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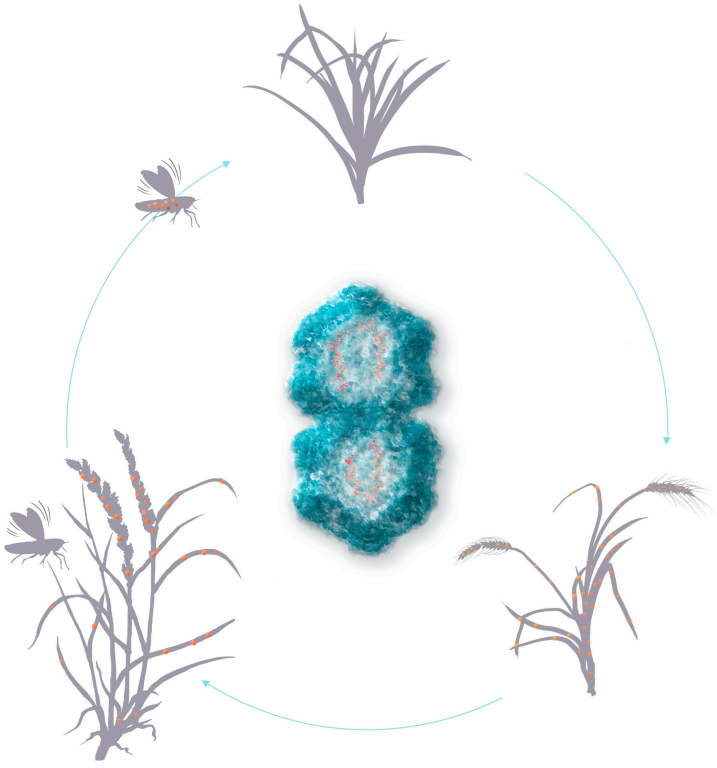
Elham Yazdkhasti



Epidemiology of wheat dwarf virus

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Cover: Transmission of WDV by leafhoppers to wheat. Geminivirus geminate particle. (Illustration: Elham Yazdkhasti)

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Abstract

In Sweden and many other parts of Europe, wheat dwarf is one of the most important diseases induced by a plant-infecting virus. Wheat dwarf disease is caused by wheat dwarf virus (WDV), transmitted by the leafhopper *Psammotettix alienus.* Our study provides the evidence that WDV, barley yellow dwarf viruses (BYDVs) and cereal yellow dwarf virus (CYDV) infect ryegrass growing in or around winter wheat fields. Phylogenetic analysis showed that a ryegrass isolate of WDV was a typical WDV-E isolate closely related to isolates infecting wheat and the isolate of BYDV-PAV grouped in a clade together with other BYDV-PAV isolates, suggesting that the same virus genotypes infecting the ryegrass can be found in wheat as well as in other host plants and the insect vector. Inoculation experiments confirmed that various genotypes of annual ryegrass can be infected with WDV to a very low titre. The results showed that leafhoppers also can acquire a low titre of WDV from infected ryegrass plants, and efficiently transmit it to wheat. Moreover, domesticated bread wheat (*Triticum aestivum*) and its wild relatives responded differently to WDV infection. *Aegilops tauschii*, *Triticum urartu* and the bread wheat cultivar Tarso exposed to the viruliferous leafhoppers had different virus content in roots and in each leaf at different time points. Between the accessions, *Ae. tauschii* stood out as the WDV accumulation and symptom development started later compared to *T. urartu* and bread wheat. As a result, *Ae. tauschii* followed a normal growth pattern and developed milder symptoms. Infected plants of *T. urartu* and wheat could not develop normally as compared to non-infected plants. In addition, infected plants of *Ae. tauschii* showed less reduction in leaf fresh weight over time compared to the other accessions. This finding introduces *Ae. tauschii* as a candidate for further studies on WDV-resistance. PCR test results for presence of WDV in leafhoppers collected from different counties in Sweden, together with known disease incidence and records of weather conditions, showed that disease occurrence and severity in winter wheat fields varied considerably between years, regions, and locations. The results suggest a strong correlation between the number of *P. alienus* leafhoppers

and disease incidence in Uppsala, Stockholm and Västmanland. For the autumn, the number of leafhoppers per field was found to be positively linked to weekly average temperature as well as average weekly maximum and minimum temperature. In conclusion, the results of this thesis has uncovered the elements influencing the epidemiology of WDV which is important for control of the disease.

Keywords: Epidemiology, Geminivirus, *Mastrevirus*, *Psammottetix alienus,* ryegrass, wheat, *Wheat dwarf virus*.

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"Seek the sound that never ceases. Seek the sun that never sets..."

Rumi

To my dearest father and mother….

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Elham Yazdkhasti in the text:

I. Yazdkhasti, E., Hopkins, R. J. and Kvarnheden, A. (2021) Reservoirs of plant virus disease: Occurrence of wheat dwarf virus and barley/cereal yellow dwarf viruses in Sweden. Plant Pathology, 70(7), 1552-1561. 10.1111/ppa.13414.

II. Nygren, J., Yazdkhasti, E., Lundquist, P. O., Kvarnheden, A., Westerbergh, A. (2022) Plants of wheat D genome donor *Aegilops tauschii* show delayed infection of *Wheat dwarf virus* and mild symptoms compared to A genome donor *Triticum urartu* during early growth. (submitted/manuscript)

III. Yazdkhasti E., Eriksson I., Ramsell J., Beuch U., Kalyandurg P. B., Nasirzadeh L., Pettersson J., Poimenopoulou E., Santos L., Sigalla J., Hopkins R., Kvarnheden A. (2022) Parameters influencing wheat dwarf disease incidence and leafhoppers abundance in Sweden. (Manuscript)

The contribution of Elham Yazdkhasti to the papers included in this thesis was as follows:

I. Highly involved in planning the project, carried out most of the experimental work, analysed the data and wrote the manuscript.

II. Participated in planning the experiments and carried out part of the experimental work and commented the manuscript.

III. Highly involved in planning the project, carried out part of the experimental work, analysed the data and wrote the manuscript.

List of tables

List of Figures

Abbreviations

ACP	Antigen-coated-plate
BYDV	Barley yellow dwarf virus
CP	Coat protein
cv	Cultivar
CYDV	Cereal yellow dwarf virus
DAS	Double-antibody sandwich
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
IC-RT-PCR	Immunocapture-reverse transcription-polymerase chain reaction
ICTV	International Committee of Taxonomy of Viruses
IPM	Integrated pest management
kb	kilobases
LAMP	Loop-mediated isothermal amplification
LIR	Long intergenic region
MP	Movement protein
MSV	Maize streak virus
NGS	Next-generation sequencing
nt	Nucleotide
ORF	Open reading frame
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RCA	Rolling circle amplification
Rep	Replication-associated protein
RepA	Replication-associated protein A
RNA	Ribonucleic acid
SIR	Short intergenic region
ssDNA	Single-stranded DNA

WDV	Wheat dwarf virus

1. Introduction

1.1 Wheat dwarf virus

Wheat dwarf disease mainly appears in wheat and barley. It is caused by infection with wheat dwarf virus (WDV) and may have devastating effects on winter cereals. In a situation with high incidence of wheat dwarf disease, huge yield losses can be expected. The virus has a wide geographic distribution. In Europe, WDV has been reported in many countries, including the Czech Republic and Bulgaria (Stephanov and Dimov, 1981; Tóbiás et al., 2009; Kundu et al., 2009), Hungary (Bisztray et al., 1989), France (Bendahmane et al., 1995), Romania (Jilaveanu and Vacke, 1995), Germany (Huth, 2000), Poland (Jeżewska, 2001), Sweden (Lindsten and Lindsten, 1999), Finland (Lemmetty and Huusela-Veistola, 2005), Spain (Achon et al., 2006), Ukraine (Tobias et al., 2011), Austria (Schubert et al., 2014), UK (Schubert et al., 2014) and Slovenia (Marn and Plesko, 2017). Besides Europe, WDV has been identified in Asian countries including Turkey (Köklü et al., 2007), China (Xie et al., 2007), Iran (Behjatnia et al., 2011) and Syria (Ekzayez et al., 2011). In addition, WDV has been found in two African countries: Tunisia (Najar et al., 2000) and Zambia (Kapooria and Ndunguru, 2004). The first symptoms similar to wheat dwarf disease were described in 1863 in a region, which is now part of Poland (Jungner, 1906). In Sweden, symptoms of wheat dwarf disease were observed in the early 1900s (Lindsten and Lindsten, 1999). However, it was not until 1961 that the causal agent of wheat dwarf disease was identified as WDV by Vacke (1961)

from the former Czechoslovak Socialist Republic. Since then, there were very few reports of wheat dwarf for three decades, until it reappeared in the 1990s in Czech Republic and Sweden (Lindsten and Lindsten, 1999).

