Transmission risks of Beet Yellows Virus by *Myzus persicae* and *Aphis fabae* aphids in diverse environmental conditions

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Abstract

The economically significant Beet yellows virus (BYV) negatively affects sugar beet yields. For the past 30 years, effective control of BYV relied on neonicotinoid insecticides. However, the European Union's ban on outdoor use of these insecticides since 2018, driven by concerns over biodiversity and human health, led to widespread losses in the sugar beet industry due to BYV outbreaks. The main vectors of this virus are the green peach aphid (*Myzus persicae*) and the black bean aphid (*Aphis fabae*). Understanding the virus-vector relationship is crucial for developing new control methods. To address this, our study investigates viral transmission efficiency in sugar beet plants based on aphid density, species, and morphs (winged/apterous) in laboratory, greenhouse, and field conditions. Laboratory experiments were based on the virus transmission from an infected plant to a vector and from a vector to a healthy plant, while greenhouse experiments observed viral transmission changes when both vector species coexisted on the same infected plants simultaneously (i.e. by analogy to the same patch in the fields). Field experiments examined whether the first flying aphids that arrived in the fields after seed germination were viruliferous. In the laboratory experiment, it was shown that the best vector of BYV at low density was the winged *M. persicae* with a 25% of transmission probability with only three aphids present. In the greenhouse experiment, a significantly higher transmission probability was observed when both vector species were present at the same time at the same patch. Collecting this data provides valuable insights into how aphid species, density, and morphs affect the transmission of the virus, setting a solid foundation for future studies and the discovery of new pest control methods.

Keywords: Beet yellows virus, aphids, viral transmission, virus-vector relationship, sugar beet

Résumé

Le virus de la jaunisse grave de la betterave (BYV) a une importance économique car il affecte négativement les rendements de la betterave sucrière. Au cours des 30 dernières années, la lutte efficace contre le BYV a reposé sur les insecticides néonicotinoïdes. Cependant, l'interdiction par l'Union européenne de l'utilisation de ces insecticides à l'extérieur depuis 2018, motivée par des préoccupations relatives à la biodiversité et à la santé humaine, a entraîné des pertes généralisées dans l'industrie de la betterave sucrière en raison des épidémies de BYV. Les principaux vecteurs de ce virus sont le puceron vert du pêcher (*Myzus persicae*) et le puceron noir de la fève (*Aphis fabae*). La compréhension de la relation virus-vecteur est cruciale pour le développement de nouvelles méthodes de contrôle. Pour répondre à cette question, notre étude examine l'efficacité de la transmission virale dans les plants de betterave sucrière en fonction de la densité des pucerons, des espèces et des morphes (ailé/aptère) en laboratoire, sous serre et sur le terrain. Les expériences en laboratoire sont basées sur la transmission du virus d'une plante infectée à une plante saine via un vecteur, tandis que les expériences en serre ont permis d'observer les changements de transmission virale lorsque les deux espèces de vecteurs étaient présentes simultanément sur les mêmes plantes infectées (par analogie au champ, dans le même groupe de plantes). Les expériences sur le terrain ont examiné si les premiers pucerons volants arrivés dans le champ, après la germination des graines, étaient virulifères. L'expérience en laboratoire a montré que le meilleur vecteur du BYV à faible densité était le puceron ailé de l'espèce *M. persicae*, avec une probabilité de transmission de 25 % en présence seulement de trois individus. Dans l'expérience en serre, une probabilité de transmission significativement plus élevée a été observée lorsque les deux espèces de vecteurs étaient présentes en même temps dans la même parcelle. La collecte de ces données fournira des informations précieuses sur la manière dont les espèces, la densité et les morphes de pucerons affectent la transmission, posant ainsi des bases solides pour les études futures et la recherche de nouvelles méthodes de lutte contre les ravageurs.

Mots-clés: Virus de la jaunisse de la betterave, pucerons, transmission virale, relation virus-vecteur, betterave sucrière.

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1. Introduction

The sugar beet industry plays a significant role in the global economy, creating jobs and driving economic activity, especially in rural areas where employment opportunities are limited. In 2022, the global sugar beet market was valued at US$4.31 billion and is expected to reach US$6.34 billion by 2028, with an annual compound growth rate of 5.7% between 2023 and 2028 (Market Data Forecast, 2023). The industry primarily focuses on the cultivation of sugar beet plants (scientifically known as *Beta vulgaris subsp. vulgaris*), and the European Union (EU) stands as the world's leading producer, accounting for over 50% of the total production (European Commission, 2022). The majority of sugar beets in the EU are grown in the northern half of Europe, including northern France, Germany, the Netherlands, Belgium, and Poland. (European Commission, 2019). While the main objective of sugar beet cultivation is sugar production, the plant offers various valuable by-products such as molasses, beet tails, bio-ethanol, bioplastics, betacal, beet soil, and beet pulp. As a result, sugar beet has gained significance due to the rising demand for sugar and its contributions to the circular economy, green chemistry, and sustainability (Bak, 2022).

Despite the growing demand for sugar beet and its by-products, its production has been declining in recent years. This decrease can be attributed to poor harvests caused by dry spring weather and aphid attacks and consequently disease outbreaks, leading EU producers to reduce their sowings (UKRSUGAR, 2020). The significant decrease in production appeared following the ban on some neonicotinoid insecticides (NNIs), which had been used in sugar beet production for over three decades (Hossain et al., 2020). Neonicotinoids were applied as seed coatings to prevent aphid attacks and the potential transmission of plant diseases such as viruses. However, due to concerns about their impact on pollinators and non-target organisms, the outdoor use of NNIs has been restricted in the EU since 2018 (LAW n° 2016-1087). These NNIs were especially effective in controlling two aphid species, *Myzus persicae* and *Aphis fabae*, which are the main vectors of the economically significant Beet yellows virus (BYV) (Limburg et al., 1997). The prohibition of NNIs has led to numerous outbreaks of BYV, resulting in reduced yields and industry revenue. For example, in the United Kingdom, the loss of the national sugar beet crop due to the ban of NNIs was estimated at 25% in 2020, leading to approximately £67 million in total economic loss across the industry (UK, Department for Environment Food & Rural Affairs, 2023). Similarly, in France, the loss was estimated at 30% of the national sugar beet yield (Audran, 2022).

To ensure the continued survival and growth of the sugar beet industry, it is crucial to deepen our understanding of the relationship between the virus and its vectors. This knowledge will provide valuable insights for the development of new methods to control these pests without causing harm to the environment or non-target organisms in the fields.

