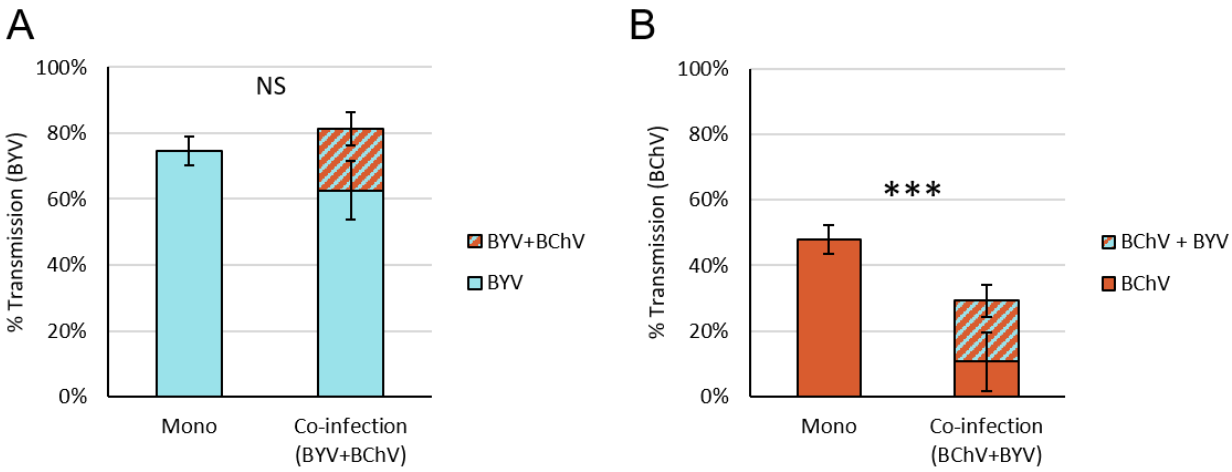



**Figure 1. Efficacité de la transmission par les pucerons du BYV (A) et du BChV (B) à partir de plantes mono ou co-infectées**. *Myzus persicae* a acquis le virus à partir de feuilles mono ou co-infectées pendant 24 h. Ensuite, trois pucerons ont été transférés par plante test pour une inoculation de 72 h. Un test DAS-ELISA a été réalisé 3 semaines plus tard pour détecter l'infection. Le pourcentage de plantes infectées est indiqué (% Transmission). La différence est statistiquement significative pour la transmission du BChV mais pas pour celle du BYV (GLMER, BYV : p-value = 0,060 ; BChV : p-value< 0,001 ; n = 75, cinq expériences indépendantes ; df = 1 ;). NS = non significatif, *** = valeur p < 0,001.

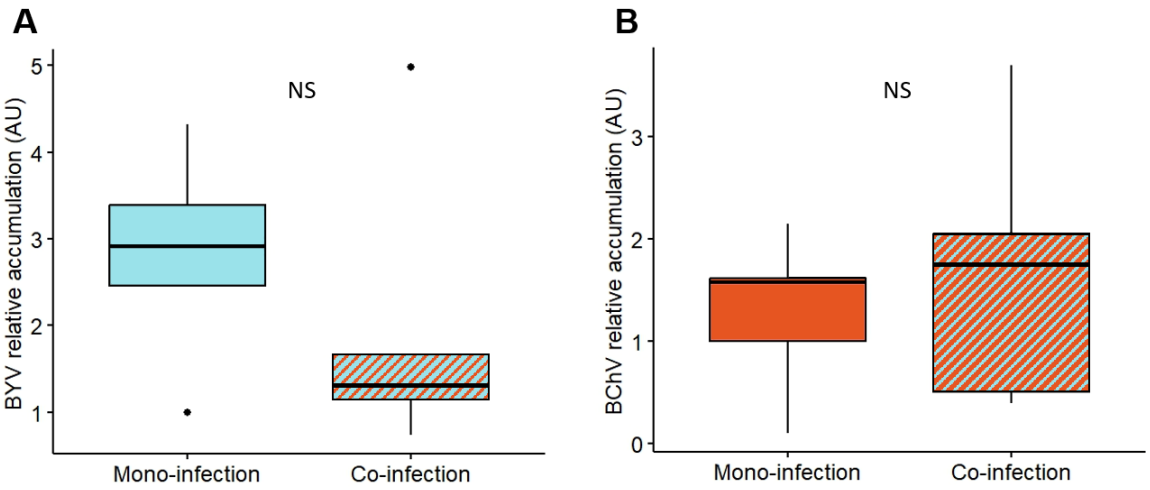



**Figure 2. Accumulation relative du BYV et du BChV dans les plantes mono- et co-infectées à 21 dpi**. L'accumulation du virus dans les plantes sources a été mesurée par RT-qPCR TaqMan multiplex comme décrit dans la section matériel et méthodes. Les diagrammes en boîte montrent la médiane (ligne noire), les percentiles 25%-75% (boîte) et les percentiles 10%-90% (moustaches). Les différences ne sont pas statistiquement significatives (test t de Student, BYV : t = -0,90817, p-value = 0,390 ; BChV : t = 0,59456 p-value= 0,569 ; n = 5 ; df = 8). NS= non significatif.