In Sweden, the first symptomatic plants affected by wheat dwarf were spotted as early as 1902 although the cause of the symptoms was unknown. Since then, the country has been through several outbreaks (Lindsten and Lindsten, 1999). In Swedish winter wheat the first experimental identification of WDV was in 1969 (Lindsten et al., 1970). Since then, there were no reports of the disease in Sweden until it reappeared in 1997 (Lindsten and Lindsten, 1999). Since 1997, the disease has been reported almost every year in large plains and open fields in central and southern Sweden (Figure 1), but the highest incidence of the disease was recorded in 2009 and 2017 (data from Swedish Board of Agriculture).

Figure 1. Map of Sweden showing the counties where wheat dwarf virus has been reported according to data from the Swedish Board of Agriculture.

1.1.1 Symptoms

Appearance of the symptoms on virus-infected plants can be due to various reasons including a side-effect of the infection, such as effects of the expression of viral suppressors of RNA silencing. Symptoms can be also the effect of plant defence (necrosis) when the plant over-reacts to the infection (Jones, 2021). Infections with WDV, during the early growth phase in cereal plants, especially in wheat and barley, result in symptoms that are caused by inhibition of normal plant growth. Symptom intensity depends on the

vegetative phase of plant growth and development at the time of infection (Nygren et al., 2015), because plants are more susceptible to the infection during their early growth phase (Lindblad and Sigvald, 2004). Infected plants are often stunted and have chlorotic, reddish or discoloured leaves (Figure 2). Additional symptoms include head stunting or no heading (Vacke, 1972). In WDV-infected oat plants, reddish discolouration can be observed. Grasses infected by WDV are often symptomless, but WDV infected ryegrass plants can show yellowing symptom (Mehner et al., 2003).

Figure 2. Symptoms of wheat dwarf disease. Photo by Kvarnheden A.

1.1.2 Host, Vector and Transmission

As a grass generalist virus, WDV has a wide range of hosts among economically important crops such as wheat, barley, rye, oats and triticale. WDV infection has been also detected in noncultivated grasses as well as cultivated grasses, including *Lolium* spp. (Vacke, 1972; Schubert et al., 2007; Ramsell et al., 2008). Wild wheat relatives *Triticum* spp. and *Aegilops* spp. have been found to be hosts for WDV in inoculation experiments (Nygren et al., 2015). Thus, WDV-infected grasses are often a potential reservoir of the virus (Ramsell et al., 2008).

WDV transmission to host plants occurs via its leafhopper vectors *Psammotettix alienus* and *P. provincialis* (family Cicadellidae). *P. alienus* transmits the virus in a persistent, circulative and non-propagative manner (Lindsten et al., 1980; Lindsten and Vacke, 1991; Lindblad and Sigvald 2004). The insect vector (*P. alienus*) is mostly attracted to cereal fields and grasslands (Lindblad and Arenö, 2002).

After feeding on the infected plants, a short latency period is often required for the leafhopper vector to be able to transmit a mastrevirus (Storey, 1928; Wang et al., 2014a). WDV may go through two pathways inside the leafhopper body (Wang et al., 2014a). The first path is more similar to persistent virus transmission of other insects. Here the virus reaches the salivary gland via movement through the anterior, middle gut and the hemocoel of the insect vector (Hogenhout et al., 2008). In the second pathway, five minutes after the virus enters the insect's esophagus, it moves to the lumen of the filter chamber and next to midgut lumen. Now, the virus can be found also in the filter chamber sheath. Ten minutes after the first feed, the virus enters the cells of the insect midgut. After 20 minutes, the viral particles have stablished themselves in the whole filter chamber, midgut, hemocoel and salivary gland. Four hours after the first feeding, there will be no trace of the virus in the filter chamber and its sheath, but the virus has accumulated in the midgut, hemocoel, salivary glands and stays there for the rest of the leafhopper's life without being replicated. At this point, the insect may inject the virus to another plant while feeding on the phloem (Wang et al., 2014a). This indicates that viruliferous leafhoppers are capable of infecting healthy plants shortly after feeding on an infected plant. The same event occurs for a more studied mastrevirus, maize streak virus (MSV), and its vector, *Cicadulina mbila* (Ammar et al., 2009). Monitoring the distribution and accumulation of MSV by qPCR has shown that viral particles accumulate mostly in the leafhoppers gut and salivary glands (Lett et al., 2001). At the beginning of autumn, adult leafhoppers that may have fed on WDV-infected plants (volunteer wheat plants and grasses) already during the summer, transmit the virus to newly sown plants. By migration in autumn, leafhoppers can spread the virus widely leading to primary infection (Lindblad and Sigvald, 2004). The area under virus infection can be quite extensive during this period as the leafhoppers can fly and spread the virus. The primary infection is a requirement for secondary infection in spring. In

the secondary infection, viruliferous nymphs move from infected plants to healthy plants and spread the virus infection in patches in the field. As a result, secondary infection in the spring can be more widespread than primary infection in the autumn. In autumn, female leafhoppers lay egg on the leaves of plants in cereal fields. Leafhopper activity decreases as the temperature, at the end of autumn, drops below the favourable temperature (<10℃) for leafhoppers (Lindblad and Arenö, 2002). The eggs overwinter in the fields and nymphs hatch in the spring. Leafhopper nymphs go through five developmental stages until they mature, which can take up to 32 days (Manurung et al., 2005). At the beginning of the summer, adult leafhoppers can be found in the fields. Autumn generation of leafhoppers are the progeny of these leafhoppers. The leafhopper vector often has two to three generations per year depending on the geographical location (Schiemenz, 1969).

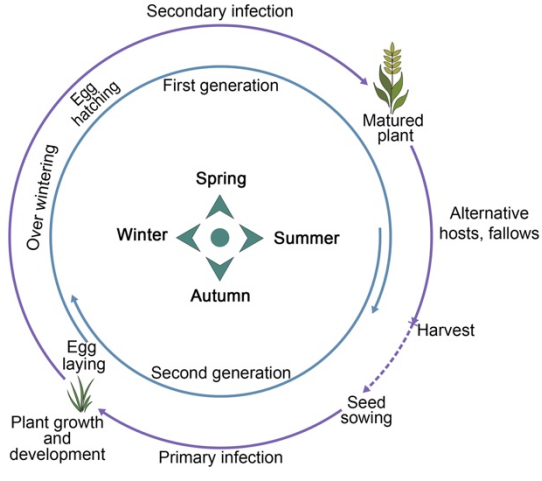

Figure 3. Schematic life cycle of winter wheat and *Psammotettix alienus*. The purple circle shows the developmental stages of winter wheat. The life cycle of *P. alienus* is presented in the blue circle for two generations. Leafhoppers lay eggs at the end of autumn. The eggs overwinter on the leaves of cereals and hatch in the spring. During the summer period, adults of this generation will feed on alternative hosts or volunteer wheat plants in the fallows. If these plants are infected by WDV, leafhoppers will pick up the virus and spread it to the newly sown winter wheat and cause the primary infection. Subsequently, nymphs of the next generation will feed on the infected winter wheat in the spring and cause the secondary infection.

1.2 Infection and Defence Mechanisms

Phloem-feeding insects inject the virus into the plant through the epidermis. Cell-to-cell movement starts from the sieve element in the phloem. Subsequently, the virus replicates its genome with the help of the DNA replication system of the host cell and to do that it manipulates the cell and gene expression within mesophyll cells and in cells of the phloem. However, many geminiviruses do not infect mesophyll cells (Bowdoin et al., 2013; Hipper et al., 2013; Blystad et al., 2020). Long-distance movement continues through the phloem until the virus reaches systemic tissues. Successful infection occurs when the virus can overcome the barriers between cells to build up a systemic infection in the whole plant (Hipper et al., 2013). The study by Liu et al. (2020b) indicated that WDV interference of host plant processes during infection affects metabolism and signalling of plant hormones, which results in symptom development. Any disruption caused by plant defence mechanisms during infection can stop the infection process (García and Pallás, 2015). RNA silencing strategies (such as DNA methylation) and resistance (*R*) gene-mediated, have been implemented by plants as antiviral defence mechanisms (Mandadi and Scholthof, 2013), nevertheless RNA silencing can be overcome by WDV (Wang et al., 2014b). In addition, mutations in genes which are required for viruses to build up infection, may result in resistance against them (Liu et al., 2017).

1.2.1 Defences

Wheat cultivars with partial resistance to WDV implement defence mechanisms to interfere with virus replication or movement (Benkovics et al., 2010). However, how host plants react to WDV infection varies depending on the extent of their susceptibility to the infection. According to symptoms and virus titre, studies on response to WDV infection in bread wheat and its close relatives have categorized the plants as resistant, tolerant or susceptible (Nygren et al., 2015). Highly susceptible plant may die as a result of extensive infection caused by the virus, while tolerant plants can survive the infection and continue to live. Tolerant genotypes show no or reduced symptoms since there might be no response to the infection among them or that a few genes react to the virus infection. Interestingly, recovery

from the virus infection can occur for some geminiviruses when RNA silencing efficiently suppresses the virus accumulation (Raja et al., 2008).