2. Literature review

Global crop production is annually reduced by approximately 15% due to plant diseases, underscoring the persistent threat to plants worldwide and consequently reducing both economic productivity and global nutritional security (FAO, 2021). Over one-third of these diseases are caused by phytopathogenic viruses (Boualem et al., 2016). Despite the use of various chemicals and insect vector management, direct control of plant virus infections is challenging. These chemical treatments may only partially restrict virus population and distribution, while usually

having detrimental effects on both human health and the environment (Islam et al., 2017). Plant viruses are characterized by an obligate intracellular lifestyle that provides a platform for host-virus interaction (Pallas and Garcia, 2011). The ability of the virus to induce infection is contingent upon its production of virulence factors that can effectively counteract the host's defense mechanisms (Jones and Dangl, 2006). Viral infections in plants can lead to a variety of symptoms such as leaf rolling, wilting, yellowing, stunting, necrosis, and mosaic pattern formation (Dekker et al., 1993). Typically, viruses do not cause the death of the host plant because they require living tissue for both their replication and completion of their life cycle. Plant pathogenic viruses can be transmitted in several manners: mechanically, via seeds, or by vectors (Sacristan et al., 2011; Gray and Power, 2018; Sui et al., 2019).

Vector-borne viruses are the dominant group, and they are mainly transmitted by insects, specifically aphids, which have a wide range of hosts from agriculture to forest plants (Brault et al., 2010; Jayasinghe et al., 2011; Vuorinen et al., 2011). The viral transmission by vectors is completed in four steps. First is the acquisition of viruses from an infected plant. Second, is the retention of the virus in the vector by binding the virion to specially adapted mouthparts for feeding (stylets) or receptor-like elements in the digestive tract or circulation from the gut to the salivary gland. Third, virions are delivered from the retention sites to a new site. Finally, virions are deposited in a susceptible cell of the host plant (Brault et al., 2010). The transmission mode can be persistent or semi-persistent (the virus has a relatively long acquisition time and remains in the host's body for an extended period) or non-persistent, and the virus can either be circulative (it replicates in the host's circulatory system) or non-circulative (remains localized to the mouthparts or foregut) (James and Keith, 2004). The efficiency of virus transmission varies depending both on the vector density and the type of virus. Semi-persistent viruses typically exhibit an increased transmission efficiency, while nonpersistent viruses generally show a decreased transmission efficiency (Kennedy et al., 1962; Harris 1997).

Aphids are unique insect vectors that mainly transmit non-persistent viruses (non-persistent transmission, NPT), some of which are responsible for severe crop damage. The epidemiology of NPT viruses depends on the density and behavior of aphid vectors, particularly on their ability to acquire and inoculate the virus during sampling probing (i.e. when aphids “taste” a potential host plant for assessing its quality) and their propensity for moving among plants (Ng and Perry, 2004). Therefore, a greater number of aphids, especially winged can increase the chances of probing and feeding on infected plants, which can lead to a higher probability of acquiring and transmitting the virus on both shorter and longer distances (Shi et al., 2021; Carr et al., 2018). For example, field studies on potato virus Y (PVY) showed the existing positive correlation between the number of winged aphids and the spread of PVY (Harrington and Gibson, 1989; Sigvald, 1989). Moreover, a study conducted in Japan showed that the overall increase in the number of winged aphids feeding on plum pox virus (PPV) increased prune infections and the enhanced movement of viruliferous aphids over the non-viruliferous ones in the fall resulted in a peak of viral transmission (Madden et al., 1990; Madden et al., 2000). However, controlling the spread of such viruses through insecticide treatments is difficult because both acquisition and inoculation occur during feeding exclusively.

Aphids can be classified as "residents," which spend most of their living on the same host plant individual under favorable conditions, or "transients," which land and probe numerous plants on the same day (Fereres and Moreno, 2009; Van Emden and Harrington 2007). Although

transient aphids are commonly thought to be the primary vectors of NPT viruses, resident aphids may also successfully transfer NPT viruses in response to crowding or changes in plant nutrient levels (Müller et al., 2001). Indeed, mathematical modeling indicates that both reduced and extended settling can contribute to epidemic development, via density-dependent production of winged forms on crowded host plants (Donnelly et al., 2019; Carr et al., 2020).

Controlling the aphids is challenging due to both their complex life cycle with alternating asexual and sexual phases and their remarkable phenotypic plasticity (Peccoud et al., 2010; Simon et al., 2010). One of the most efficient methods for managing aphids in agriculture has been the use of NNIs, such as imidacloprid, clothianidin, and thiamethoxam. These systemic insecticides are taken up by the plant and can provide control of aphids through contact and ingestion (Hossain et al., 2020). However, due to their potential negative impacts on pollinators and other non-target organisms, NNIs have been restricted for outdoor use in the EU since 2018 (LAW n° 2016-1087). This prohibition has resulted in a large number of issues, including numerous outbreaks of economically important plant viruses. Infection of sugar beet by viruses can decrease sugar yield by up to 25% (Clover et al., 1999) or even up to 47% (Smith and Hallsworth, 1990) depending on virus species and time of infection. One of the viruses that caused the biggest economic loss after the restriction of NNIs is the Beet Yellows virus (*Closterovirus*, BYV). The BYV is a phloem-limited virus belonging to the family of Closteroviridae Infections with BYV lead to yellow discoloration of the older leaves, and subsequently to red necrosis (De Koeijer and van der Werf, 1999). However, despite BYV being one of the economically most important pathogens of sugar beet, there are significant knowledge gaps in both virus-vector and vector-plant interactions.

The main vectors of BYV are two aphid species: *Myzus persicae* and *Aphis fabae* (Hemiptera: Aphididae), which transmit the virus in an NPT manner. Both vector species are residents of sugar beet (Limburg et al., 1997) and good BYV vectors. However, it is shown with the electrical penetration graph (EPG) that *M. persicae* has suitable feeding behavior which can imply its better vector capacity than *A. fabae* (Jiménez et al., 2018). Theoretical studies suggest that the simultaneous presence of multiple vector species on a single plant can have both positive and negative effects on NPT, as it may either enhance or impede the spread of the virus through the production of aphids chemical repellents or attractants (Chisholm et al., 2019; Crowder et al., 2019; Thaler et al., 2010; Van den Bosch and Jeger, 2017). There is currently no empirical research available on the relationship between coexisting vector species in general either on *M. persicae* or *A. fabae* when simultaneously present in the same field or plant. However, it is known from previous studies that resident aphids can interfere directly through the production of allelochemicals that can have a repelling and/or attracting effect on other aphid species (Crowder et al., 2019). Several experimental studies have shown that after a plant infestation with a species-adapted aphid biotype, the plant defense mechanism is decreased, which allows better nutrition of a non-adapted biotype that was previously unable to feed on that plant species (Shih et al., 2023; Varenhorst et al., 2015; ten Broeke et al., 2017). For example, co-infestation of the lettuce aphid, Nasonovia ribisnigri, on resistant lettuce, revealed that avirulent aphids placed among virulent aphids boosted their phloem feeding time and survival rate. However, the avirulent biotype induced weak defense on both susceptible and resistant lettuce plants, which resulted in reduced phloem feeding time of the virulent biotype (ten Broeke et al., 2017).