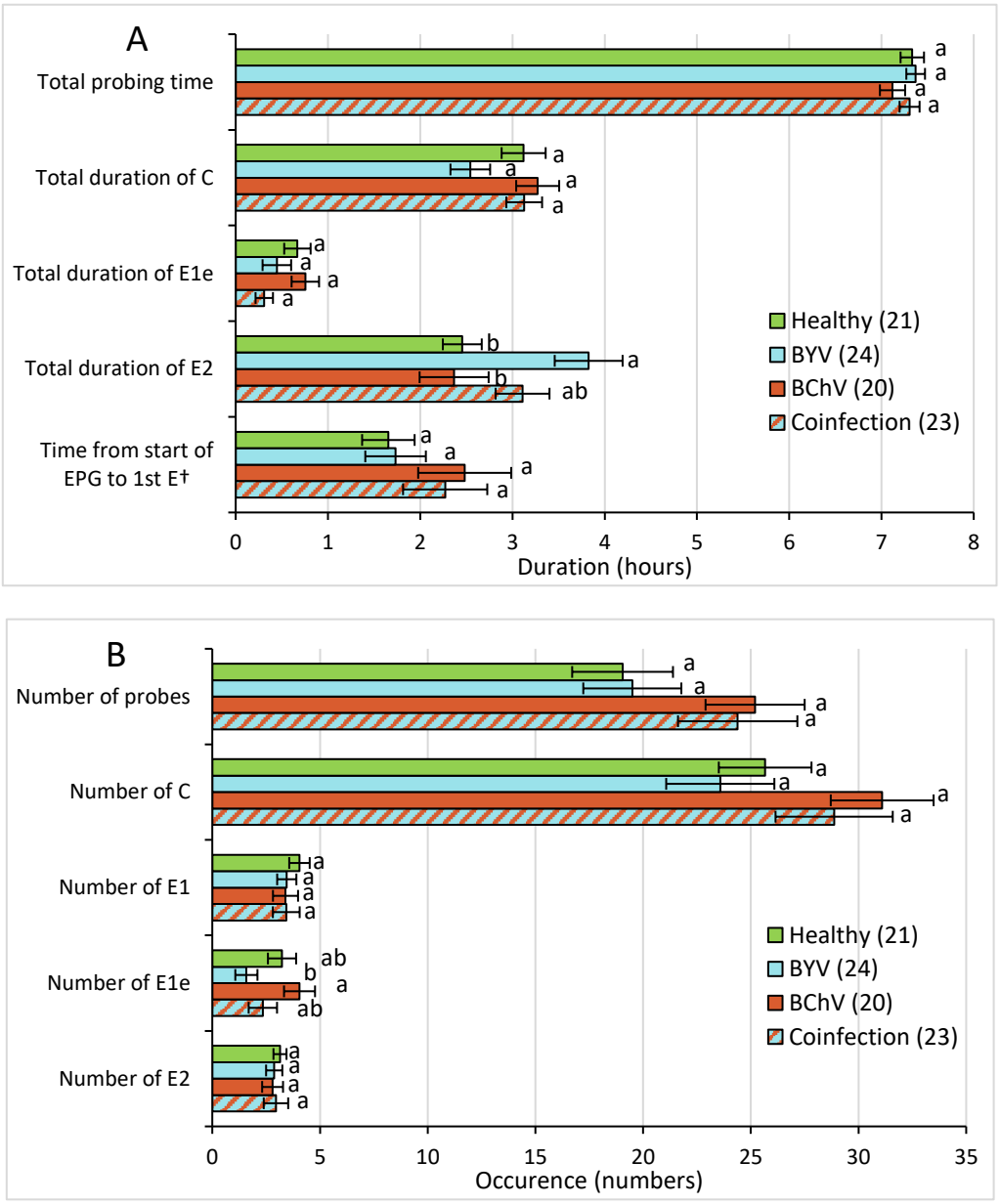
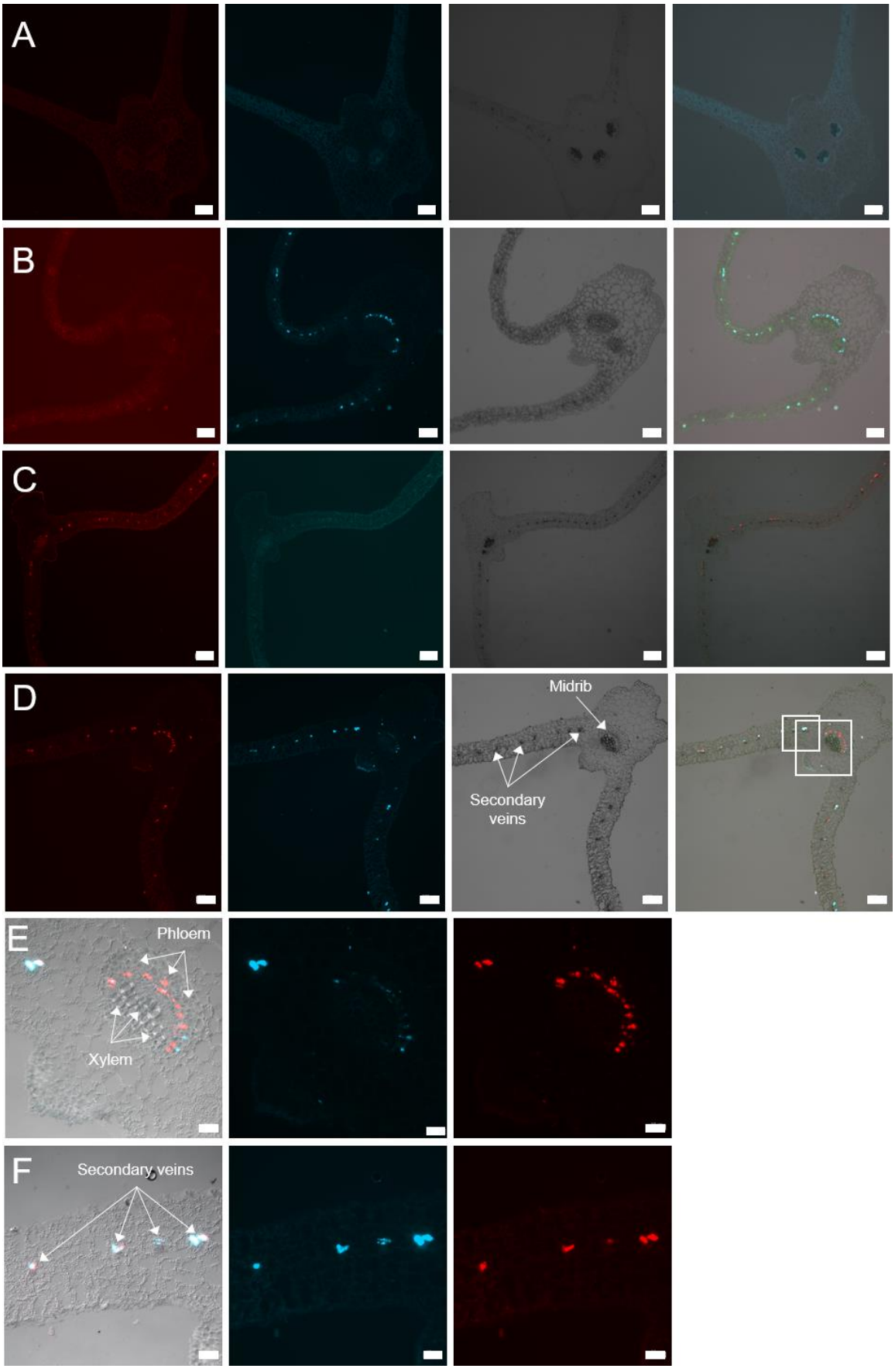



**Figure 3. Comportement alimentaire de *Myzus persicae* sur des betteraves saines, mono-infectées par le BYV ou le BChV ou co-infectées par le BYV et le BChV (N=20-22).** (A-B) Le comportement des pucerons individuels a été enregistré par la technique d'électropénétrographie (EPG) pendant 8 heures sur la troisième feuille supérieure. Les paramètres EPG sélectionnés sont présentés selon (A) la durée ou (B) l'occurrence. Les barres de l'histogramme indiquent les moyennes et les erreurs standard (SEM). Des lettres différentes indiquent des différences significatives entre les statuts d'infection des plantes, testées par GLM (modèle linéaire généralisé) suivi de comparaisons par paire à l'aide du paquet R : emmeans (méthode P<0,05 : Turquie). L'analyse statistique de la durée des événements indique une différence significative pour la durée de l'ingestion du phloème (E2) sur les plantes infectées par le BYV par rapport aux plantes saines ou infectées par le BChV (GLM, Df = 3, X2 = 15,98, P = 0,001) mais aucune différence pour la durée totale des pénétrations du stylet (temps de sondage) (GLM, Df = 3, X2=2. 585, P = 0,460), la durée totale des phases de cheminement (C) (GLM, Df = 3, X2 = 6,962, P = 0,073), la durée totale de la salivation intercellulaire (E1e) (GLM, Df = 3, X2 = 5,645, P = 0,130) et le temps avant la première ingestion de sève du phloème (Cox, Df = 3, p = 0,526). L'analyse statistique de l'occurrence des événements a révélé des différences significatives pour la salivation intercellulaire entre les plantes infectées par le BYV et le BChV (GLM, Df = 3, X2 = 8,581 ,P = 0. 035) mais aucune différence pour le nombre d'insertions des stylets (GLM, Df = 3, X2 = 4,725,P = 0,193), les phases de cheminement (C) (GLM, Df = 3, X2 = 4. 954, P = 0,175), la phase de salivation (E1) (GLM, Df = 3, X2 = 1,006, P = 0,800) et l'ingestion de sève du phloème (GLM, Df = 3, X2 = 0,459, P = 0,928).


A
B
C
D
Midrib
Secondary veins
E
Phloem
Xylem
F
Secondary veins

**Légende de la Figure 4 (page précedente). Distribution tissulaire du BChV et du BYV dans les feuilles mono-infectées par le BChV, le BYV ou co-infectées par les deux virus.** (A-D) montrent des sections transversales de (A) feuilles saines, (B) mono-infectées par le BYV, (C) mono-infectées par le BChV, et (D) co-infectées par le BChV/BYV. La première colonne montre le marquage BChV (rouge), la deuxième le marquage BYV (turquoise), la troisième les acquisitions en champ clair et la dernière colonne présente les superpositions. (E-F) Agrandissements des régions décrites en (D) montrant la distribution du virus dans (E) la nervure centrale et (F) le limbe de la feuille. La première colonne présente les superpositions d'images, la deuxième le signal BYV (turquoise) et la dernière colonne le marquage BChV (rouge). Barres d'échelle = 200 µm (A-D) et 50 µm (E-F).