1.3 Taxonomy and Genome

Family *Geminiviridae* is the largest and one of the most diverse groups of plant-infecting viruses. Viruses of this family cause devastating diseases in crop production all over the world. Geminiviruses have a genome of circular single-stranded DNA (ssDNA). This circular DNA is encapsidated in one or two geminate particles of 22 × 38 nm. So far, based on the genome structure, vector, host range and phylogeny, 14 genera (Table 1) of 520 species of geminiviruses have been established (https://talk.ictvonline.org/ictv-reports/ictv_online_report/ssdna-viruses/w/geminiviridae, Fiallo-Olivé et al., 2021).

Table 1. Genera in family *Geminiviridae*

Genus	Exemple species	Size of the genome (kb)	No. of species	Vector
Becurtovirus	*Beet curly top Iran virus*	3.0	3	Leafhopper
Begomovirus	*Bean golden mosaic virus*	2.6–2.8	445	Whitefly
Capulavirus	*Euphorbia caput-medusae latent virus*	2.7	4	Not known
Citlodavirus	*Citrus chlorotic dwarf associated virus*	3.7	4	Not known
Curtovirus	*Beet curly top virus*	2.9–3.0	3	Leafhopper
Eragrovirus	*Eragrostis curvula streak virus*	2.7	1	Not known
Grablovirus	*Grapevine red blotch viru*	3.2	3	Treehopper
Maldovirus	*Apple geminivirus 1*	2.9	*3*	Not known
Mastrevirus	*Maize streak virus*	*2.7*	45	Leafhopper
Mulcrilevirus	*Mulberry crinkle leaf virus*	*2.9*	*2*	Not known
Opunvirus	*Opuntia virus 1*	2.9	1	Not known
Topilevirus	*Tomato apical leaf curl virus*	2.8	2	Not known
Topocuvirus	*Tomato pseudo-curly top virus*	2.8	1	Treehopper
Turncurtovirus	*Turnip curly top virus*	2.9	2	Leafhopper

The genus *Begomovirus* is the only one that includes bipartite and monopartite viruses while viruses of other genera have only a single genome component. Different insect species are accountable for virus transmission of geminiviruses. The genus *Mastrevirus* has 45 species (Fiallo-Olivé et al., 2021), and the monopartite mastreviruses are transmitted by leafhoppers from family Cicadellidae. The most studied member of the genus is MSV, which is a serious threat for maize production in Africa (Martin et al., 2008; Monjane et al., 2020). WDV has a small genome of around 2750 nt, similar to viruses of all the species in the genus *Mastrevirus*. The genome has four known open reading frames (ORF) encoding four multifunctional proteins. Furthermore, ORF finding program identify ORFs, which may encode proteins of the similar size, for geminiviruses of different genera. Additional

ORFs, that may encode proteins, have been identified in the WDV genome, but the exact functions of these putative proteins are not known yet (Kvarnheden et al., 2002; Gong et al., 2021). The two ORFs on the complementary strand (C1, C2) encode the replication-associated proteins Rep and RepA (Figure 4).

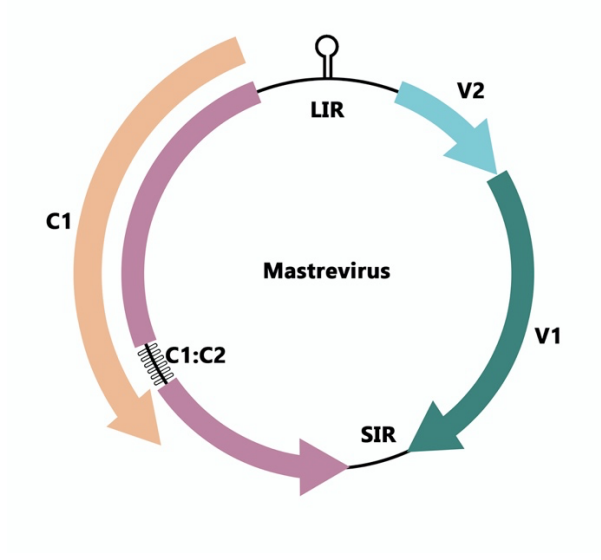

Figure 4. Genomic organization of mastreviruses. LIR: long intergenic region, V2 encodes the movement protein (MP), V1 encodes the coat protein (CP), SIR is a short intergenic region, C1 encodes replication-associated protein A (RepA). C1 and C2 encode the replication-associated protein (Rep).

At the early stages of infection, these two genes are transcribed and translated. Rep is formed after a transcript-splicing event between C1 and C2 regions, while RepA is translated from the C1 transcript without splicing. RepA helps the virus to induce S-phase of the cell cycle by binding to retinoblastoma-related protein whilst Rep is responsible for initiation of rolling-circle replication in order to reproduce copies of ssDNA. The virion strand encodes the other two proteins: gene V1 encodes the coat protein (CP) and gene V2 encodes the movement protein (MP) (Schalk et al., 1989; Wright *et al*., 1997; Abt and Jacquot, 2015). The MP is responsible for cell-to-cell movement, whereas CP functions as a nucleocytoplasmic shuttling protein besides its function in encapsidation and vector transmission. Between the genes for Rep and MP, there is a long intergenic region (LIR),

which contains promoters for transcription and is also involved in replication. LIR contains the TAATAT/AC motif, which is highly conserved across geminiviruses species. At this sequence, the Rep protein unwinds LIR to initiate rolling-circle replication (Laufs *et al*., 1995). Short intergenic region (SIR) located between the 5′ ends of C2 and V1, launches the synthesis of the positive strand. SIR also contains the transcription termination sequence (Boulton, 2002).

1.3.1 Diversity

WDV isolates have been categorized into two groups of wheat- or barley-infecting isolates (Lindsten and Vacke, 1991; Commandeur and Huth, 1999). Based on the demarcation criterion for the genus *Mastrevirus*, 78% and 94% genome nucleotide identity for species and strains respectively (Muhire et al., 2013), wheat and barley isolates are currently considered as members of the same species, sharing 83–84% genome-wide nucleotide sequence identity. However, the sequence identity among barley-infecting isolates of WDV is at least 89% while among the wheat-infecting isolates of strain WDV-E the identity is more than 98% (Köklü et al., 2007; Ramsell et al., 2008; Ramsell et al., 2009; Muhire et al., 2013; Abt and Jacquot, 2015). Comparing WDV-E wheat isolates over the years demonstrates a low degree of variation regardless of the isolate source (Ramsell et al., 2008; Schubert et al., 2014), however, a number of mutations leading to diversity within this group has been found, predominantly in the LIR, SIR and C1 genomic regions (Kvarnheden et al., 2002; Wu et al., 2008). According to Muhire et al. (2013), the species of WDV has been divided into five strains (A to E) with strains A, B and D containing barley-infecting isolates, and strains C and E containing wheat-infecting isolates. In Sweden, all the isolates from different hosts and the vector belong to the strain WDV-E (Kvarnheden et al., 2002; Ramsell et al., 2008). The areas of divergence in barley-infecting isolates (WDV-A) are located in LIR and SIR (Schubert et al., 2007). Since a mix of WDV genotypes can be found in a single host, recombination events can occur. Among wheat-infecting isolates, two Chinese isolates are suspected to have genomes of recombinant origin (Wu et al., 2008). One recombination event has also been found in the genome of a barley-infection isolate from Turkey (Ramsell et al., 2009). There has been some controversy over the ability of wheat isolates to infect barley plants and barley isolates to

infect wheat. While isolates of the barley strains of WDV (A, B, D) have been occasionally found in wheat plants, the efficiency in inoculation of wheat plants by barley-infecting isolates has been found to be low in controlled experiments (Schubert et al., 2007; Ramsell et al., 2009). However, a study by Abt et al. (2020) demonstrated that infection can occur in wheat and barley plants in mixed infection (trans-complementation) by wheat-infecting and barley-infecting isolates of WDV in an inoculation experiment using leafhoppers.

A new species of mastreviruses closely related to WDV has been discovered in Germany and Iran (Schubert et al., 2007; Kamali et al., 2017). Oat dwarf virus causes a dwarf disease in oat plants and the genome shares 69-70% sequence identity with those of both wheat and barley strains of WDV (A, B, D) (Schubert et al., 2007).