Since BYV is transmitted in an NPT manner, the density of the vector population is one of the key factors when it comes to transmission, as mentioned before. A recent study has shown that 10 viruliferous wingless *M. persicae* were sufficient for efficient BYV transmission (Hossain et al., 2020). Also, it is known that in order to perform the most efficient transmission, aphids (both apterous and winged) need 24 hours for the acquisition of the virus on the infected plant (German and Martelli, 1999). The minimal number of winged aphids required for efficient virus transmission is currently unknown, as most studies have focused on apterous forms. However, some studies have suggested that winged aphids may be more efficient in transmitting viruses compared to apterous aphids, possibly due to their ability to disperse within and between fields (Inswell et al., 2012; Blua and Perring, 1992; Ben-Ari et al., 2015). Additionally, the feeding behavior of winged and apterous aphids may differ, with winged aphids probing and moving between different plant tissues more frequently compared to apterous aphids, which may affect the efficiency of virus transmission (Boquel et al., 2011). The ability of the virus to replicate and move within aphids may also differ between winged and apterous forms, potentially affecting their ability to transmit the virus (Boquel et al., 2011; Huijuan et al., 2023).

The first aim of our study was to test the effect of aphid density, for both apterous and winged morphs of both vector species, on the probability of virus infection. We expect that the transmission is more efficient with winged morphs (Blua and Perring, 1992; Huijuan et al., 2023) of *M. persicae* (Jiménez et al., 2018; Symmes et al., 2008; Limburg et al., 1997; Watson, 1946; Heathcote and Cockbain, 1964) implying that even small populations or densities are sufficient for virus transmission. Secondly, due to the lack of data regarding the relationships between i) vectors of the same virus that are present at the same time on the same plant, and ii) *M. persicae* and *A. fabae* as resident aphids on the same plant, our aim was to test for any potential changes in virus transmission when apterous forms of these aphid species coexisted or not on the same patch, at various time frames. It is expected that the virus transmission is better when the vector species coexist in the same area due to the potential facilitation effect of one species to the other (Shih et al., 2023; Varenhorst et al., 2015; ten Broeke et al., 2017) and due to a significant level of cooperation between the vectors in terms of viral transmission (Petrović-Obradović et al., 2023; Kershaw, 1965). Finally, to measure the prevalence of the virus in winged aphid populations arriving at the beginning of the season on the susceptible stages of sugar beet, a field survey was conducted.

3. Material and Methods

Experiments were divided into three parts: laboratory, greenhouse, and field experiment. The laboratory experiment was conducted under controlled conditions and aimed to determine the minimum population size required for efficient transmission of the BYV virus by both winged and apterous vector morphs of both vector species. Greenhouse experiments were carried out in a controlled greenhouse environment to understand how the primary vectors of BYV, *M. persicae* and *A. fabae* interact with each other in terms of potentially facilitating the virus transmission. The field experiment involved a screening of both winged vectors *M. persicae,* and *A. fabae* in sugar beet fields in order to determine the actual prevalence of the virus in current field conditions.

3.1. Laboratory and greenhouse experiments

3.1.1. Biological materials

Two different aphid clones were used for both *M. persicae A. fabae*, received from INRAE Colmar, France. The *M. persicae* clone was DNV^{-}/ (cleaned up from Densovirus in 2010), named the MPCOL strain, collected in 1974 in Colmar (France) and maintained on sugar beet-pepper plants. The *A. fabae* clone was collected in Laon (France) in 2022 and maintained on sugar beet plants. For both laboratory and greenhouse experiments, the aphid clones were reared separately on sugar beet plants in separate glass cages (30x30x30cm). The cages were maintained in growth chambers under a constant temperature of 21°C±1°C, a photoperiod of 16:08 Light:Dark hours, and a relative air humidity of 60%±10%. To ensure continuous production of apterous forms, a new sugar beet plant was introduced into the cages every ten days. However, for experiments involving winged aphids, the addition of new plants was stopped after two weeks to encourage the density-dependent development of winged forms.

For laboratory and greenhouse experiments, both healthy and BYV-infected sugar beet plants of the SESVANDERHAVE Arum variety were utilized. These plants were produced in a greenhouse under the same experimental conditions as the ones under which the aphids were kept. The first two leaves were marked on all produced plants with a crochet thread in size 10. The soil in which plants were sown was potting soil (Universal Soil, Botanic, France). Before sowing, all used soil was sterilized in a microwave at the maximum temperature for 15 min. A total of 30l of soil was sterilized for every 20 seeds. After sterilization, the soil was cooled for 5h before sowing the seeds. The seeds were sown in trays, with each tray containing 20 seeds. The plants were watered every other day, resulting in a 100% of germination rate. Healthy plants served for the experimental setup and the aphid feed. However, the infected plants were exclusively used for virus acquisition of the aphids in the laboratory experiments, while in the greenhouse experiments, they were used both for the virus acquisition and experimental setup.

Healthy plants were sown prior to the experiment and used to rear both aphid vectors. This resulted in three groups of plants at the beginning of the experiment: the first group with 15 plants at the two-leaf stage (three-week-old), the second group with 15 plants at the four-leaf stage (6 weeks old), and the third group with plants at the six-leaf stage (8 weeks old). The infected plants were obtained by intentionally infesting the produced healthy plants with 10 viruliferous *M. persicae* (the same aphid clone used in the experiment). After the infestation, the plants were moved from the greenhouse and placed under a glass cage (glass box with glass sides 30x30x30cm) in the growth chamber, maintaining the same experimental conditions as in the greenhouse

3.1.3. Serological methods

The virus transmission was verified by measuring the viral content in sugar beet plants with a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Beet yellows virus 07012C/480 Standard Kit DAS-ELISA, LOEWE, Germany). Leaf samples (400mg) from each plant were analyzed individually in triplicate (no dissimilarity found between triplicates). The test was done in accordance with the protocol given by the LOEWE company. After the collection, leaf samples were kept frozen at -80°C in the extraction bags (Extraction bag Universal 12x15 cm, Bioreba, Switzerland) in which they were homogenized upon the beginning of the protocol by hand homogenizer (Hand model, Homogenizer, Bioreba, Switzerland). In each plate, there were eight wells with controls: four wells with the healthy plant sample, one well

with sample buffer, fifth and sixth well with positive and negative control provided with the kit. Absorbance was measured at 450 nm in a spectrophotometer (Helios Omega UV-VIS Spectrophotometer, Thermo Scientific, United States). The threshold was calculated in the following manner: (average of healthy controls) +3*(standard deviation of healthy controls) (Clark and Adams, 1977). The samples with an absorbance two times above the threshold were considered positive, while samples with an absorbance lower than the threshold were considered negative.