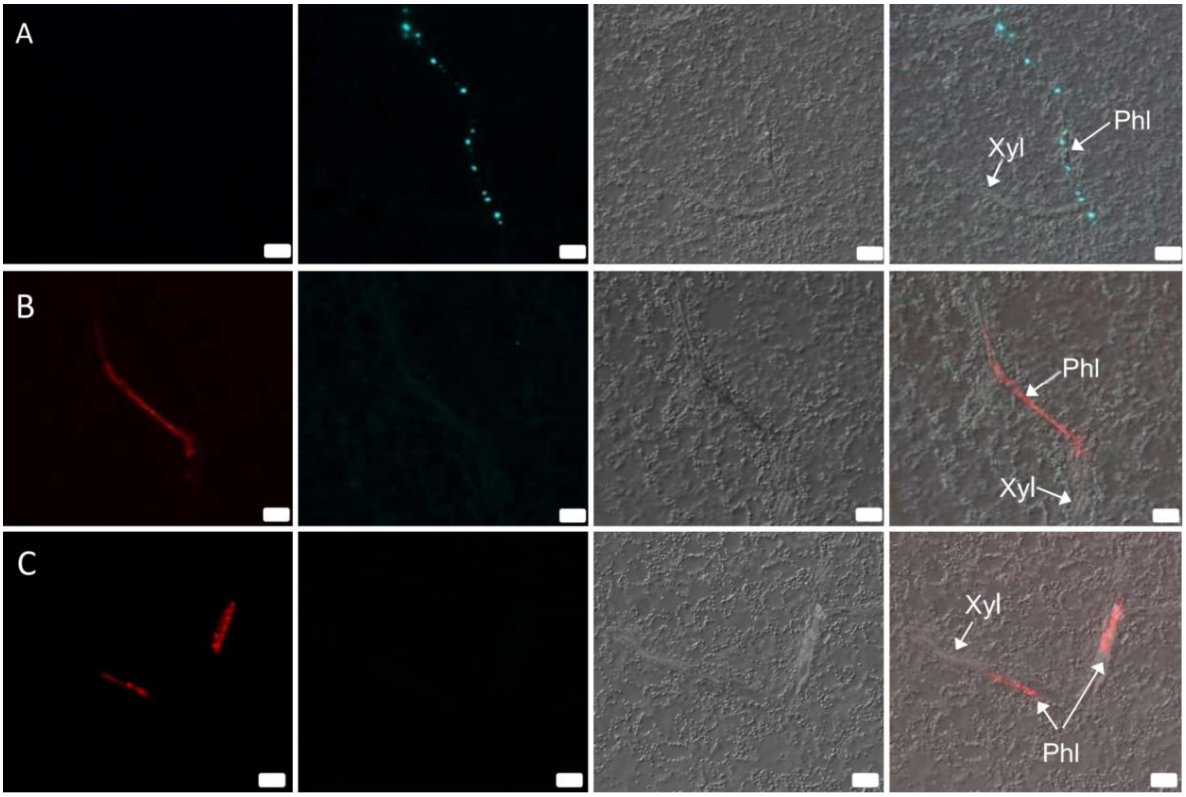



**Figure 5. Distribution intracellulaire du BYV et du BChV dans les feuilles mono-infectées**. Les images montrent des sections longitudinales du phloème de (A) une feuille mono-infectée par le BYV et (B-C) une feuille mono-infectée par le BChV avec (B) le BChV dans un tube criblé et (C) dans une cellule compagne. La première colonne représente le marquage du BChV (rouge), la deuxième le marquage du BYV (turquoise), la troisième les images de contraste interférentiel différentiel, et la dernière colonne présente les superpositions d'images. Xyl, xylème ; CC, cellule compagne ; ST, tube criblé.

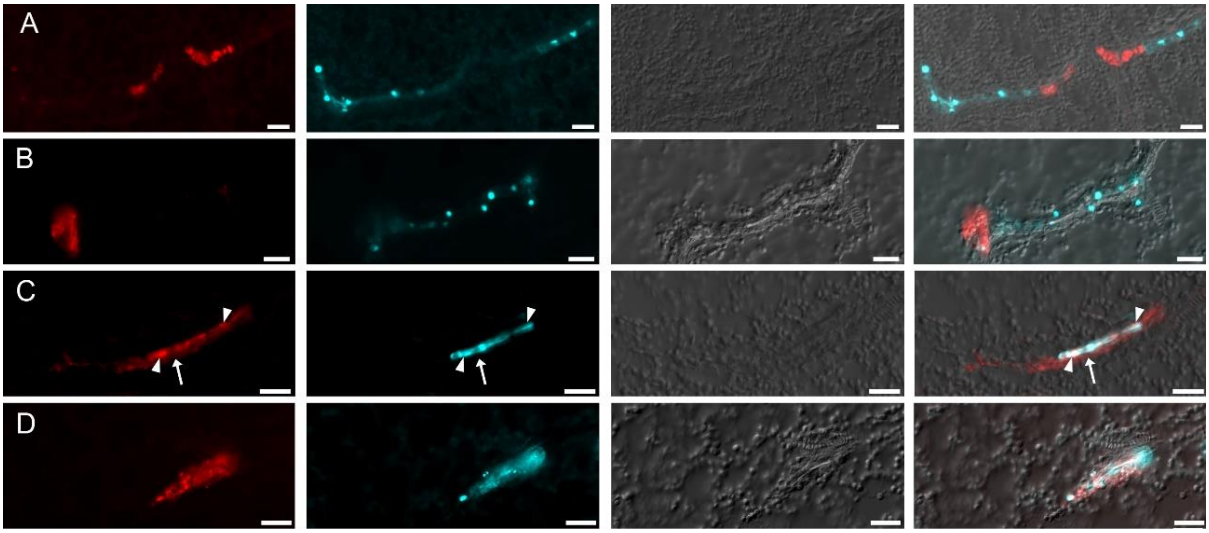



**Figure 6. Distribution intracellulaire du BYV et du BChV dans les feuilles co-infectées.** Les images montrent des sections longitudinales de phloème avec (A-B) des cellules compagnes mono-infectées, (C) une cellule compagne mono-infectée par le BChV (flèche), et une cellule adjacente co-infectée par les deux virus (têtes de flèches), et (D) une cellule du parenchyme phloémien co-infectée. La première colonne présente le marquage du BChV, la deuxième le marquage du BYV, la troisième les images de contraste interférentiel différentiel et la quatrième les superpositions d'images.

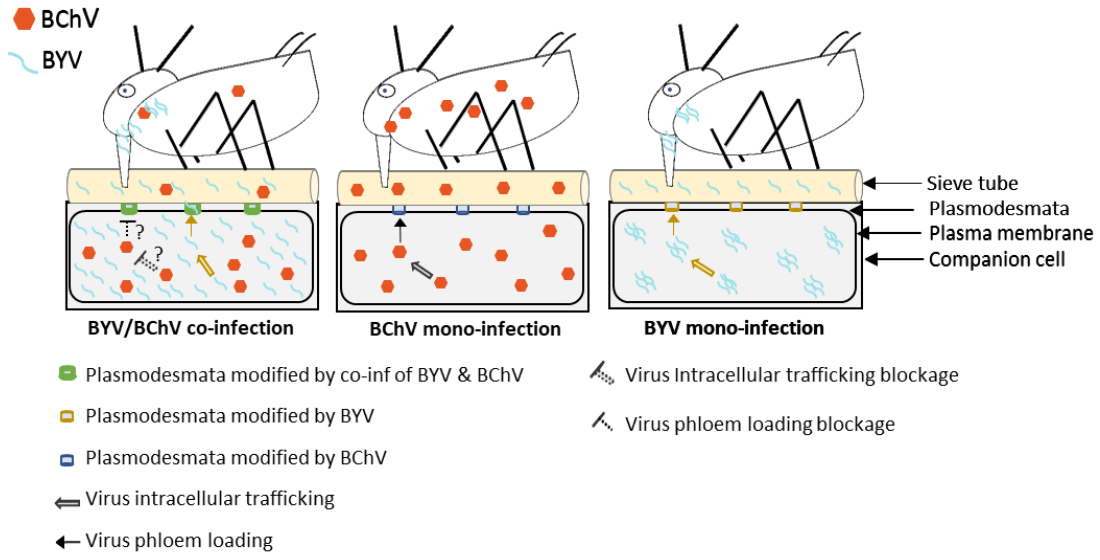



**Figure 7 : Modèle permettant d'expliquer la réduction de la transmission du BChV à partir de plantes co-infectées par le BChV/BYV**. Dans les plantes mono-infectées (cellules du milieu et de droite), les particules de BYV et de BChV sont transportées à partir des cellules compagnes infectées à travers les plasmodesmes dans la lumière des tubes criblés et sont acquises par les pucerons qui ingèrent la sève du phloème dans les tubes criblés. Dans les plantes co-infectées, environ un tiers des cellules du phloème infectées sont co-infectées. La co-infection n'affecte pas l'accumulation des virus de manière significative, et les deux virus peuvent s'accumuler à des niveaux similaires dans les cellules mono- et co-infectées, comme le montre le schéma (cellule de gauche). Le BYV et le BChV interagissent directement ou indirectement dans les cellules co-infectées. Nous proposons que cette interaction inhibe le passage du BChV vers et/ou à travers les plasmodesmes et affecte la libération du BChV dans les tubes criblés. A l'inverse, la libération des particules du BYV dans la sève du phloème reste inchangée. Par conséquent, la quantité de particules du BChV dans les tubes criblés serait significativement plus faible, comparé à une plante mono-infectée ce qui entrainerait une acquisition réduite du BChV par les pucerons à partir des plantes co-infectées.