1.4 Diagnostics

To prevent and minimize viral disease damages in crop fields, the first step is to determine the cause of disease. The subsequent phase is to identify the source of infection both in the plant and in the vector. The use of diagnostic methods is not only important for controlling plant disease but also can indirectly help to gain a better knowledge on virus evolution as well as virus epidemiology (Varma and Kumar Singh, 2020). Various diagnostic methods are being implemented today to detect viral pathogens (Blystad et al., 2020). However, it is important to choose the right method according to the aim of the study as the accuracy of the result is crucial for choosing the appropriate management strategy (Rubio et al., 2020). As virus-infected plants are not always symptomatic, the detection method must be capable of identifying the virus even in non-symptomatic plants. In addition, diseased plants can be suffering from mixed infections, which must be considered when selecting the appropriate approach. The first step in identifying the causative agent of a diseased plant is symptom observation. However, the symptom range of different viruses can overlap and the symptoms may have been caused by mixed infection or even abiotic stresses. Therefore, there is a strong need for more sophisticated testing methods to identify the specific virus or even variant of the same virus in the infected plant (Rojas et al., 2018). One of the

first tools, which allowed researchers to study viral particles, was the electron microscope that allowed the visualization of virus morphology. Using electron microscopy, Lindsten et al. (1980) showed that WDV is a geminivirus with a typical particle shape. In 1989, the first record of the presence of WDV in Hungary was determined by Bisztray and Gáborjányi, when they showed that WDV has "geminate" particles of geminiviruses by using electron microscopy. The next widely used methods are serological tests, such as enzyme-linked immunosorbent assay (ELISA). This robust, handy and sensitive technique has been massively used in many laboratories and fields surveys to detect plant viruses (Boonham et al., 2014; Blystad et al., 2020). There are various types of ELISA that can be used to detect viruses. Depending on the aim of the study as well as specificity and availability of antibodies, the right approach must be chosen. The basis of the assay is to set up a coating on a polystyrene microtitre plate with an antibody against a specific virus antigen. The most common version of ELISA used for the detection of WDV in high number of samples is double-antibody sandwich (DAS) ELISA (Ramsell et al., 2008; Varma and Kumar Singh, 2020). Recent advances have shown that antigen-coated-plate ELISA (ACP-ELISA) and dot-ELISA using monoclonal antibodies are efficient types of ELISA for WDV detection (Zhang et al., 2018). The polymerase chain reaction (PCR) technique has made a significant improvement in identification of many plant viruses as well as revealing the sequence of their genetic components. The PCR method has been sensitive and specific enough to identifying WDV and its variants (Kvarnheden et al., 2002). The approach has been often followed by DNA sequencing and analysis of the result, which has been an advance in characterization of geminiviruses (Rojas et al., 1993). Besides PCR, rolling circle amplification (RCA), which is based on amplification of circular ssDNAs, has been demonstrated to be efficient in identification of ssDNA, even in detection of previously unknown geminiviruses (Inoue-Nagata et al., 2004). RCA has been used also for WDV detection (Schubert et al., 2007; Ramsell et al., 2009).

More recently, as detection methods of plant viruses progressed, relative and quantitative real-time PCR were used for efficient and sensitive detection of both RNA and DNA viruses. The method has been used also for identification of WDV in infected plants as well as in the leafhopper vector (Zhang et al., 2020). With the help of this method, it is possible to quantify

the number of virus DNA genome copies inside tissue of plants and leafhoppers. The quantification can help to determine the virus titre in different hosts and this information may be used for control strategies (Zhang et al., 2010). The assay has been successfully used for differentiating and comparing wheat and barley variants of WDV (Gadiou et al., 2012).

A simple, accurate and cost-effective method is real time loop-mediated isothermal amplification (LAMP), which only needs simple equipment, such as a water bath (Notomi et al., 2000). The method has been also developed for detection of WDV (Hao et al., 2021). The virus DNA is amplified rapidly in less than 60 minutes using four to six sets of specific primer pairs (Nagamine et al., 2002). A DNA polymerase carries out the amplification processes. At the same time, the protocol has been modified and adapted to be used for detecting RNA viruses (Zhao et al., 2010). The positive result can be visualized by gel electrophoresis or by using SYBR green dye. To some extent the use of dilution series can help with quantitative detection. Hao et al. (2021) demonstrated that a shorter amplification time until a positive test is visible, is a sign of high virus titre in the samples, whereas a longer amplification period is the indication of lower virus titre.

Next-generation sequencing (NGS) is undoubtedly the most efficient advance in diagnostics of uncharacterized geminiviruses (Rojas et al., 2018). Combined with bioinformatic analyses, the method is a powerful tool to detect different variants of a virus (Ho et al., 2014; Kamali et al., 2017; Villamor et al., 2019; Sõmera et al., 2021), which helps in detection of SNPs and subsequently studying the changes in the protein sequence of the virus that may affect the virus infection mechanism and its virulence.

1.5 Epidemiology and Control

Being considered as a major threat to wheat production, wheat dwarf disease can cause a drastic yield loss. In a study by Lindblad and Waern (2002), farmers reported an average total yield loss of about 35%, while the loss was estimated to be up to 90% in severely affected fields. The severity and extent of the damage that WDV infection can cause depend on a number of biotic and abiotic factors. For an outbreak of wheat dwarf disease to occur, virus, vector, host and environment factors interrelate in complex ways. To

customize efficient management of wheat dwarf disease, a complete knowledge of the disease and the factors involved in disease outbreaks is required. The population dynamics of the vector *P. alienus* is hugely dependent on the weather, especially temperature and precipitation. A high insect population is one of the risk factors for wheat dwarf disease incidence. A high temperature of above 15°C during autumn can be a warning for wheat growers as increased temperature favours leafhopper activity (Lindblad and Arenö, 2002). On the other hand, leafhoppers become less active as the temperature falls below 10ºC (Lindblad and Arenö, 2002). For their replication, viruses are dependent on their host plants. Volunteer cereal plants and grasses are often suitable reservoir hosts. The presence of infected reservoirs and their density can increase the incidence of infection with many mastreviruses including WDV and MSV (Ramsell et al., 2008; Shepherd et al., 2010). Grasses are often present around or within cultivated cereal fields (Blystad et al., 2020) as a catch crop, forage crop, grass weeds or wild grasses (Lindsten et al., 1999; Ramsell et al., 2008), including WDV hosts such as *Lolium* spp., *Avena fatua, Poa pratensis*, *Apera spica-venti, Bromus rubens, B. arvensis, B. commutatus, B. hordeaceus* and *B. japonicus* (Mehner et al., 2003; Ramsell et al., 2008; Pouramini et al., 2019). The presence of volunteer plants in the cultivated area (Abt and Jacquot, 2015) and irregular seedling emergence can increase the risk of wheat dwarf disease. In addition, reports of the disease show that leafhoppers are attracted by nonuniform stands (Lindblad and Waern, 2002). Their feeding behaviour, population density and activity have a great impact on the intensity and frequency of disease (Abt and Jacquot, 2015). Population dynamics of the insect vector is strongly linked to the climate. Accordingly, climate change is affecting the leafhoppers' life cycle and population size, which eventually has a great impact on vector distribution and the incidence of wheat dwarf disease (Habekuβ et al., 2009). While warm weather during spring and autumn favours the leafhopper, an increase in precipitation can negatively influence the leafhopper population. At the same time, sensitivity of nymphs to cold weather during a cold spring may decrease population size of their next generation and subsequently influence the primary infection in the autumn (Lindblad and Arenö, 2002). The correlation between weather fluctuations, *P. alienus* population dynamics and disease incidence has been previously shown in Sweden (Lindblad and Arenö, 2002).

Wheat genotypes which are susceptible to WDV differ in their response to the infection. The susceptibility of these crops to WDV ranges from very susceptible to partially resistant cultivars (Lindblad and Waern, 2001; Benkovics et al., 2010; Nygren et al., 2015; Pfrieme et al., 2022). In addition, leafhopper vectors might show a variable tendency toward feeding on different wheat cultivars (Thresh, 1982). During the last decade, several cultivars of winter wheat and its wild relatives have been found to be tolerant or partially resistant to infection by WDV, which can be beneficial for wheat cultivation (Benkovics et al., 2010; Nygren et al. 2015). At the same time, as with most plants, wheat is mostly susceptible to WDV infection when the plants are young. Wheat dwarf disease develops in wheat plants if they are infected at an early stage. As the wheat plants grow, they will be less affected and will eventually develop mature plant resistance when the first node appears (Vacke, 1972; Lindblad and Sigvald, 2004). When adult leafhoppers fly out in late spring/early summer, the wheat plants have reached a developmental stage where they are resistant to WDV.

Cultivation practices are an indispensable part when studying epidemiology of plant virus diseases. They are strongly linked to fluctuation in the incidence of wheat dwarf disease and the extent of yield loss. Any change in the agricultural practices can impact the crop fields and volunteer plants inside the cultivated fields in different ways as well as population dynamics of the leafhopper vector (Lindsten and Lindsten, 1999; Lindblad and Arenö, 2002; Abt and Jacquot, 2015). Spread of WDV is strongly influenced by sowing time. Often severe yield loss is an outcome of early sowing of winter wheat. Simultaneous presence of viruliferous leafhoppers and early sown winter wheat may result in devastating disease as it provides an extended time span with a temperature suitable for leafhoppers to feed on plants and increases the area affected by the infection. Due to this situation, there will be a greater risk of primary infection during autumn, and subsequently secondary infection during the following spring (Lindblad and Waern, 2001; Lindblad and Arenö, 2002). Similar to WDV, MSV infection is more severe in early sown maize (Shepherd et al., 2010). It is likely that leafhoppers stay in fields with reduced tillage. Furthermore, volunteer plants in fallows may act as reservoir increasing the risk of infection (Lindblad and Arenö, 2002).