3.2. Laboratory experiments: experimental design

The laboratory experiment was conducted from February to May 2023 in two parts. The first part of the experiment was set with apterous individuals from both vector species, while in the second part, winged individuals were used. Each experimental part was divided into three rounds of tests. In each round, 12 sugar beet plants were used. Twenty-four hours before setting up the experiment, plants were brought from the growing platform (greenhouse) to the room where the experiment took place, for acclimatization. Each of the 12 plants was placed in one individual cage (cage for the observation of insects and other animals-30x30x30cm, Le Club biotope, France). Additionally, 24h before the experiment, healthy aphids, either apterous or winged, were placed on the infected plants to acquire the virus. After 24h on the infected plants, the exact number (3, 8, or 13) of the aphids was allocated in one of the 12 cages (respecting the order shown in Figure 1). After 24h, aphids were counted and manually killed. Following the aphid removal, each plant was marked and moved from individual cages to the collective one. Three weeks after, the first two leaves previously marked, were cut off and stored at -80°C in the extraction bags, until the ELISA test was performed to confirm whether the virus had been transmitted or not.

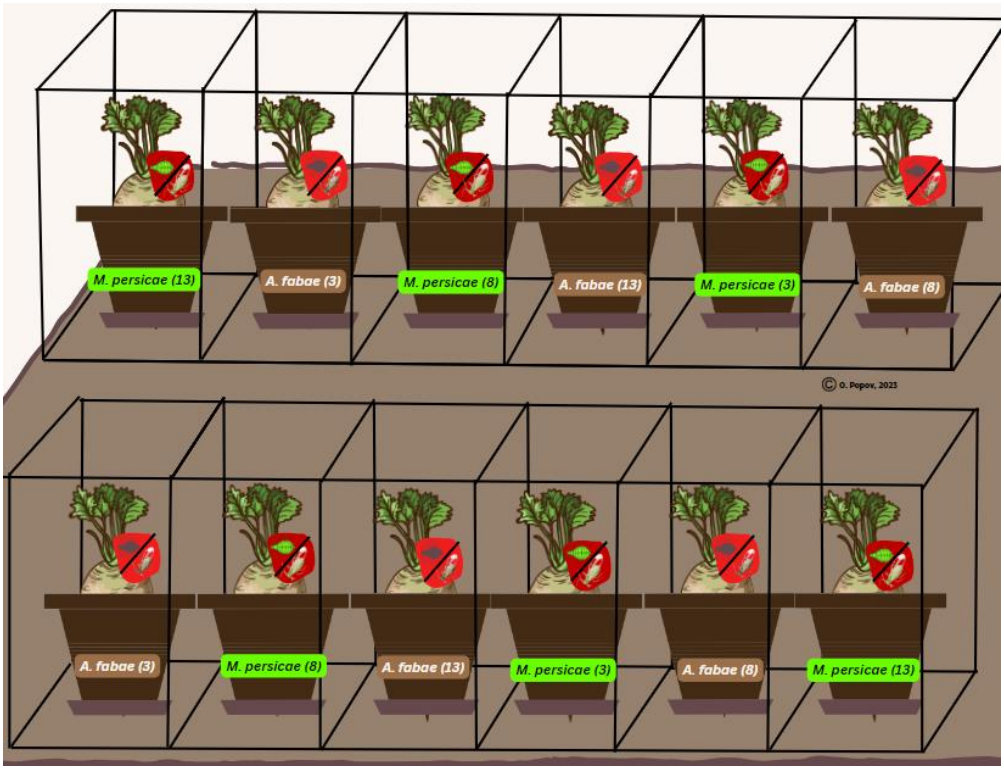

Figure 1. Laboratory experiment set up. Each plant had viruliferous aphids of either morph (winged or apterous) of one of the two species (*A. fabae* or *M. persicae*). The experiment was divided into two parts, the first part with apterous and the other with winged aphids, each consisting of three identical rounds. In each round, a total of 12 plants were infested with apterous/winged morphs, with 6 plants infested with one species and the remaining 6 with the other species. Among these 12 plants, two of them were infested with 3, two with 8, and two with 13 aphids of each species, following the order shown in the figure.

3.3. Greenhouse experiments: experimental design

The greenhouse experiments were conducted from May to June 2023 in 48 arenas, aiming to simulate field conditions in terms of the spatial arrangement between plants. Only apterous aphids were used, as they are the primary agents responsible for spreading the infection that was initially introduced in the fields by winged morphs on distant individual plants. The number of aphids used for the experimental setup was determined based on previous studies. Therefore, 10 viruliferous wingless *M. persicae* aphids were used in the experiment as demonstrated by Hossain et al. (2020). However, there was no available data on the sufficient number of wingless viruliferous *A. fabae* aphids for BYV transmission. Hence, 11 individuals of wingless viruliferous *A. fabae* were used since it is known that *M. persicae* has better vector capacity (Jiménez et al., 2018).

The experiment took place in a greenhouse within arenas, which were rectangular containers (60x40x10.5cm) filled with soil. These arenas were isolated in separate breeding cages (60x60x90cm, Vermandel, The Netherlands). Each breeding cage contained an arena with five six-week-old plants: one in the center and one in each corner (Figure 2). The plants in the corners of the arena were sown directly from the seeds, while the middle plant was introduced after the infection had developed. At three weeks old, the BYV was transmitted to the middle plant by viruliferous aphids, ensuring that by the time it reached the sixth week after sowing, the infection had already developed. When the plants were six-week-old, the infected plant was placed in the center of the arena and healthy aphids were placed on it. In 24 cages, 10 individuals of *M. persicae* were placed on the middle plant in each arena, while in the remaining 24 cages, a combination of both 10 individuals of *M. persicae* and 11 individuals of *A. fabae* was used. After introducing the aphids, the cages were divided into three groups of 16 cages each, containing 8 cages infested with *M. persicae* and the remaining 8 cages infested with the combination of *M. persicae* and *A. fabae* (Figure 3). The aphids, in the first group, were kept on the plants for 4 days, in the second group for 8 days, and in the third group for 12 days, after which the aphids were killed by a spray with a mixture of 0.14 kg/ha of flonicamid (Teppeki; ISK Biosciences) and 1L/ha of Actirob B. Three weeks after the application of the aphicide mixture, the two marked leaves from each plant were collected and placed in the extraction bags (Extraction bag Universal 12x15 cm, Bioreba, Switzerland). These bags were stored in the refrigerator for two days at 4°C until ELISA was performed to verify the occurrence of transmission, following the same procedure as previously described.