## Chapitre 2 : Nouvelles propriétés biologiques des polérovirus infectant la betterave et impact de la co-infection sur leur transmission par puceron.

La deuxième combinaison étudiée a été la co-infection avec les deux polérovirus BChV et BMYV. Nous avons analysé la sensibilité de la betterave sucrière à ces virus en fonction du stade de développement et étudié la distribution des virus dans les plantes. Comme les deux virus peuvent être présents en même temps dans la betterave sucrière et qu'ils sont tous deux transmis par *M. persicae*, nous avons également étudié l'effet de la co-infection de la betterave sucrière sur l'accumulation du virus, la localisation tissulaire et cellulaire du virus et la transmission par les pucerons. J'ai pour ma part réalisé dans cette étude les analyses de localisation des virus ainsi qu'une partie des expériences de transmission à partir des plantes co-infectées, ce travail ayant à l'origine été amorcé par Hélène Schlaefli pendant son stage de M2.

Nous avons montré que le BMYV et le BChV se colocalisent dans environ 40 % des cellules infectées, ne révélant aucune exclusion virale au niveau cellulaire **Fig.1**. La co-acquisition des deux virus par les pucerons a entraîné une augmentation de l'efficacité de la transmission, ce qui suggère un effet coopératif des deux virus à l'étape de l'inoculation **Fig.2.**

Une hypothèse de coopération entre les deux virus pour supprimer les défenses de la plante dans les cellules primo-infectées a été émise.

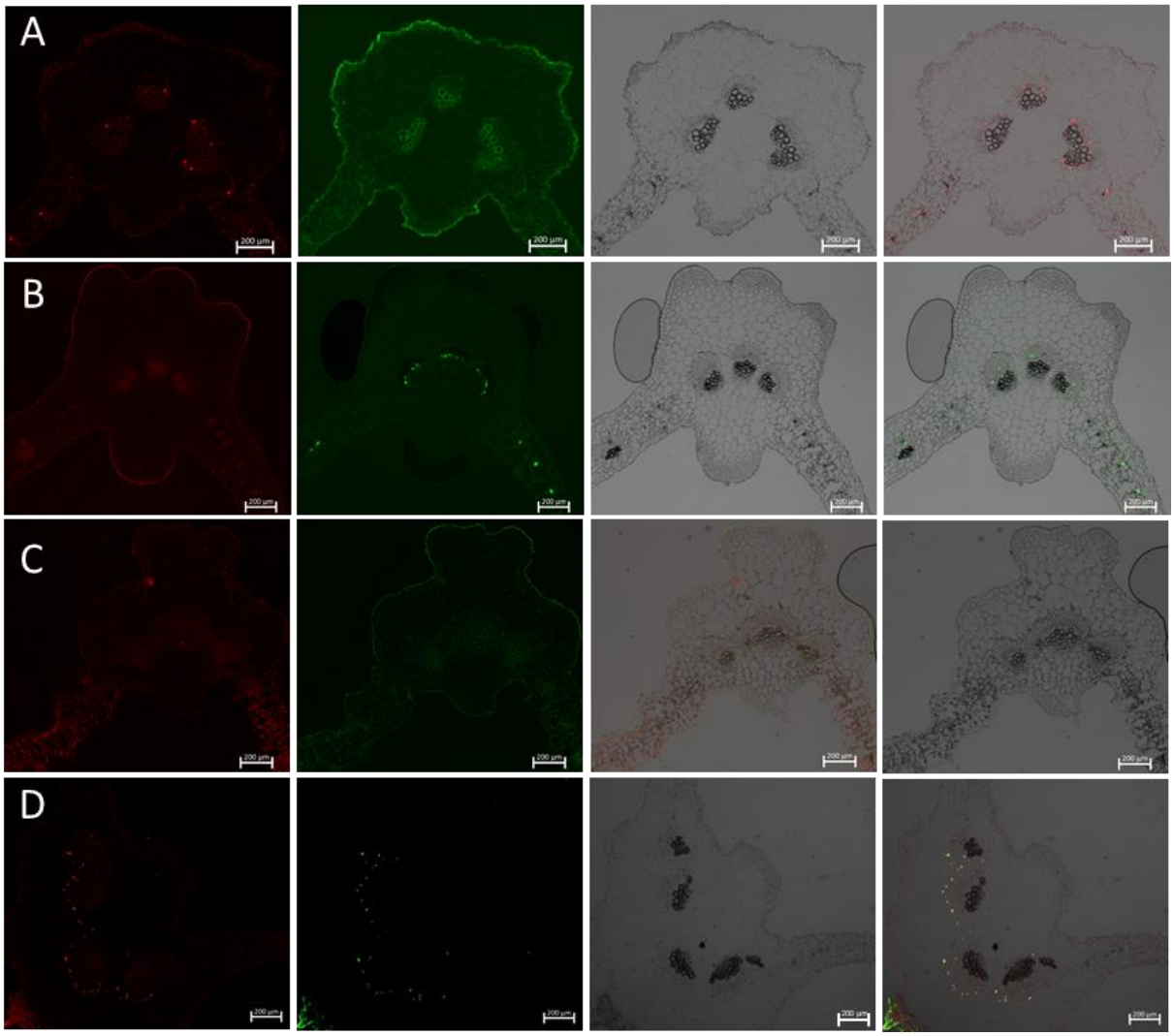



**Figure 1 : Détection par SABER-FISH des génomes ARN du BChV et/ou du BMYV dans des betteraves sucrières mono-infectées par le BChV ou le BMYV ou co-infectées par les deux virus.** Images représentatives de coupes transversales de feuilles de betteraves sucrières infectées par le BMYV (A), le BChV (B), le BMYV et le BChV (D) ou non infectées (C). La première colonne montre le marquage du BMYV (en rouge), la deuxième le marquage du BChV (en vert), la troisième l'acquisition en champ clair et la dernière colonne montre les superposisions d'images.

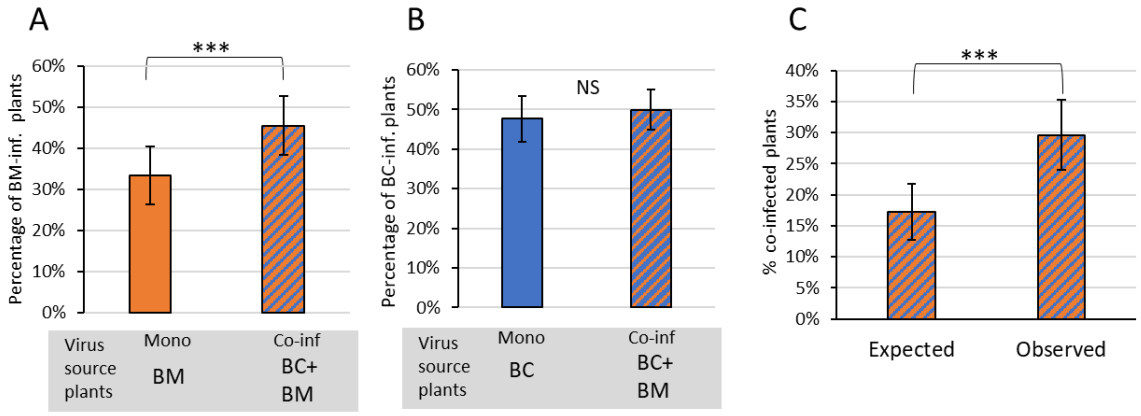



**Figure 2 : Efficacité de la transmission du BMYV et du BChV par les pucerons lorsqu'ils sont acquis à partir de betteraves sucrières mono- ou co-infectées.**

## Chapitre 3 : Les interactions synergiques entre le potyvirus responsable de la mosaïque de la betterave et le clostérovirus responsable de la jaunisse de la betterave diminuent la transmission du closterovirus

La troisième combinaison étudiée a été la co-infection du closterovirus BYV et du potyvirus, le beet mosaic virus (BtMV). Les co-infections BYV et BtMV ont provoqué des symptômes aggravés chez les betteraves sucrières, conduisant à l'apparition d'une mosaïque sévère sur les feuilles supérieures, un jaunissement internervaire des feuilles plus âgées et une forte réduction de la croissance des plantes ce qui suggère un effet synergique entre les deux virus pour le développement des symptômes dans les plantes co-infectées **Fig.1**.