Variation in virus distribution and severity of infection is determined by different epidemiological factors, environment, and years (Jones et al., 2010). To predict and prevent the incidence and outbreak of virus disease, extended knowledge of epidemiology is required. This knowledge will help to design and establish an optimized approach, so called integrated pest management (IPM), to control the infection and minimizing yield loss. Therefore, appropriate agricultural practices should be deployed based on a deep and comprehensive study over the various factors contributing to rise of an epidemic. The main means of insect vector management is still chemical insecticides (Aranda and Freitas-Astúa, 2017) for seed treatment or spraying on the growing crop (Shepherd et al., 2010; Abt and Jacquot, 2015). However, the fact is that relying on pesticides has negative consequences for the environment. Hence, the most efficient and environmentally friendly way to control the spread of WDV is to build up improved agricultural practices supported by a minor use of insecticides (Rojas et al., 2018). This is now used in Sweden to manage wheat dwarf disease together with limited use of insecticide in spring. In conjunction with the latter, supporting natural enemy populations as a biological control means to reduce leafhoppers populations is desirable (Rojas et al., 2018). To put this method into practice, experiments have been carried out showing *P. alienus* to be preyed upon by the spider *Tibellus oblongus* (Samu et al., 2013). Additionally, further studies on wheat cultivars with resistance or partial resistance to WDV, which can be used in breeding or directly in the field, will be a great asset in order to control and manage the disease (Vacke and Cibulka, 2000; Benkovic et al., 2010; Nygren et al., 2015; Pfrieme et al., 2022), although resistance may be overcome by new variants of the virus (Gómez et al., 2009). Forecasting wheat dwarf disease based on improved knowledge about leafhopper population size and activity together with relevant weather data, virus and host plant features compiled into a model can be beneficial (Jones et al., 2010). However, this needs a large specific and precise data set which must include both biotic and abiotic factors involved in epidemiology of wheat dwarf disease (Rojas et al., 2018). Furthermore, deep insight into genetic diversity of viruses is essential knowledge (Olarte-Castillo et al., 2011), not only for prediction models, but also to adapt efficient control methods.

It is difficult to avoid having grasses close to a wheat field. Grasses can often be a potential reservoir for WDV and act as a green bridge. For

instance, cultivation of ryegrass (*Lolium* spp.) should be avoided in risk areas as a catch crop since they have been found to be susceptible to WDV infection (Lindsten and Lindsten, 1999; Ramsell et al., 2008). Thus, it is of importance to clear the cultivation area from any potential source of infection by ploughing and crop rotation (Blystad et al., 2020). It is important to keep in mind that not all symptoms are a result of virus infection, therefore, regular testing of symptomatic plants and leafhoppers in areas with a previous history of infection has a great value in recognizing vulnerable geographical locations and deploying suitable preventing measures for the coming seasons (Bukvayová et al., 2006). Adoption of fast-growing cultivars can be beneficial as the plants will develop mature resistance in a shorter time. Likewise, growing wheat after wheat should be avoided. However, since this is still common, it is then very important to follow the other recommendations, for example to avoid early sowing. Additional studies are needed to develop a deeper knowledge of the epidemiology of WDV as global climate change and change in agricultural practices can expose winter wheat production to a higher risk of disease outbreaks resulting in devastating yield losses.

1.6 Barley/cereal yellow dwarf viruses

One of the most devastating viral diseases of cereals, yellow dwarf diseas, is caused by yellow dwarf viruses (YDVs) belonging to the genus *Luteovirus* (family *Tombusviridae*) and genus *Polerovirus* (family *Solemoviridae*) (Sõmera et al., 2021). The disease was shown to be caused by barley/cereal yellow dwarf viruses first in 1951 (Oswald and Houston, 1953). These viruses have been threatening grain production globally, which has led to huge yield reductions and created economical losses in production of spring and winter cereal crops (Perry et al., 2000). In a designed experiment, the yield loss due to infection by barley yellow dwarf-PAV (BYDV-PAV) reached up to 84% in wheat and 64% in barley compared to control plants (Nancarrow et al., 2021). However, the level of yield loss can vary according to the choice of cultivar and environmental factors. YDVs have a genome of positive-sense linear single-stranded (ss) RNA (Miller and Rasochova, 1997). They are phloem-limited viruses (Walls et al., 2019) transmitted by

over 25 different aphid species in a persistent manner (Svanella-Dumas et al., 2013; Walls et al., 2019). The most well-studied species of genus *Luteovirus* is *Barley yellow dwarf virus PAV* (D'Arcy, 1995), and isolates of this species have been found to be common in wild and cultivated grasses (Bisnieks et al., 2004). As a result of infection by YDVs, the flow in the sieve elements will be disturbed, and diseased plants exhibit a variety of symptoms, such as leaf discolouration, stunting and reduced headings (Ali et al., 2014; Van den Eynde et al., 2020), which can be mistaken for symptoms caused by WDV or induced as a result of abiotic stress. However, the extent of the manifested symptoms depends on the virus titre in the infected plant, the virus strain and the vector. Both genera *Luteovirus* and *Polerovirus* include viruses capable of infecting crop plants and wild grasses. Examples of cereal-infecting poleroviruses are cereal yellow dwarf virus-RPV, cereal yellow dwarf virus-RPS, maize yellow dwarf virus RMV, maize yellow mosaic virus, and sugarcane yellow leaf virus as well as the two new poleroviruses barley virus G (Tamborindeguy et al., 2013; Wang et al., 2016; Zhao et al., 2016; Sõmera et al, 2021) and wheat leaf yellowing-associated virus. Barley yellow dwarf virus PAV, barley yellow dwarf virus PAS (Kundu, 2008), barley yellow dwarf virus MAV (BYDV-MAV), barley yellow dwarf virus kerII, and barley yellow dwarf virus kerIII are luteoviruses (Svanella-Dumas et al., 2013) as well as the newly identified barley yellow dwarf virus OYV, for which isolates have has been found in grasses of *Festuca pratensis* in Sweden (Sõmera et al, 2021). These viruses do not only differ in their molecular characteristics, but also in the severity of induced symptoms, host range and aphid vectors (Jarosova et al., 2013). In Sweden, BYDV-PAV and BYDV-MAV have been the most frequently occurring YDVs, and they have been found in both *Festuca* spp and *Lolium* spp as well. However, the extent of infection varied among these hosts (Bisnieks et al., 2004). Periodical appearance of the disease in many parts of the world as well as Sweden is linked to the biology and dynamics of the vectors plus climate condition (Walls et al., 2019), not to mention the vast host range, which all together have made control and management of the yellow dwarf disease difficult.

2 Aim of the study

The incidence of wheat dwarf disease has been sporadically reported during the last decades in Sweden. The aim of the study was to obtain a basic understanding of the complex epidemiology of WDV and in general cereal-infecting viruses in Sweden, to reduce the extent of disease and the damage it can cause in cereal crops. The knowledge will be of use for integrated pest management of wheat dwarf disease.

The specific objectives of the study were:

- To uncover the role of various virus sources for WDV in Sweden
- To determine the potential of ryegrass as a virus reservoir for WDV and B/CYDV
- To characterize the virus isolates of B/CYDV and WDV occurring in ryegrass
- To evaluate the difference in virus accumulation, symptom development and WDV spread pattern between different wheat genotypes as a potential source for breeding
- To investigate the role of vector in incidence of wheat dwarf virus
- To determine the role of weather fluctuations for the vector population

3 Results and Discussion

3.1 Occurrence of WDV and B/CYDVs in Ryegrass (Paper I)

To complement previous studies, which have been carried out to identify potential reservoirs for WDV and B/CYDVs, including ryegrass in Sweden (Bisnieks et al., 2006; Ramsell et al., 2008), a collection of randomly selected ryegrass samples from the counties of Västra Götaland, Stockholm and Uppsala were tested for WDV infection. Choice of sampling sites was based on previous occurrence of wheat dwarf disease. Of 845 tested samples, WDV infection was detected in five plants when tested by DAS-ELISA, with 0.2% from the county of Västra Götaland and 1.0% of the samples from the county of Uppsala. An amplicon of 1.2 kb produced by PCR confirmed the ELISA result. The same sets of samples were serologically tested by DAS-ELISA for presence of B/CYDVs. In the test, 0.5% of the samples from Västra Götaland were found to be infected by BYDV-PAV. This result was confirmed by IC-RT-PCR. In the same set of samples from Västra Götaland, 4.0% of the samples were infected by BYDV-MAV, while 3.8% proved to be infected by CYDV-RPV. None of the samples from Uppsala or Stockholm were found to carry B/CYDVs. These findings demonstrate the presence of both WDV and B/CYDVs among ryegrass plants found in and around cereal fields in different part of Sweden. Since the area under ryegrass cultivation as a mean to prevent nutrient leakage or as a forage crop can be extensive, together with conditions favourable for wheat dwarf disease, it can

lead to great damage in cereal fields, even though our results showed that the extent of infection is low in ryegrass. WDV presence in other grass species, such as *Poa annua*, *P. pratensis*, and *A. fatua* had been reported before (Lindsten and Lindsten, 1999; Ramsell et al., 2008), which shows the broad range of reservoir plants for WDV. At the same time our negative results for WDV infection in couch-grass and timothy is consistent with older studies on WDV reservoirs, where these grasses were found not to be the host for WDV (Lindsten and Lindsten, 1999; Ramsell et al., 2008). B/CYDVs have long been abundant in cereals and grasses all around the world (Clarke and Eagling, 1994; Bisnieks et al., 2006; Delmiglio et al., 2010). Therefore it was no surprise to detect BYDV-PAV, BYDV-MAV, and CYDV-RPV in ryegrass in Västra Götaland. Negative test results for B/CYDVs in counties of Uppsala and Stockholm can be linked to the fact that virus titre is often low in grasses and can be easily missed by ELISA (as we have shown for WDV in ryegrass) which is not sensitive enough to detect viruses with low titre or the ryegrass was not infected.