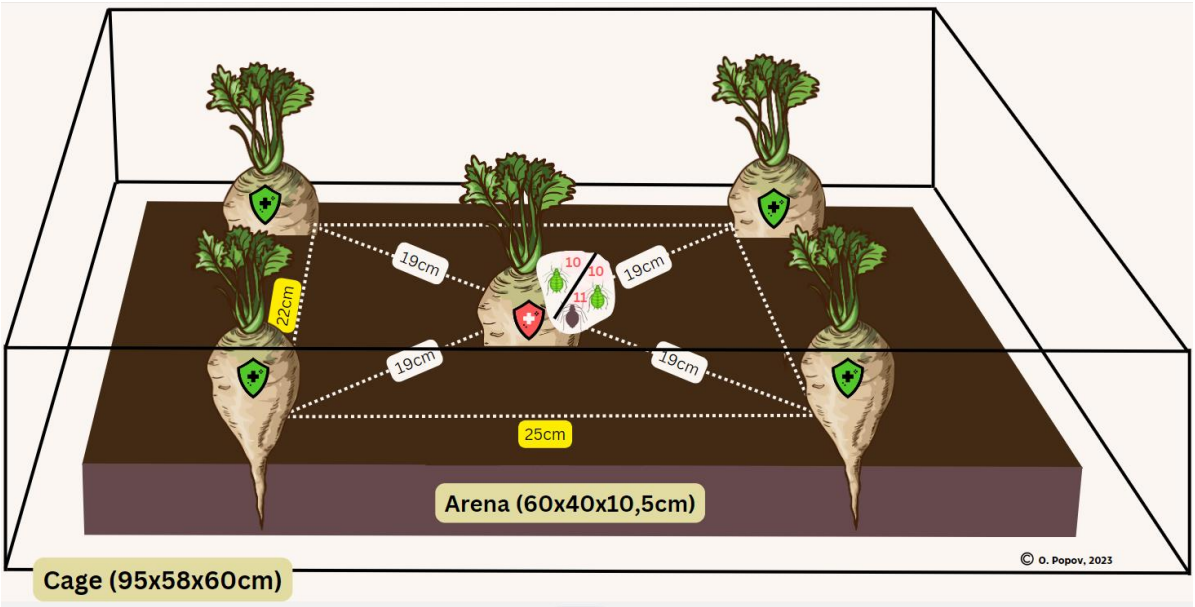

Figure 2. Arena set up used in the greenhouse. The breeding cage with the arena is containing five six-week-old plants: four healthy plants (green symbols) and one infected plant (red symbol). The infected plant was infested with 10 healthy apterous *M. persicae* or with a combination of 10 healthy apterous *M. persicae* and 11 healthy apterous *A. fabae.* In total 48 cages i.e. arenas were used during the greenhouse experiments, within 24 cages i.e. arenas of each modality.

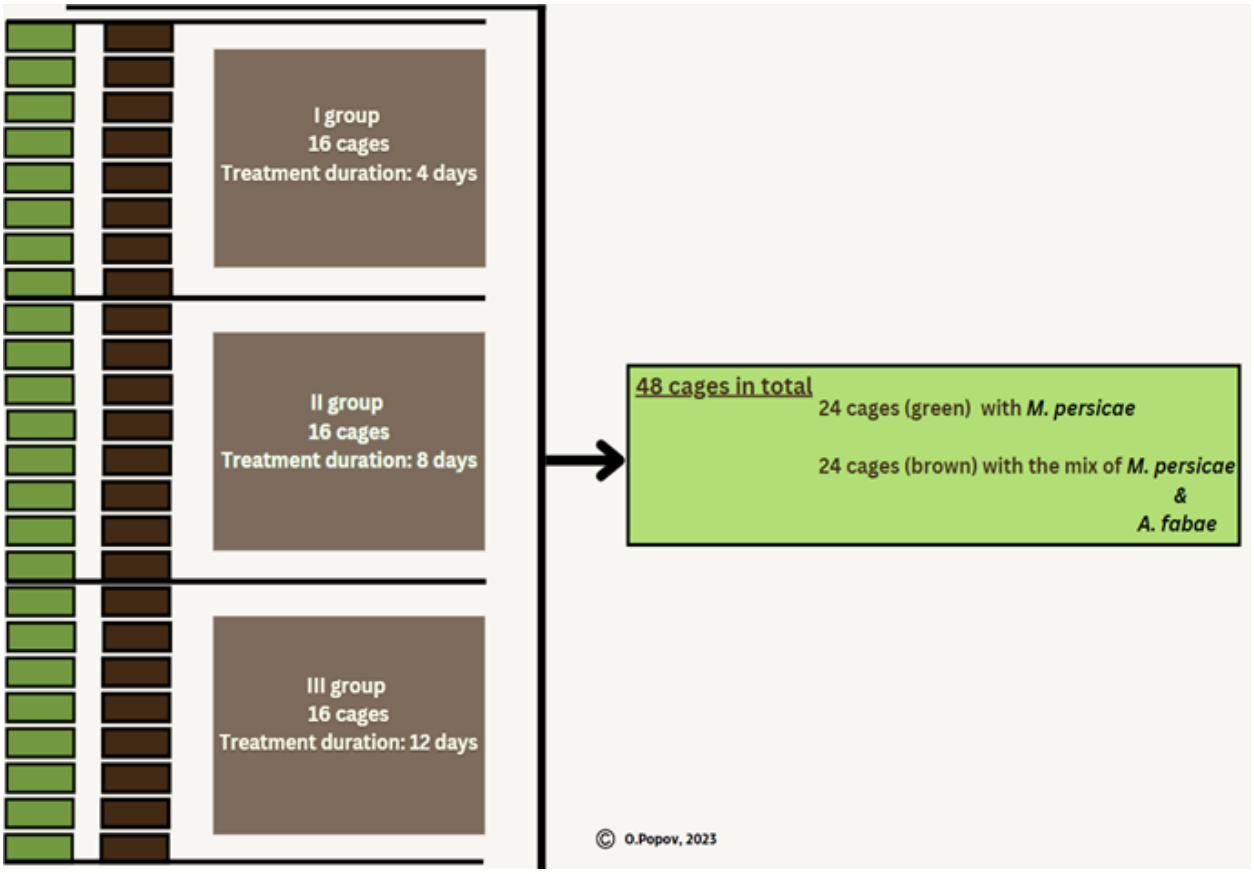

Figure 3. The greenhouse set up. In 24 cages, 10 healthy *M. persicae* were put on the center infected plant in each arena, while the remaining 24 cages were infested by a mix of 10 healthy *M. persicae* and 11 healthy *A. fabae*. After the aphids were

introduced, the cages were separated into three groups of 16 cages each, with 8 cages infested with *M. persicae* and the remaining 8 cages infested with both *M. persicae* and *A. fabae*. The aphids in the first group were kept on the plants for 4 days, in the second group for 8 days, and in the third group the aphids were kept for 12 days.

3.4. Field experiments

3.4.1. Aphid collection

The field experiment was conducted in May 2023 in three different sugar beet fields located in Saint-Just-en-Chaussée, Cires-lès-Mello, and Fontaine Lavaganne (Hauts-de-France region, France). The objective of this experiment was to collect winged aphids from both vector species; specifically, to screen vector populations in order to determine the prevalence of viruliferous aphids at the beginning of the sugar beet growing season. Since it is known that winged aphids are the ones that introduce the infection in the fields on individual plants located in distant patches. Winged aphids of *M. persicae* and *A. fabae* were collected from the field with the small painting brush from the leaves of six-week-old sugar beet plants. They were transferred to vials filled with 15ml of 95% ethanol, and stored at -80°C until the PCR was performed. Each vial contained five winged aphids of a single species, all collected from different plants chosen randomly in each experimental field. In total, 165 aphids were collected, with 55 aphids obtained from each field. While both vector species were sought, only winged *A. fabae* was found in all three experimental fields.