L'effet de la co-infection BYV et BtMV sur la transmission des virus par puceron a tout d'abord été analysé. Nous avons observé une transmission plus faible qu'attendue du BYV et aucune modification de la transmission du BtMV **Fig.2**. Pour déterminer si la réduction drastique de la transmission du BYV est due à une moindre accumulation du BYV dans les plantes co-infectées, nous avons quantifié les titres de virus par RT-qPCR sur des betteraves mono- ou co-infectées. Nous avons détecté une augmentation significative de l'accumulation du BtMV et du BYV dans les plantes co-infectées, par rapport aux plantes uniquement infectées par le BtMV ou le BYV **Fig.3**.

Pour déterminer si la co-infection a affecté la distribution tissulaire du BtMV ou du BYV, le SABER-FISH a été réalisé sur des sections longitudinales de plantes infectées à 21 dpi. Sur les coupes transversales des feuilles mono-infectées par le BtMV, le marquage du BtMV a été observé dans des cellules de différents types cellulaires, comme attendu pour les virus

systémiques (cellules du phloème, de l'épiderme et du mésophylle) **(Fig. 4A)**. Dans les feuilles mono-infectées par le BYV, la fluorescence du BYV a été observée exclusivement dans les cellules du phloème, comme attendu pour les virus limités au phloème **(Fig. 4B)**. La co-infection n'a pas affecté la localisation tissulaire du BYV ou du BtMV : le marquage BYV reste limité aux cellules du phloème, et aucune sortie vers les cellules non phloémiennes n'a été remarquée ; le BtMV est détecté dans les cellules des différents tissus comme dans les plantes mono-infectées **Fig.4**. Nous avons aussi observé dans les cellules co-infectées par le BYV et le BtMV une co-localisation partielle dans le cytoplasme avec une redistribution cellulaire du BYV identique à celle observée pour la co-infection BYV/BChV **Fig.5**. Cela indique encore une fois une interaction directe, ou indirecte, entre les deux virus qui pourrait être responsable de la diminution de la transmission du BYV par puceron.

Des études sur le comportement alimentaire des pucerons sur les plantes co-infectées a montré un effet « BYV » sur l'augmentation de la durée E2 (ingestion de phloème) avec ou sans BtMV. Ceci suggère que la baisse de transmission du BYV en condition de co-infection avec le BtMV n'est pas liée à une diminution du temps d'ingestion de phloème par les pucerons. En comparant les conditions BYV et BYV-BtMV par rapport à Healthy (non-infecté), nous avons également observé une diminution du temps E1e (salivation extra-cellulaire) qui est plutôt considérée comme un comportement de stress du puceron bien que son rôle exact ne soit pas encore complètement élucidé **Fig.6**.

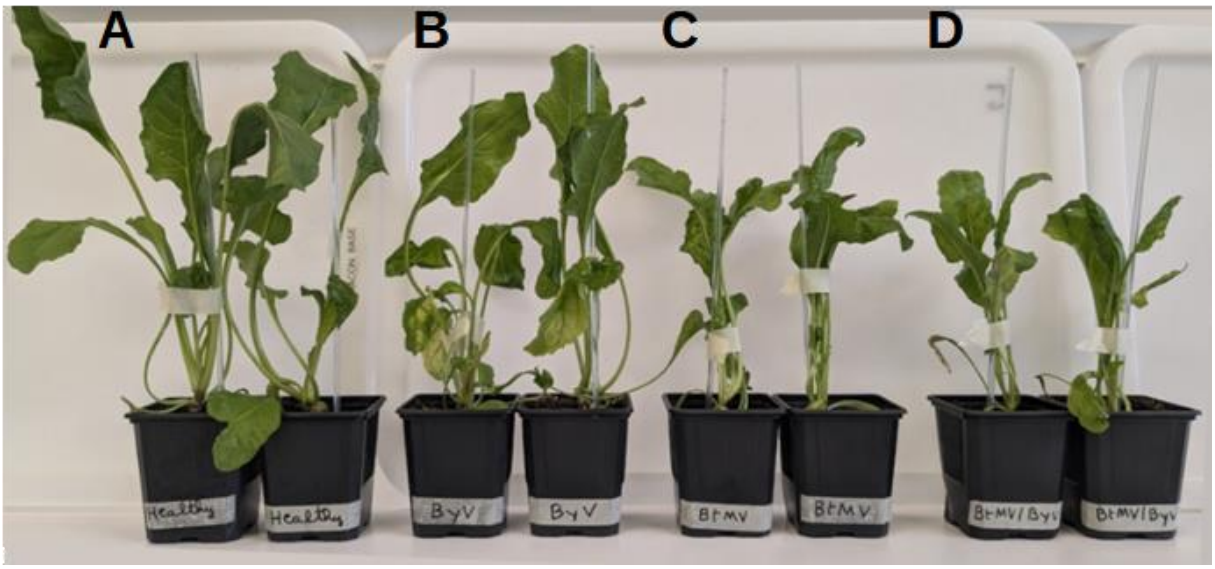



**Figure 1. Symptômes induits par la mono- ou la co-infection par le BYV et le BtMV 28 jours après l'inoculation.** (A) plantes témoin saines (Healthy), (B) plantes infectées par le BYV, (C) plantes infectées par le BtMV, et (D) plantes co-infectées par le BYV et le BtMV.

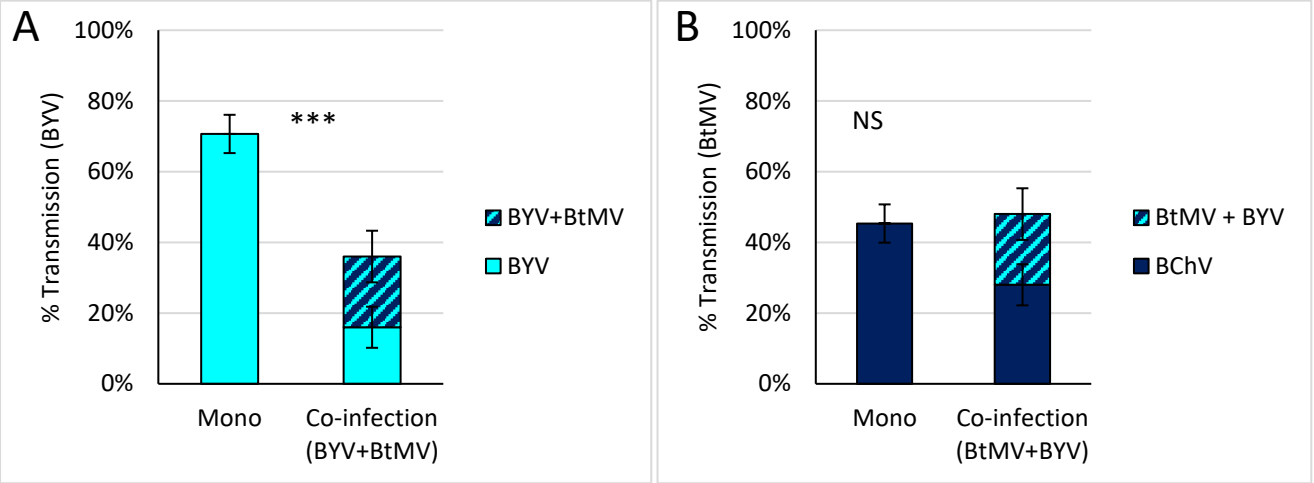



**Figure 2. Efficacité de la transmission du BYV (A) et du BtMV (B) par les pucerons à partir de plantes mono ou co-infectées.** *Myzus persicae* a acquis le(s) virus à partir de feuilles mono ou co-infectées pendant 24 h. Ensuite, trois pucerons ont été transférés par plante test pour une inoculation de 72 h. Un test DAS-ELISA a été réalisé 3 semaines plus tard pour détecter l'infection. Le pourcentage de plantes infectées est indiqué (% Transmission). La différence est statistiquement significative pour la transmission du BYV mais pas pour celle du BtMV (GLMER, BYV : p-value = 1 ; BtMV : p-value< 0,001 ; n = 75, cinq expériences indépendantes ; df = 1 ;). NS = non significatif, *** = valeur p < 0,001.