3.2 Sequence and Phylogenetic Analyses (Paper I)

A cloned PCR-fragment of 1.2 kb was sequenced for one WDV isolate from ryegrass from Västra Götaland. The result demonstrated that the isolate belongs to strain WDV-E with at least 97.9% identity to previously sequenced wheat-infecting isolates found in Sweden (Kvarnheden et al., 2002; Ramsell et al., 2008). Phylogenetic analysis of the partial sequence obtained in this study (WDV-E[SE:ryegrass:2012]) as well as available WDV sequences in GenBank demonstrated that the ryegrass sequence forms a well-supported clade together with other WDV-E isolates, while no strict geographical or host species grouping was observed. The diversity within this clade was shown to be low. The close relationship between the isolate originating from ryegrass and ones from different hosts as well as the vector *P. alienus*, suggests that the closely related WDV genotype infecting ryegrass can be found in wheat (Ramsell et al., 2008), this was also confirmed by WDV-transmission experiments (Paper I).

3.3 WDV Detection in Inoculated Ryegrass (Paper I)

As ryegrass may be growing in field borders for extended periods and it is commonly used as a forage or catch crop, it is likely to be an important virus reservoir (Lindsten and Lindsten, 1999; Ramsell et al., 2008). Therefore, the ability of ryegrass to act as a green bridge for the spread of WDV was evaluated in transmission experiments. Negative results were obtained by ELISA tests after plants of different ryegrass species were exposed to viruliferous *P. alienus.* All the ryegrass test plants inoculated by WDV in the greenhouse experiment remained symptomless during the experiment. However, when testing the plants with PCR, three individual plants were found to be infected by WDV. It has been shown before as well that grasses infected by viruses often do not develop clear symptoms although they are infected, which may be due to the low virus titre in the plant (Mehner et al., 2003; Parry et al., 2012). Moreover, by using qPCR assay we could detect WDV in inoculated ryegrass plants of different species although the ELISA test result was negative. The qPCR result confirmed that the virus level in infected ryegrass was much lower when compared to infected wheat. Accordingly, it can be assumed that ELISA is not an optimal method to be used when it comes to virus detection in grasses. Then it is best to use more sensitive tools such as PCR or qPCR (Ingwell and Bosque-Pérez, 2015). This result was an indication of the ability of WDV to infect plants of different ryegrass species, but to a lower degree in comparison with wheat, which is expected as it has been shown before for BYDV-PAV (Delmiglio et al., 2010). For more than two years after inoculation, ryegrass plants remained infected by WDV. These plants had a low virus titre and did not display any symptoms but could still be used for transmission of WDV by *P. alienus* leafhoppers to healthy wheat plants, even though the transmission rate was low (Figure 5). These observations are important as they confirm the potential role of ryegrass as a reservoir for WDV. As weeds and grasses are commonly found in and around arable land, recognizing them as a potential source of virus infection is key knowledge in the studies on plant virus epidemiology (Lindsten and Lindsten, 1999) and for adapting efficient control measures against wheat dwarf disease. Detection of WDV and B/CYDVs in ryegrass not only confirms the ability of grasses to act as a common reservoir for different plant viruses, but also shows that the host

range of these viruses is wide. This broadens the area affected by these viruses. The extensive use of perennial ryegrass will guarantee the persistence of WDV and B/CYDVs in the field (Duffus, 1971) and cause subsequent infections during the following seasons (Clarke and Eagling, 1994).

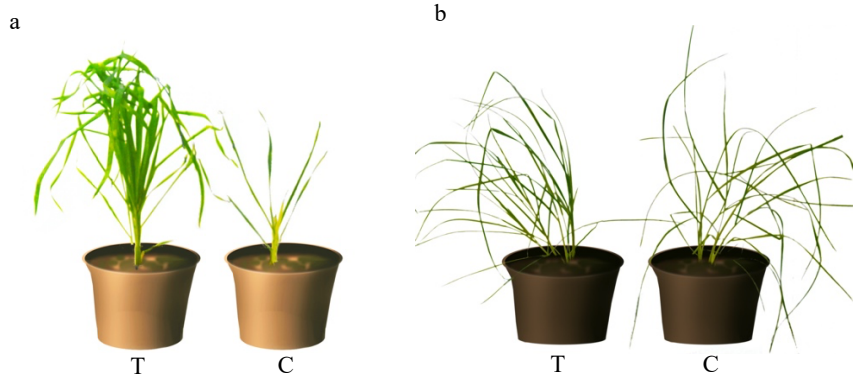

Figure 5. Response of wheat (a) and ryegrass (b) plants to infection by wheat dwarf virus (WDV). T, plant infected with WDV; C, healthy plant.

3.4 Assessment of Development of WDV Infection in Host Plants (Paper II)

A delay in the spread of WDV infection can enable the plants to have a normal growth pattern and reach tillering stage before virus accumulation, resulting in only mild stress responses and tolerance (Nygren et al., 2015). Symptom development and systemic spread of WDV were evaluated in three different wheat genotypes: bread wheat (*Triticum aestivum* spp. *aestivum*) cv. Tarso, and its two wild relatives *T. urartu* and *Aegilops tauschii*. Plants of *T. urartu* were previously found to be very sensitive to infection to WDV, while plants of *Ae. tauchii* showed tolerance (Nygren et al., 2015). The hexaploid genome of bread wheat includes A, B and D genomes originating from its ancestors. While the A genome comes from *T. urartu* (Dvorak et al., 1993), *Ae. tauschii* is considered as the D genome doner (Petersen et al., 2006). The assessment was done on accumulation and spread of WDV in roots and leaves of the selected host plants during the initial stages of plant

growth and development. Accumulation of WDV was observed in the roots of all the accessions already at the first leaf stage and continued to increase during the early growth stages. However, *T. urartu* stood out among the accessions by higher virus titre at the third leaf stage compared to *Ae. tauschii* and wheat cv. Tarso. Elevated WDV content was later observed for *Ae. tauschii* and wheat cv. Tarso as well (Figure 6). Compared to roots, the presence of WDV in the leaves was seen later for all the accessions included in the study. This is in agreement with studies where systemic movement within an infected plant was shown to take place through external phloem and internal phloem. Spread of the virus via external phloem occurs toward the root and via internal phloem toward the younger leaves (Cheng et al., 2000; Gosalvez-Bernal et al., 2008). The starting points of virus accumulation in the shoot also varied between the accessions, as it started at the second leaf stage for *T. urartu*, and the fourth leaf stage for wheat cv. Tarso and *Ae. tauschii* (Figure 6).

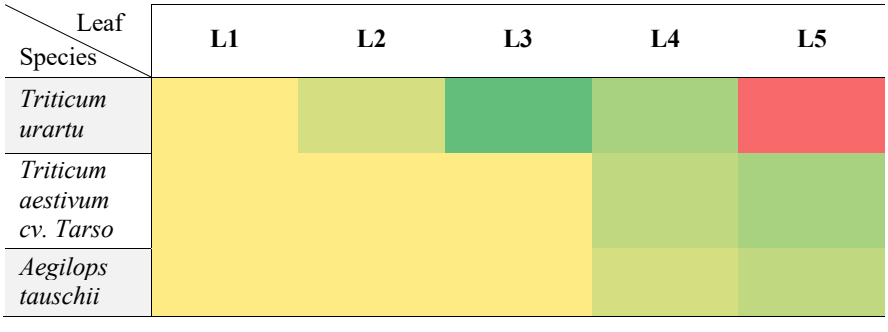

Figure 6. Leaf stages and time-course of change in WDV content in leaves. ELISA absorbance values for WDV content in leaves at different leaf stages in *T. urartu*, *T. aestivum* cv. Tarso and *Ae. tauschii*

Abs 0.00 Abs. 0.37-0.73 Abs. 1.25> Abs. 0.37-1.25> Plant did not survive

In the inoculation experiments, *T. urartu* was the first accession with high accumulation of WDV both in root and leaf resulting in severe symptoms already at the fourth leaf stage. The plants of this accession could not develop the fifth leaf and died. For wheat cv. Tarso and *Ae. tauchii*, presence of WDV

was detected in all the leaves during the fifth leaf developmental stage. Compared to *Ae. tauschii*, the average WDV titre in all the leaves was shown to be higher for wheat cv. Tarso. Plants of all the accessions developed WDV-induced symptoms but plants of *T. urartu* demonstrated more pronounced symptoms with significantly higher extent of leaf chlorosis. The percentage of leaf chlorosis was similar in plants of wheat cv. Tarso and *Ae. tauschii.* Infected plants of these three accessions varied substantially also in their leaf fresh weight, with wheat cv. Tarso having highest fresh weight among the accessions and *T. urartu* being significantly lower in fresh weight. The results complement a previous study by demonstrating that wild and domesticated wheat show noticeable variation in susceptibility at earlier time points due to genome differences with *Ae. tauschii* being a candidate for further studies on WDV resistance and breeding of resistant or tolerant cultivars (Nygren et al., 2015). In the present study, it was shown that during the early stages of development, *Ae. tauschii* was the only accession that followed normal growth despite infection with WDV.