3.3.2. Molecular analyses

Aphids were taken from alcohol and transferred in 2ml Eppendorf tubes. Total RNA was extracted from the whole *A. fabae* (5 aphids per sample) as described by Mulot et al. (2016). Then the quantification at 260 nm of extracted viral RNA was performed (Nanodrop 2000; Thermo Fischer Scientific). The viral RNA was converted into cDNA using the reverse primer 1192* (Table 1) and the M-MLV reverse transcriptase kit (Promega, United States). The forward primer 1194* and the reverse primer 1192* (Table 1) were used to amplify cDNA by Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) using the same set-up as described by Mulot et al. (2016). Final visualization of the qRT-PCR products was done by gel electrophoresis using BenchTop 100bp DNA Ladder (Promega, United States).

Primers	Sequences
Forward 1194*	GTC TAC TTT CAT CAA AGC TTC G
Reverse 1192*	GGC AGA AAC ACC GCA ATT TG

Table 1. BYV forward and reverse primers

3.5. Statistical analyses

To test whether aphid species (*M. persicae*/*A. fabae*), morphs (winged/apterous), and aphid density (covariate) had an effect on plant infection probability, a generalized linear mixed-effect model (GLMM) with a binomial distribution (infected/healthy) and a logit link function was fitted to the data. Two-way interactions between the three factors were tested, and the round number (1, 2, or 3) was added as a random factor in the model. To test whether *M. persicae* alone or in combination with *A. fabae* (factor with two levels) and the time spent in the arena (covariate) had an effect on plant infection probability, a binomial generalized linear model (GLM) with a logit link function was fitted to the data, and the two-way interaction between the

two factors was included. The significance of each term in the models was tested using a type II ANOVA (package car, Fox et al. 2019). Post-hoc tests were performed using pairwise comparisons of estimated marginal means (package emmeans, Lenth et al., 2018).

Note that the results of the field experiment are not yet complete, at the time of writing, and will therefore not be presented.

4. Results

4.1. Laboratory experiments

Infection probability was 1.9 times higher with *M. persicae* than with *A. fabae*, all morphs and densities confounded (LR $\chi^2 = 8.6$, df = 1, $p < 0.01$). However, this difference was dependent on the morph (interaction effect: LR $\chi^2 = 6.6$, df = 1, $p < 0.05$); the difference in infection probability between species was true for winged morphs only ($z = -3.69$, $p < 0.001$), but not for apterous morphs ($z = -1.54$, df = 1, $p = 0.25$). This also means that winged morphs of *M. persicae* ($z = -3.69$, $p < 0.001$) but not *A. fabae* ($z = 0.78$, $p = 0.44$) had higher virus transmission probabilities than apterous morphs. For winged aphids, there was a difference between *M. persicae* and *A. fabae* at a density of 3 ($z = -3.4$, $p < 0.001$) and 8 ($z = -3.6$, $p < 0.001$), and marginally at 13 ($z = -1.9$, $p = 0.06$). There was no difference between *M. persicae* and *A. fabae* for apterous aphids, marginally at a density of 3 ($z = -2.0$, $p = 0.05$) and at 8 ($z = -1.2$, $p = 0.25$) and 13 ($z = 1.4$, $p = 0.16$). For both species and morphs, higher aphid density increased the infection probability by the BYV (LR $\chi^2 = 16.6$, df = 1, $p < 0.001$, correlation of 23%) (Figures 4a and 4b).

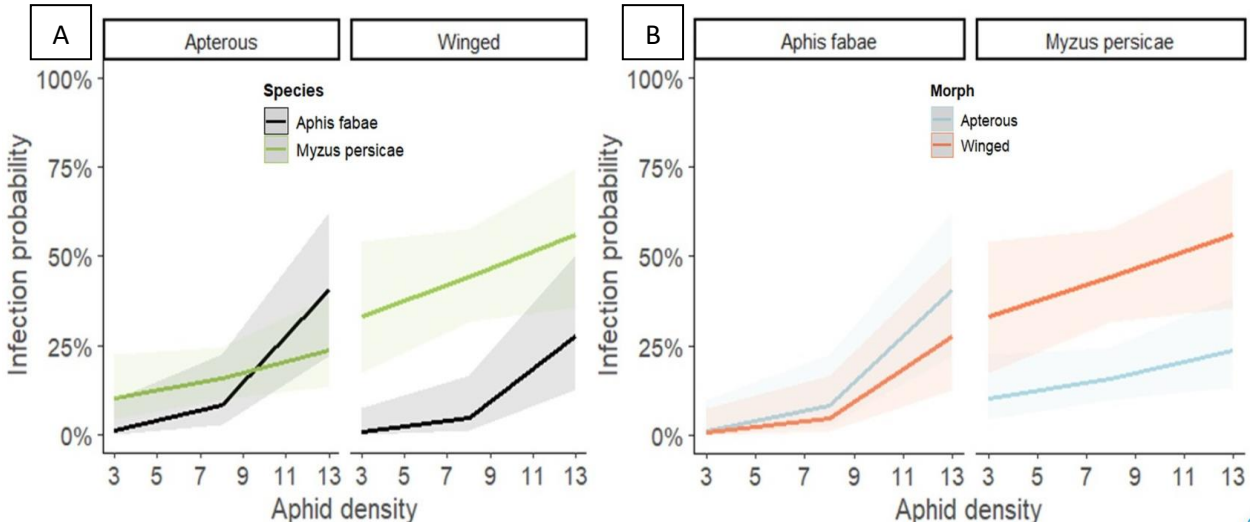

Figure 4. Effect of aphid density on BYV transmission. (A) Infection probability between morphs of aphids (winged and apterous), based on aphid density, for both vector species (*A. fabae* and *M. persicae*). (B) Infection probability between aphid species (*A. fabae* and *M. persicae*), based on aphid density, for both vector species. N = 6 per treatment (density, morph, species). Line represent estimated values from the model and the envelope represents 95% confidence intervals

4.2. Greenhouse experiments

For all treatments, time spent by the aphids in the arena increased the infection probability (LR $\chi^2 = 43.6$, df = 1, $p < 0.001$, correlation of 28%). There was a higher infection probability when both *A. fabae* and *M. persicae* were present on the plant, compared to *M. persicae* alone

(LR χ^2 = 10.1, df = 1, p < 0.01), although this effect depended on the time spent in the arena (interaction effect: LR χ^2 = 13.0, df = 1, p < 0.001); the difference between treatments was significant at 4 days (z = -4.6, p < 0.001), 8 days (z = -0.58, p < 0.01) but not 12 days (z = 0.24, p = 0.41). In addition, the dynamics of virus infection (i.e., the rate of increase by a unit of time) was higher for *M. persicae* alone (correlation of 42%) than for both species combined (correlation of 14%) (z = 3.55, p < 0.001) (Figure 5A and Figure 5B).