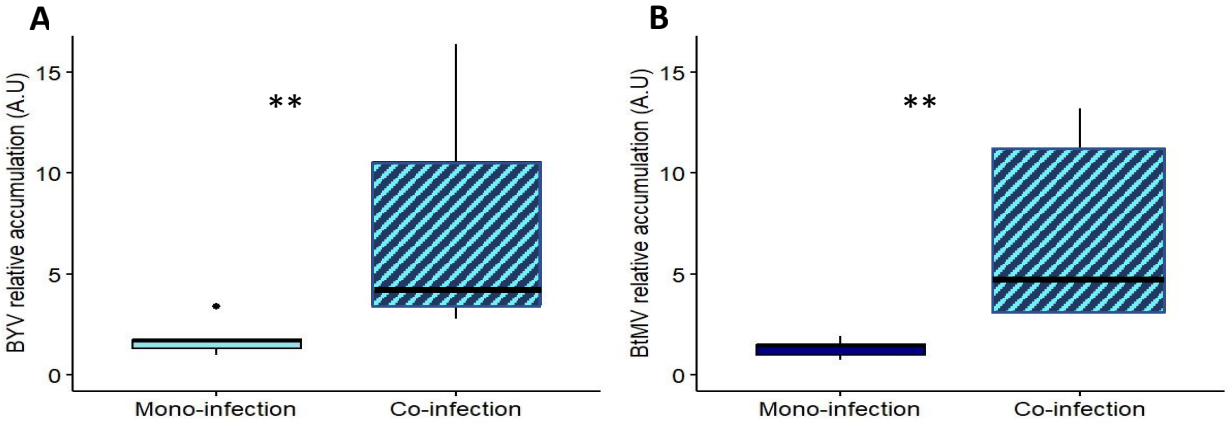



**Figure 3. Accumulation relative du beet yellows virus (BYV) et du beet mosaic virus (BtMV) dans les plantes mono- ou co-infectées 21 jours après inoculation.** L'accumulation de virus dans les plantes sources a été mesurée par RT-qPCR TaqMan multiplex, comme décrit dans la section matériel et méthodes. Les graphiques bBox montrent les médianes (ligne noire), les percentiles 25 %-75 % (boîte) et les percentiles 10 %-90 %. Les différences sont statistiquement significatives (test t de Student, BYV : t = -0,90817, p-value = 0,00341 BtMV : t = 0,59456 p-value=0,0005761 n = 5 ; df = 8). ** significatif.

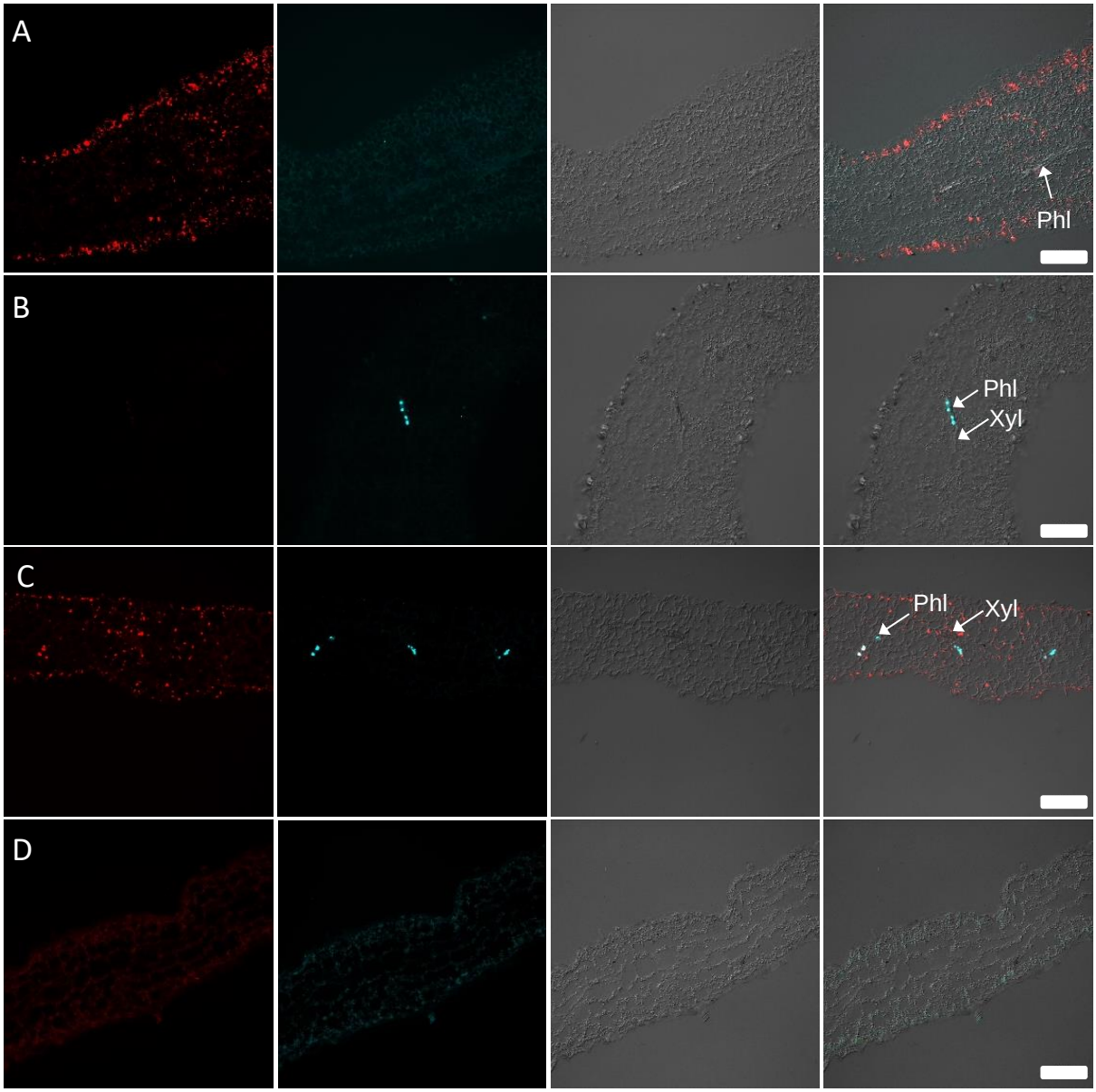



**Figure 4. Distribution tissulaire du BtMV et du BYV dans les feuilles mono-infectées par le BtMV, le BYV ou co-infectées par les deux virus**. Les feuilles ont été traitées trois semaines après l'inoculation par SABER-FISH pour la détection du BtMV et du BYV. (A-D) montrent des sections transversales de (A) feuilles mono-infectées par le BtMV, (B) feuilles mono-infectées par le BYV, (C) feuilles co-infectées par le BtMV/BYV, et (D) feuilles saines. La première colonne montre le marquage du BtMV (rouge), la deuxième le marquage du BYV (turquoise), la troisième les acquisitions en champ clair et la dernière colonne présente les fusions. Barres d'échelle = 100 µm ; Phl, phloème ; Xyl, xylème.