The reactions of host plants to virus invasion differ based on the intensity of selection during their coevolution. Therefore, susceptibility of *T. urartu* to WDV infection can be explained by a weak reciprocal selection interaction during the coevolution of the host plant and the virus. On the other hand, as a result of strong reciprocal selection interaction, a balanced mix of resistant and susceptible host plants can be formed in the population. In an equilibrium of susceptible and resistant host plants, resistance is preferred by selection as long as it does not cost (Roy and Kirchner, 2000). Thereby tolerance will be favourable by natural selection as it will not suppress virus colonization, yet virus infection will not induce severe symptoms in the host plant resulting in higher incidence of infection. Nygren et al. (2015) have shown the presence of a tolerant accession (*Ae. tauschii*) among bread wheat ancestors. The potential of *Ae. tauschii* as a tolerant genotype was confirmed in this study by showing delayed spread of WDV.

The observation from this study (Paper II) can introduce the wild relatives of bread wheat as potential sources of tolerance/resistance to WDV and emphasizes the importance of studies on the relation between the host plant and virus defence mechanisms deployed in the interaction.

3.5 Abundance and Dynamics of *P. alienus* in Sweden (Paper III)

The dynamics of leafhopper populations can influence wheat dwarf disease incidence. During autumn (week 36-42) of the years 2002-2020, leafhoppers (*P. alienus*) were collected in yellow traps placed in cereal fields in the counties of Uppsala, Stockholm, Västmanland, Södermanland, Östergötland and Västra Götaland in Sweden. These are regions with previous problem of wheat dwarf disease. Analysis of the data showed that the leafhopper population size varied considerably between years and different counties in winter wheat fields (Figure 7). In addition, our observation showed that *P. alienus* had been persistently present in catches of leafhoppers from yellow traps in the counties of Uppsala and Stockholm during all years of study. A fitted line plot showed a declining pattern in the number of leafhoppers during 2002-2020 in the counties of Uppsala, Stockholm, Västmanland and Östergötland. The same result was observed when we looked at the data for all the counties combined. However, the slope of the line was relatively gradual, and there was not a significant decrease for Västra Götaland and in Södermanland.

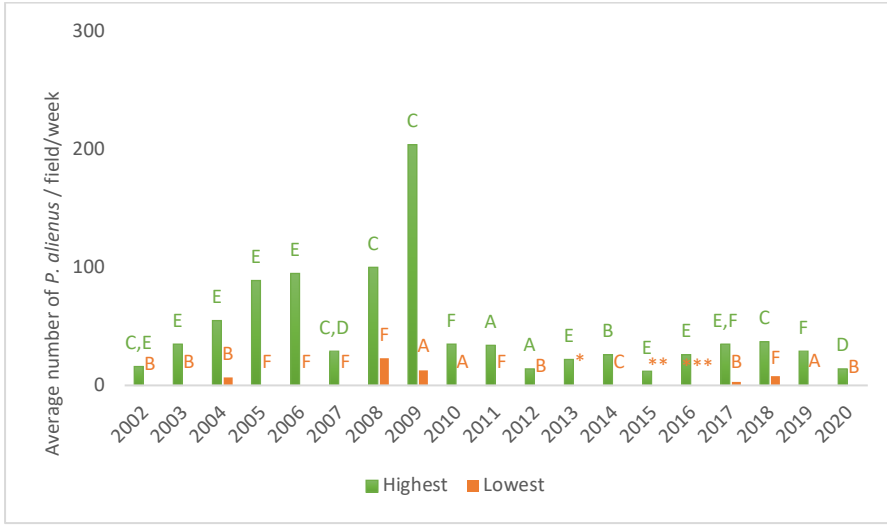

Figure 7. Highest and lowest average number of *P. alienus* leafhoppers per field and week during autumn (week 36-42) in surveyed counties in Sweden during 2002-2020. Counties: (A) Östergötland, (B) Västra Götaland, (C) Uppsala, (D) Västmanland, (E) Stockholm, (F) Södermanland, (*) counties A, B and F, (**) Counties A, B, C and F, (***) Counties A, C and F. Data was missing for Västra Götaland (in 2002, 2003), Södermanland (in 2007, 2015) and Östergötland (in 2010).

3.6 Incidence of Wheat Dwarf Disease and Presence of WDV in *P. alienus* Leafhoppers in Sweden (Paper III)

Wheat dwarf disease occurs in winter wheat fields in several parts of Sweden, however, the severity and incidence of disease varies considerably between years, regions and fields. During the study period, the incidence of wheat dwarf disease was reported regularly by plant protection centres in Sweden and it showed a substantial variation among years and regions. PCR tests were carried out on leafhoppers collected in different counties during 2002-2020, except for 2007. The PCR result confirmed the presence of WDV in leafhoppers in the years with high disease incidence (2002, 2003, 2009, 2010, 2017). This result suggests that WDV never disappeared in Sweden and may had been present in reservoir plants and reappeared when the optimum conditions were met (Lindblad and Waern, 2002; Ramsell et al.,

2008). One important finding of this study was a statistically significant correlation between the number of leafhoppers and wheat dwarf disease incidence the following year, in the counties of Uppsala, Stockholm and Västmanland, which had been more affected by the disease. The result highlights the role of the leafhopper population size in the risk of disease outbreak, which is very important for disease forecasting. On the contrary, years with high incidence of the disease and low number of leafhoppers the year before were observed as well. It is then likely that disease incidence can be influenced by the effect of biotic and abiotic factors such as habitat diversity and variation in virus load in the insect vector. This observation suggests that the epidemiology of WDV is not solely dependant on the population size of its insect vector.

3.7 Distribution and Prevalence of *P. alienus* in Connection to Meteorological Factors (Paper III)

It has been shown that distribution and population size of the leafhopper vector is linked to abiotic and biotic factors, which can affect the incidence of wheat dwarf disease. Weather is one of the most epidemiologically important elements affecting the interaction between leafhoppers, virus and host plants (Lindblad and Arenö, 2002). A positive linear relation was found between weekly average maximum temperature in autumn and the number of leafhoppers in Uppsala, Stockholm, Västmanland and Östergötland. The same result was observed when we looked at the data for all counties combined, which is consistent with results from a survey carried out in the county of Uppsala during 1997-2000 (Lindblad and Arenö, 2002). The weekly average maximum temperature in autumn was never below 11.2 °C in any of the counties during 2002-2020, which is warm enough for leafhopper activity (Lindblad and Arenö, 2002). Further, the average weekly temperature in autumn was found to be connected with the number of leafhoppers in Uppsala, Stockholm, Västmanland and all the counties combined. Weekly average minimum temperature in autumn was found to be correlated with the number of leafhoppers only in Uppsala, Stockholm and Västmanland. In comparison to the other counties, Uppsala, Stockholm

and Västmanland had the highest weekly average minimum temperature in autumn. It is likely, but not confirmed, that a higher weekly average temperature, weekly average of maximum and minimum temperature in autumn in these three counties are some of the factors contributing to the persistence of WDV, knowing that weather fluctuations and environmental conditions are affecting the incidence of viruses infecting cereals (Bukvayová et al., 2006).

Analysis of weather data showed an increase in precipitation in autumn over the years (2002-2020) in Uppsala, Stockholm, Västmanland, Östergötland, Västra Götaland and Södermanland. Compared to the other counties, precipitation in Västra Götaland was found to be higher. Looking at the data for wheat dwarf disease incidence, the disease was found to be less of a problem in Västra Götland. It is possible that the lower incidence of wheat dwarf disease in Västra Götaland can be connected to the fact that on one hand extended periods of precipitation in autumn will delay the sowing time for winter wheat, and on the other hand leafhopper activity will decrease and population size may shrink due to the unfavourable conditions (Lindblad and Arenö, 2002; Scott, 2021). However, using regression analysis and Pearson correlation, no significant correlation between the number of leafhoppers and precipitation was found. Therefore more detailed investigations are needed to determine the exact effect of precipitation on the population dynamics of leafhoppers, the effects it has on plant cultivation and to which extent it can influence wheat dwarf disease incidence. Since WDV transmission occurs via leafhoppers, epidemiology of the virus is widely dependent on its vector biology and behaviour (Manurung et al., 2004). The results of this study suggested that the frequency of the wheat dwarf disease probably corresponded to leafhopper population size, primarily in the autumn before, which affects also the extent of infection during spring, when wingless nymphs then transmit the virus (Bukvayová et al., 2006). At the same time, wild and perennial grasses as well as self-sown cereals are other key players in the epidemiology of wheat dwarf, providing the source of virus in the autumn as a reservoir. WDV reservoirs, such as ryegrass (Paper I), have an important role in maintenance of both leafhoppers and the virus itself (Manurung et al., 2004).