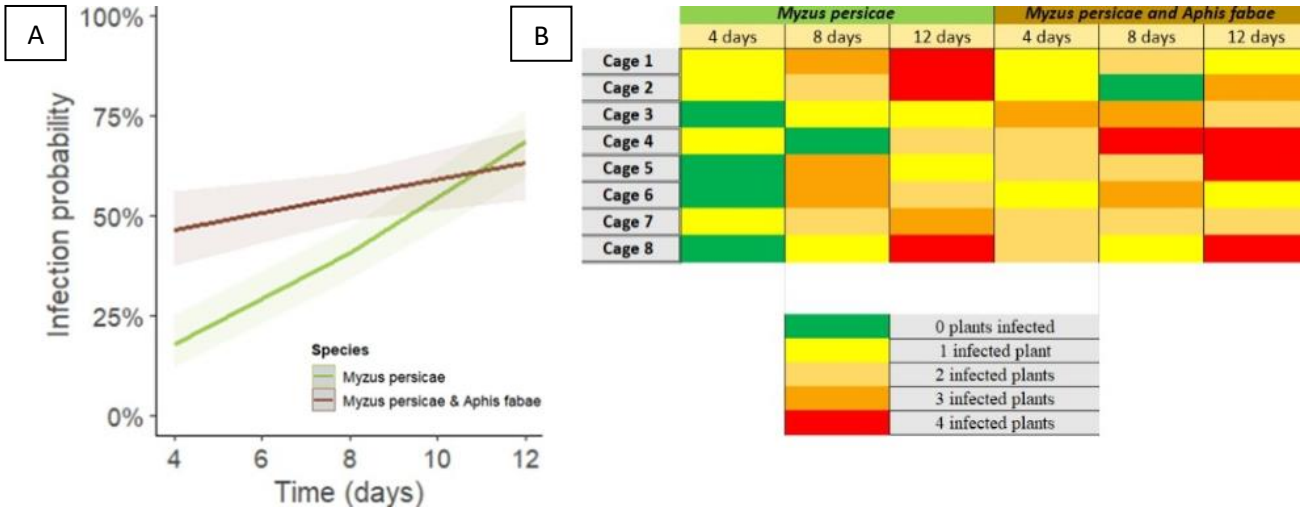

Figure 5. Impact of vector species coexistence. (*Myzus persicae* and *Aphis fabae*) on virus transmission. (A) The Difference in infection probability between the vector species *M. persicae* and its combination with *A. fabae* based on the duration of infestation. The line represents estimated values from the model and the envelope represents 95% confidence intervals. (B) The number of infected plants per cage, based on the duration of the vectors' presence on the plants and the treatment. Each cage contained four plants and the infection state was measured, four infected plants (red), three infected plants (bright orange), two infected plants (light orange), one infected plant (yellow), and zero infected plants (green)

5. Discussion

5.1. Laboratory experiments

Based on previous studies, we expected that virus transmission would be more efficient with winged morph (Blua and Perring, 1992; Huijuan et al., 2023), of *M. persicae* (Jiménez et al., 2018; Symmes et al., 2008; Limburg et al., 1997; Watson, 1946; Heathcote and Cockbain, 1964), implying that even small populations densities would be sufficient for BYV transmission. In our study, we observed that winged *M. persicae* exhibited the highest transmission efficiency at low population densities. With just three viruliferous individuals present on a single sugar beet plant, there was a 25% probability of infection. However, no significant difference was observed between the winged and apterous morphs of *A. fabae* in terms of BYV transmission. Likewise, no disparity in the transmission probability of BYV was observed between the apterous morphs of *M. persicae* and *A. fabae*.

Our main finding that winged *M. persicae* was the most efficient vector for BYV was supported by Huijuan et al. (2023). Their study demonstrated a higher vector efficiency of winged *M. persicae* compared to apterous morphs, attributing this disparity to the difference in salivary protein expression between the two morphs. Specifically, winged *M. persicae* enhance vesicle trafficking inside the plant, which in turn aids the translocation of the virus from cell to cell (Huijuan et al., 2023). On another plant-pathogen system, Chatzivassiliou et al. (2016)

monitored the transmission of the Watermelon mosaic virus (which is also an NTP virus) by different aphids in Greece. Their observations supported our findings, as they also identified winged *M. persicae* as the most efficient vector for transmission,

Secondly, our next important result was that winged *A. fabae* was not as efficient a vector as winged *M. persicae* and that there were no observed differences in infection probability between their apterous morphs nor between apterous and winged *A. fabae*. These results are consistent with the study of Cockbain et al. (1963), which showed that winged *A. fabae* were often less efficient vectors than *M. persicae* of pea mosaic virus and sugar-beet mosaic virus. Furthermore, they showed that winged mature *A. fabae* seldomly transmitted these viruses unless they had either flown (tethered) or fasted for several hours before feeding on infected plants. On the other hand, flight-mature *M. persicae* transmitted the virus before flying or fasting, though more frequently afterward (Cockbain et al., 1963). Moreover, it was also shown that apterous forms of *A. fabae* were equally effective in transmitting BYV compared to winged *A. fabae* (Limburg et el., 1997; Heathcote and Cockbain, 1964).

The difference in the transmission probability between winged *M. persicae* and *A. fabae* shown in our study could be explained by two factors: i) differences in their feeding behavior and ii) indirect manipulation of the vectors by the virus.

i) Differences in feeding behavior between the two species were shown in the study of Paprocka et al. (2023), where the probing capacity of apterous *M. persicae* and *A. fabae* was studied on three different species of grapevines. It was shown that there were behavioral differences between *M. persicae* and *A. fabae* which favor *M. persiace* as the vector. Furthermore, EPG analyses have shown that among two clones of winged *M. persicae,* the clone with a higher number of shorter punctures within a given timeframe exhibited a better transmission rate (Symmes et al., 2008). This finding underscores that although *M. persicae* is commonly regarded as the primary vector, the viral transmission rate of this species is greatly influenced by its feeding behavior (Symmes et al., 2008).

ii) Blua and Perring (1992) studied plants infected with zucchini yellow mosaic virus (ZYMV), a potyvirus that is transmitted exclusively in a stylet-borne manner by several species of aphids. They showed that the production of winged aphids was the highest on infected plants suggesting that characteristics specific to the early phases of viral infection encouraged wing development through the changes in plant physiology. Then, if the virus is spread efficiently by the activities of winged, its dispersal would be greater (Blua and Perring,1992). Additionally, it was found by Macias and Mink (1969) that twice as many *M. persicae* winged went directly on yellows-virus-infected plants 24h after releasing in an enclosed area than on healthy plants or non-infected plants with BYV. Such virus manipulation of vectors is quite widespread in many pathosystems (Mauck et al., 2018).