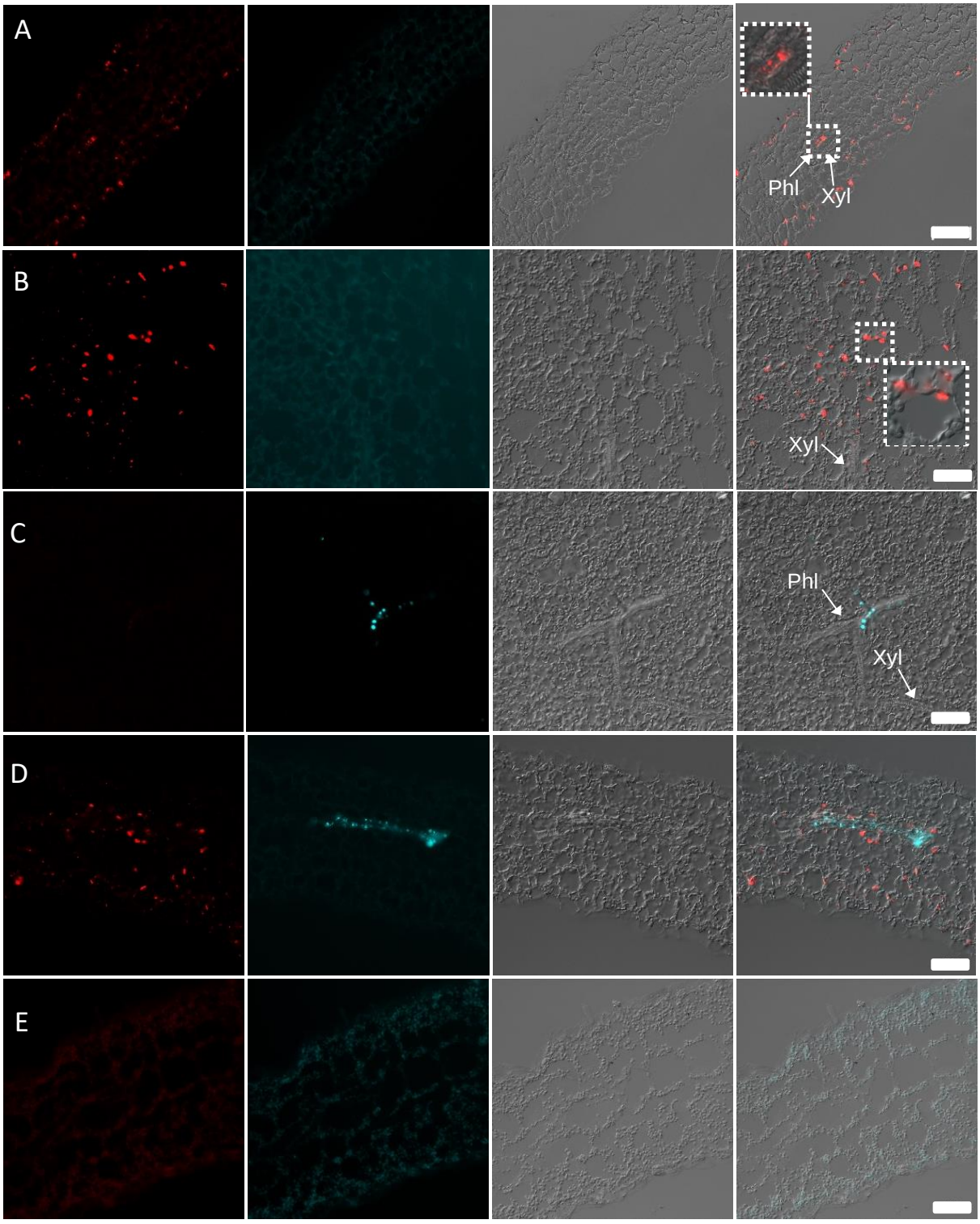



**Figure 5. Distribution intracellulaire des génomes du BYV et du BtMV dans les feuilles mono et co-infectées.** Les feuilles ont été traitées trois semaines après l'inoculation par SABER-FISH pour la détection du BtMV (rouge) et du BYV (turquoise). Les images montrent des sections longitudinales (A&B) d'une feuille mono-infectée par le BtMV, (C) d'une feuille mono-infectée par le BYV, (D) d'une feuille co-infectée par le BtMV et le BYV, et (E) d'une feuille saine. La première colonne représente le marquage du BtMV (rouge), la deuxième le marquage du BYV (turquoise), la troisième les images de contraste interférentiel différentiel, et la dernière colonne présente les fusions d'images. Xyl, xylème ; Phl, phloème. Barres d'échelle = 50 µm.

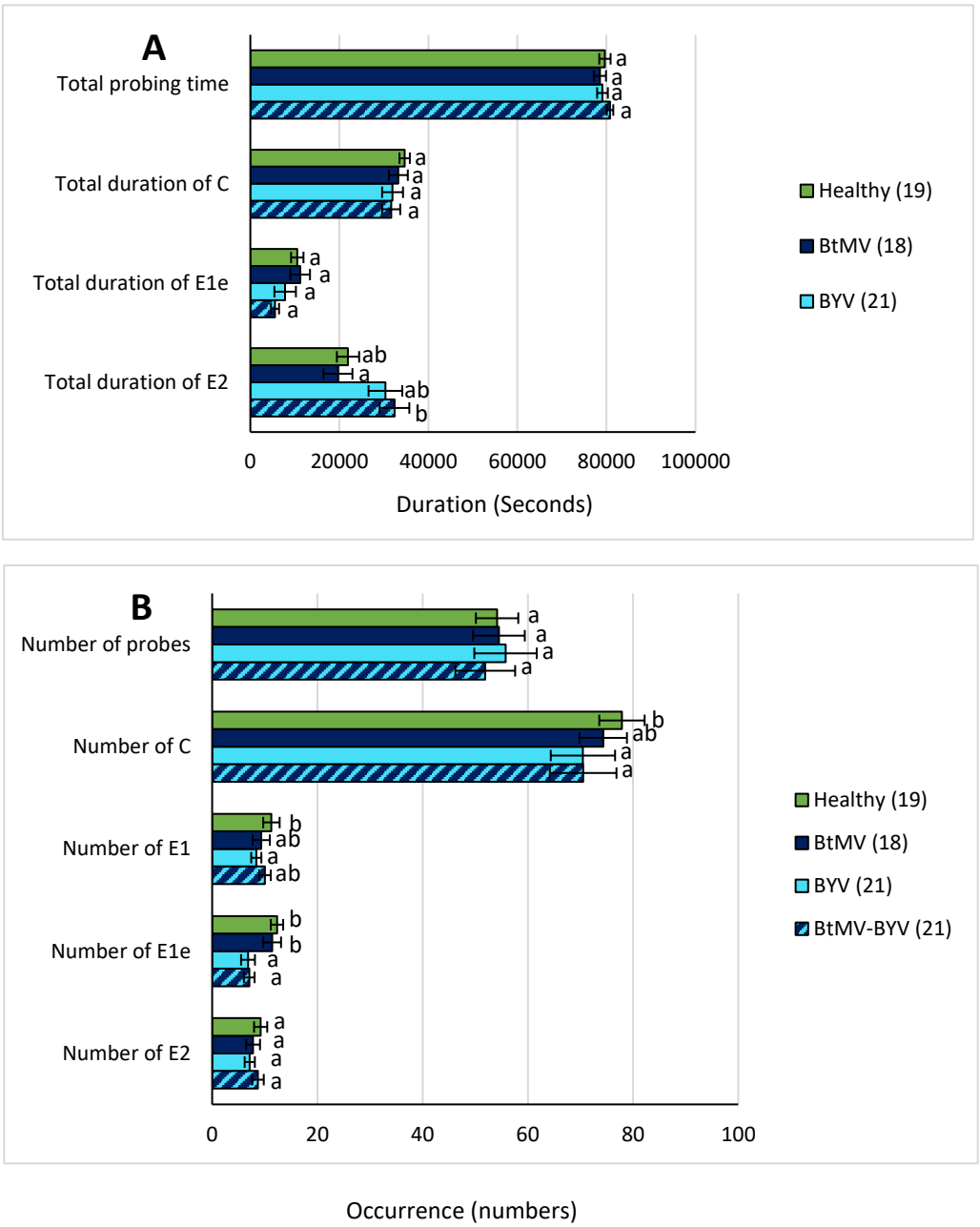



**Figure 6. Comportement alimentaire de *Myzus persicae* sur des betteraves saines, mono-infectées par le BYV ou le BtMV ou co-infectées par le BYV et le BtMV (N=18-22).** (A-B) Le comportement des pucerons individuels a été enregistré par la technique d'électropénétrographie (EPG) pendant 8 heures sur la troisième feuille supérieure. Les paramètres EPG sélectionnés sont présentés selon (A) la durée ou (B) l'occurrence. Les barres de l'histogramme indiquent les moyennes et les erreurs standard (SEM). Des lettres différentes indiquent des différences significatives entre les statuts d'infection des plantes, testées par GLM (modèle linéaire généralisé) suivi de comparaisons par paire à l'aide du paquet R : emmeans (méthode P<0,05 : Turquie).