From our scope of knowledge, there is a lack of studies that compared viral transmission rates and feeding behavior between winged and apterous morphs of *M. persicae* and *A. fabae*. We were able to find only one study with that topic done by Heathcote and Cockbain, (1964), in which they compared transmission rates of winged and apterous morphs of both *M. persicae* and *A. fabae*. Their results indicated that winged and apterous *M. persicae* were better vectors than those of *A. fabae*, but the difference in transmission between winged and apterous for the same species was not significant. Other studies focused only on the combination of apterous *M.*

persicae and winged and apterous *A. fabae* (Limburg et al., 1997) or on the combination between winged and apterous *M. persicae* and apterous *A. fabae* (Jiménez et al., 2018), and based on feeding behavior (probing capacity), revealed that the best vector with high transmission rates, was apterous *M. persicae*. It is important to note that these studies compared different morphs, and used various aphid clones, different plant varieties, and different viral strains. Therefore, differences in findings were expected when comparing them to our study.

Thus, it can be suggested that aphid clones of *M. persicae* and *A. fabae* used in our study might have differential transmission ability, compared to other clones cited in literature. Such a difference between aphid clones could be the result of independent co-evolution of aphid and virus populations under different environmental conditions and selection pressures, revealing genetic diversity in both partners, as has been discussed for other Luteoviridae members, such as species of the Barley yellow dwarf complex (Gray 1999). Moreover, differences in our results are probably not due to an insufficient virus uptake from BYV-infected plants, since the virus content of plants was verified by ELISA test, and aphids were acquiring the virus for 24h as in all mentioned studies. Thus the lack of difference between the two species could therefore be explained by molecular aspects where different helper proteins (HP) are present to link the virus to the aphid stylet acrostyle (Gildow, 1999; Brault et al., 2007). Finally, differences in aphid probing might affect our results particularly due to the variations in leaf morphology between plant species or even varieties (Nalam et al., 2021).

5.2. Green house experiments

The greenhouse experiments aimed to provide insights into two relationships: a) the interaction between vectors of the same virus present simultaneously on the same plant, and b) the interaction between *M. persicae* and *A. fabae*, both resident aphids on the same plant. The objective was to examine potential changes in virus transmission when apterous forms of these aphid species coexisted on the same patch, compared to when only the main vector species was present. The investigation was conducted at various time frames.

It was expected that BYV transmission would be enhanced when vector species coexisted in the same area, based on the facilitation effects of aphid colonization observed in other pathosystems (Shih et al., 2023; Varenhorst et al., 2015; ten Broeke et al., 2017), and potentially due to a significant level of cooperation between the vectors in terms of viral transmission (Petrović-Obradović et al., 2023; Kershaw, 1965)

Our hypothesis was confirmed as we observed a significant difference in virus transmission during the early stage of infection between trials with only apterous *M. persicae* individuals and those with a combination of apterous *M. persicae* and *A. fabae*. The infection probability doubled, in the early stage of aphid infestation, when both vector species were present. However, as the vector populations, including both *M. persicae* and the vector combination, remained on the plants for an extended period of time, the infection probability continued to increase. Notably, when the vectors were present for twelve days, the infection probability was identical in both treatments. This result can be explained by examining the interspecific relationship between the two aphid species. Coexistence of both aphid species on the same plant triggers a competitive relationship as they compete for limited nutrient resources (Muller and Godfray, 1997); forcing the aphids to exhibit heightened mobility in their quest for alternative food sources, including neighboring plants. Consequently, the presence of the combined aphid species may have led to an escalated rate of virus transmission (Crowder et al.,

2019). Moreover, an important finding from an EPG study conducted by ten Broeke et al. (2017) demonstrates that aphids, when feeding in a group, display an increased frequency of probing behaviors compared to their solitary counterparts. This elevated probing activity is likely attributable to the disturbance caused by the presence of other aphids within the group. Importantly, this increased probing behavior could potentially promote a positive influence on the virus transmission rate (ten Broeke et al., 2017).

Additional explanation of our results can be provided also through recent studies, which revealed that the relationship between aphids is more complex than simple competition. The coexistence of different aphid species can lead to various effects due to the production of different allelochemicals (Nishida, 2014). For instance, when aphids were present on lettuce varieties resistant to viruses transmitted by aphids, the resistance of the variety was not consistently effective in different aphid combinations (ten Broeke et al., 2017). This variation in resistance can be attributed to the production of protein effectors by aphids which have the potential to interact with plant signaling pathways, suppressing plant defense mechanisms, modulating plant processes beneficial for aphid colonization, and even triggering plant responses through the recognition of effectors by receptors involved in plant resistance (Carolan et al., 2011; Hogenhout and Bos, 2011). In recent years, several protein effectors have been identified in various aphid species (Carolan et al., 2011; Harmel et al., 2008; Nicholson et al., 2012; Vandermoten et al., 2014). Some of these effectors enhance plant susceptibility to aphids, while others induce plant defenses. For example, a protein effector, COO2 produced by *M. persicae* was shown to enhance its fecundity, providing an advantage for aphid colonization. However, the same effector had no effect on other species, such as *Acyrthosiphon pisum* (Pitino and Hogenhout, 2013). Thus the effect of this effector, for instance, would be interestingly studied on *A. fabae*.

One potential model that we can propose, for the relationship between *M. persicae* and *A. fabae* involves the role of winged morphs of *M. persicae* in initiating infections on individual plants in the field, while the concomitant presence of apterous *A. fabae* facilitates the spread of the virus between plants, leading to the formation of yellow patches. Several studies support this hypothesis, as they have demonstrated that the spread of BYV in the field is not primarily dependent on winged immigrant aphids, as their numbers are not significant at the beginning of the season and infections are not observed throughout the field (Ribbands, 1963; Kershaw, 1965). In contrast, in certain seasons, the virus spreads more rapidly when only apterous morphs of the vectors are present (Ribbands, 1963; Kershaw, 1965). Moreover, it has been observed that apterous *A. fabae* tend to be distributed in patches that correspond to subsequent yellow virus patches, indicating their key involvement in the spread (Ribbands, 1963; Petrović-Obradović et al., 2023).

This hypothesis offers further support for our laboratory experiment results, which indicated no difference in the infection probability between apterous *M. persicae* and *A. fabae*. If we consider the hypothesis, it suggests that apterous *A. fabae* plays a role in local infection spread in the field, implying its capacity as a vector is not lower than that of apterous *M. persicae*. However, further research is needed to fully investigate and validate this hypothesis.

6. Conclusion

Based on our research, we have gained some new insights into the transmission probability of different morphs of the two main vectors of BYV. We showed that winged morphs of *M. persicae* were the main responsible for BYV infection. However, we also demonstrated that *A. fabae*, even if not as efficient as a vector, is increasing transmission potential when present on the same plants as *M. persicae*. These results have great importance in terms of aphid management strategies, and we suggest increased attention should be paid to coinfection by the two BYV vectors, and to *A. fabae*, which responsibility for virus infection has long been overlooked. However, since there is limited data about the relationship between both species, particularly in field conditions, further studies are needed to achieve a better mechanistic understanding.

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