## Conclusion et discussion

Ce travail explore l'impact de la multi-infection virale de la betterave sucrière sur la biologie et la transmission de quatre virus : BtMV, BYV, BMYV et BChV. Mes travaux de recherche se sont concentrés sur cinq combinaisons spécifiques de virus qui ont montré des effets reproductibles sur les taux de transmission par les pucerons. Trois de ces combinaisons (BChV/BYV, BtMV/BYV et BChV/BMYV) ont fait l'objet d'une étude plus approfondie et ont abouti à l'écriture de 3 papiers soumis.

L'étude a révélé des résultats différents pour chaque combinaison de virus :

- La co-infection BYV/BChV a réduit la transmission du BChV de 50 % mais n'a pas eu d'impact sur la transmission du BYV.
- La co-infection BYV/BtMV est corrélé à une transmission plus faible du BYV.
- La co-infection BMYV/BChV conduit à une transmission augmentée des deux virus.

Pour comprendre ces effets, nous avons analysé différents paramètres, notamment le comportement alimentaire des pucerons, l'accumulation du virus, la distribution du virus dans les tissus et la localisation intracellulaire du virus. Pour la co-infection BMYV/BChV, environ 30 % des cellules infectées sont co-infectées, sans impact sur le phénotype cellulaire. Le mécanisme à l'origine de l'augmentation de la transmission dans ce cas est discuté au chapitre 3 de la thèse mais pourrait provenir d'une synergie entre les deux virus pour détourner les défenses de la plante au moment de l'inoculation par le puceron.

Dans les co-infections BYV/BChV et BYV/BtMV, la distribution intracellulaire du BYV passe d'inclusions sphériques distinctes dans les cellules mono-infectées à une distribution plus diffuse dans les cellules co-infectées. Ce changement de localisation subcellulaire suggère des interactions entre les virus qui diminuent l'acquisition par les pucerons et peuvent donc affecter la transmission.

Plusieurs hypothèses sont évoquées pour expliquer les changements observés sur la transmission du virus :

1) Les interactions des virus co-infectants diminuent la quantité de virus libérée dans la sève phloèmienne à partir de laquelle le BYV et le BChV sont acquis par les pucerons. Cette baisse engendrait une transmission moins efficace. Ceci pourrait être dû aux facteurs viraux et de l'hôte.

- Interactions entre protéines virales : Les protéines de mouvement (MPs) de différents virus peuvent interagir, affectant les limites d'exclusion de taille des plasmodesmes (PD) et le mouvement de cellule à cellule. Par exemple, les interactions entre les MP du

BtMV et du BYV pourraient inhiber la formation des virions « à queue » du BYV, nécessaires pour les mouvements intercellulaires.

- Implication des protéines de l'hôte : Les facteurs de l'hôte tels que la pectine méthylestérase (PME) et les composants du cytosquelette (filaments d'actine et microtubules) jouent un rôle crucial dans le mouvement du virus. La co-infection pourrait modifier ces interactions, affectant la distribution et le mouvement du virus dans les cellules.

2) Entrave au niveau du vecteur : Des interactions compétitives entre virus dans le puceron vecteur peuvent se produire, un virus pouvant bloquer les sites de liaison d'un autre. Par exemple, le BtMV pourrait obstruer l'acrostyle, empêchant le passage ou la fixation du BYV dans les stylets du puceron.

## Perspectives :

- Mesurer l'accumulation de virus dans la sève du phloème de plantes co-infectées en utilisant des techniques telles que la section des stylets du puceron ou l'exsudation de la sève du phloème facilitée par l'EDTA pour conforter l'hypothèse 1.
- Réalisation d'analyses transcriptomiques sur des plantes co-infectées et mono-infectées afin d'identifier les gènes candidats impliqués dans le transport intracellulaire, la formation et fonction PD et la charge du phloème.
- Localiser les virus dans les pucerons pour conforter l'hypothèse 2.

En conclusion, cette thèse de doctorat fournit des informations précieuses sur les interactions complexes entre plusieurs virus dans les plantes de betterave sucrière et leurs effets sur la transmission par les pucerons vecteurs. Les résultats ouvrent de nouvelles voies de recherche en virologie végétale et peuvent contribuer au développement de nouvelles stratégies de gestion des maladies virales dans les cultures.

Souheyla KHECHMAR

# Impact de la multi-infection de la betterave sucrière sur la transmission des virus de plante par puceron


## Résumé

Nous ne savons que peu de choses sur la façon dont la co-infection virale modifie la transmission des virus par leur vecteur. Nous avons analysé dans la betterave à sucre la transmission des virus, le comportement alimentaire des pucerons, la localisation et l'accumulation des virus sur trois combinaisons virales pour lesquelles la transmission des virus par *Myzus persicae* est affectée: BYV/BChV, BYV/BtMV et BMYV/BChV La co-infection BYV/BChV a réduit la transmission du BChV de 50 %, mais n'a pas eu d'impact sur la transmission du BYV, la co-infection BYV/BtMV a induit une transmission plus faible du BYV et la co-infection des deux polérovirus, BMYV/BChV, a entraîné un effet synergique sur leur transmission. La réduction drastique de la transmission du BChV ou du BYV n'est pas due à une accumulation plus faible des virus dans les plantes co-infectées, ni à une réduction de l'ingestion de sève du phloème par les pucerons sur ces plantes. Cependant, pour les co-infections BYV/BChV et BYV/BtMV, nous avons observé une distribution intracellulaire différente du BYV dans les cellules co- ou mono-infectées. Le BYV est observé sous la forme d'inclusions sphériques dans les cellules mono-infectées et se retrouve plus diffus dans le cytoplasme des cellules co-infectées par BtMV/BYV et BChV/BYV. Pour la co-infection BMYV/BChV, environ 30 % des cellules infectées sont co-infectées, sans impact sur la localisation intracellulaire des virus. Nous proposons que la diminution de la transmission par les pucerons du BChV à partir des plantes co-infectées par le BChV/BYV ou du BYV à partir des plantes co-infectées par le BtMV/BYV soit due à des interactions entre les deux virus ou avec des facteurs de l'hôte qui diminuent le chargement du phloème avec le BYV ou le BChV et, par conséquent, l'acquisition des virus par les pucerons. L'explication la plus probable pour la transmission accrue du BMYV et du BChV à partir des plantes co-infectées est une synergie pour réduire les défenses de la plante lors de l'inoculation des virus.



## Résumé en anglais

Little is known about how viral co-infection modifies the transmission of viruses by their vector. We analyzed in sugar beet virus transmission, aphid feeding behavior, virus localization and accumulation of three viral combinations for which virus transmission by *Myzus persicae* was affected: BChV/BYV, BtMV/BYV/ and BMYV/BChV BChV/BYV co-infection decreased transmission of BChV by 50 %, but had no impact on BYV transmission, BtMV/BYV co-infection induced a lower transmission of BYV and co-infection of the two poleroviruses, BMYV/BChV resulted in mutual transmission synergy. The drastic reduction of BChV or BYV transmission was not due to a lower accumulation of the viruses in co-infected plants, nor to reduced phloem sap ingestion by aphids from these plants. However, for BChV/BYV and BtMV/BYV co-infections, we observed a different intracellular distribution of BYV in co-infected vs mono-infected cells. BYV was observed in intracellular spherical inclusions in mono-infected cells and displayed a rather diffuse distribution in BtMV/BYV and in BChV/BYV co-infected cells. For BMYV/BChV co-infection, about 30 % of infected cells were co-infected, without impacting virus localization. We propose that the decrease aphid transmission of BChV from BChV/BYV co-infected plants or of BYV from BtMV/BYV co-infected plants is due to virus-virus or plant-virus interactions that decrease BYV or BChV release in the phloem sap and consequently acquisition by aphids. The most probable explanation for enhanced BMYV and BChV transmission from co-infected plants is synergy to reduce plant defenses during virus inoculation.