4 Concluding remarks

The findings of this thesis contribute to a better understanding of WDV epidemiology in Sweden. The main findings include:

- Non-crop plants such as grasses and volunteer plants, an inseparable part of the flora of crop fields, can influence virus incidence in crop plants.
- Detecting WDV, BYDV-PAV, BYDV-MAV and CYDV-RPV in ryegrass, suggest that there is exchange of virus between grasses and wheat by transmission of their vectors.
- WDV can remain in symptomless ryegrass for a long period.
- Efficient WDV transmission by leafhoppers can occur even with low virus titre.
- The delayed systemic infection in *Ae. tauschii* will allow the plants to have normal growth and reach the tillering stage before virus accumulation, which eventually results in only mild stress responses and tolerance to WDV.
- *Ae. tauschii* is a potential candidate for further pre-breeding research and a genetic resource for improvement of WDV tolerance in wheat.
- Weather conditions during autumn is an important factor affecting epidemiology of WDV.
- Wheat dwarf disease incidence is strongly linked to the vector population.
- Wheat dwarf disease incidence varies significantly between years and different environment.

5 Future perspectives

With the worldwide increasing demand on cereal production, it is important to invest in studies with focus on the improved management of virus diseases of cereals to prevent further outbreaks and crop losses. To achieve that goal, frequent surveillance or monitoring programmes are required. The survey of the occurrence of wheat dwarf disease in Sweden during 2002 to 2020 and investigations on WDV and BYDV reservoirs are too limited to draw reliable conclusions about the complex epidemiology of these viruses. There is a need for more detailed investigations on the complex epidemiology of WDV considering the behaviour of the vector and the cropping systems in different environmental conditions and geographical locations. More detailed studies on the role of different reservoirs and their interaction with the virus and the vector will be desirable. In addition, it is of interest to understand the genetic diversity of the WDV population in the leafhopper vector, and identification of potential variants with higher fitness in the population as well as any change in their virulence. Effects of climate change on crops and virus reservoirs as well as the vector are inevitable. In many studies, the influence of weather factors affecting the biology and behaviour of insect vectors and influence on the growth pattern of the cereal crop have been shown. Therefore, continued monitoring of these factors is needed. Future efforts should aim to improving the knowledge on the biology and genetics of the leafhopper *P. alienus* which is poorly studied. At the same time, our finding of a potentially WDV-tolerant species may help to create a stronger interest in further studies on resistance against cereal-infecting viruses among wild relatives of cereals and how they can be integrated

into agricultural systems. In addition, the identification of natural enemies of the vector via enhanced screening can be a promising approach.

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Popular science summary

Viruses are important pathogens on plants. To reduce their impact, it is necessary to understand how they are transmitted between plants and how they interact with their plant hosts. Wheat dwarf disease is caused by wheat dwarf virus (WDV) and transmitted by the leafhopper *Psammotettix alienus*. Diseased plants become yellow and stunted, and the seed production is reduced. In Sweden and many other parts of Europe, wheat dwarf is one of the most important diseases caused by a plant-infecting virus. During the last century, the disease has caused significant damage to wheat in Sweden. From the end of the 1990s, wheat dwarf has re-appeared as an important disease in the central parts of Sweden. The periodic re-appearance has been found to be associated with changes in agricultural practices and presence of alternative hosts. In this study, we collected ryegrass samples from different parts of Sweden which had problems with wheat dwarf disease. The results of virus tests and transmission experiments show that ryegrass growing in or around winter wheat fields is a potential reservoir for WDV. In addition, a WDV isolate infecting ryegrass was found to be closely related to typical WDV isolates present in Sweden. With the predicted global warming, the incidence and effects of WDV infection may increase, since there will be an opportunity for virus transmission by leafhoppers for a longer period during autumn. In this study, we got a deeper understanding for how the leafhopper population fluctuates and how this influences wheat dwarf disease incidence. The results suggested that the number of leafhoppers and incidence of wheat dwarf disease are connected. We analysed data of leafhoppers collected from different parts of Sweden and autumn temperature. Autumn temperature was found to influence the population size of leafhoppers. All wheat cultivars tested so far are susceptible to WDV infection. Our observation in this study showed that plant development was affected by the onset of WDV infection

in different bread wheat relatives. In addition, the load of virus in the plant is suggested to have an effect on symptom and plant development. In this study we have observed the spread of WDV in a potentially tolerant wheat ancestor. We found that systemic infection in this genotype was delayed compared to other genotypes which can enable the plants to continue to grow before virus accumulation, and thus result in mild responses and tolerance to WDV. Further studies are required to identify sources of resistance against WDV. With improved knowledge on WDV epidemiology it will be possible to use agricultural practices that reduce the risk of infection. Taken together, this thesis contributed to a better understanding of the complex epidemiology of cereal-infecting viruses, where many alternative hosts often are playing an important role. The knowledge will be possible to use for integrated pest management of wheat dwarf disease.

Populärvetenskaplig sammanfattning

Virus är viktiga växtpatogener. För att minska deras påverkan är det nödvändigt att förstå hur de överförs mellan växter och hur de interagerar med värdväxten. Vetedvärgsjuka orsakas av vetedvärgvirus och överförs med den randiga dvärgstriten (Psammotettix alienus). Sjuka plantor gulnar och stannar i växten, och produktionen av vetekorn minskar. Vetedvärgsjuka är i Sverige och många andra delar av Europa en av de viktigaste sjukdomarna som orsakas av ett växtvirus. Sjukdomen har under senaste århundrandet orsakat betydande skador på vete i Sverige. Vetedvärgsjuka har från slutet av 1990-talet åter dykt upp som en viktig sjukdom i Mellansverige. Den periodiska återkomsten har funnits vara förknippad med ändrade jordbruksmetoder och förekomsten av alternativa värdväxter. I denna studie samlades svenska prover av rajgräs in från regioner som haft problem med vetedvärgsjuka. Resultat av virustester och överföringsexperiment visar att rajgräs som växer i eller omkring fält av vintervete utgör en potentiell källa till vetedvärgvirus. Ett isolat av vetedvärgvirus fanns dessutom vara nära släkt med typiska svenska isolat av vetevärgvirus. Med den förutspådda globala uppvärmningen kan förekomsten och effekterna av infektioner med vetedvärgvirus öka eftersom virusöverföring med stritar kommer att vara möjligt under en längre tid under hösten. Vi har i denna studie fått en djupare förståelse för hur stritpopulationen fluktuerar och hur det påverkar förekomsten av vetedvärgsjuka. Resultaten tyder på att antalet stritar och förekomsten av vetedvärgsjuka är kopplade. Vi analyserade data för stritar insamlade från olika delar av Sverige och hösttemperaturer, och hösttemperaturen fanns påverka stritarnas populationsstorlek. Alla vetesorter har än så länge visat sig vara mottagliga för infektion med vetedvärgvirus. Observationer i denna

studie visade att växtens utveckling påverkades av när infektionen satte igång hos olika vetesläktingar. Mängden virus i växten verkar också påverka symptom och växtens utveckling. I denna studie har vi studerat spridningen av vetedvärgvirus hos en potentiellt virustolerant anfader till vete. Vi fann att den systemiska infektionen i denna genotyp var långsammare jämfört med andra genotyper vilket kan göra det möjligt för plantan att fortsätta växa innan viruset börjat ansamlas och då resultera i en mildare respons och tolerans för infektion med vetedvärgvirus. Det behövs fortsatta studier för att identifiera resistenskällor mot vetedvärgvirus. Med förbättrad kunskap om vetedvärgvirusets epidemiologi blir det möjligt att använda odlingsmetoder som minskar riskerna för infektion. Sammantaget har denna avhandling bidragit till en förbättrad förståelse för den komplexa epidemiologin för virus som infekterar stråsäd där många alternativa värdväxter ofta spelar en viktig roll. Kunskapen kommer att kunna utnyttjas för integrerat växtskydd mot vetedvärgsjuka.

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I

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ORIGINAL ARTICLE

Plant Pathology WILEY

Reservoirs of plant virus disease: Occurrence of wheat dwarf virus and barley/cereal yellow dwarf viruses in Sweden

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Abstract

Non-crop plants such as grasses and volunteer plants are an inseparable part of the flora of crop fields and can influence virus incidence in crop plants. The presence of grasses as virus reservoirs can lead to a higher probability of virus incidence in crop plants. However, the role of reservoirs as an inoculum source in agricultural fields has not been well studied for many viral diseases of crops. Grasses have been found to constitute potential reservoirs for cereal-infecting viruses in different parts of the world. This study revealed that cereal-infecting viruses such as wheat dwarf virus (WDV), barley yellow dwarf viruses (BYDVs), and cereal yellow dwarf virus-RPV (CYDV-RPV) can be found among ryegrass growing in or around winter wheat fields. Phylogenetic analysis showed that a WDV isolate from ryegrass was a typical WDV-E isolate that infects wheat. Similarly, a ryegrass isolate of barley yellow dwarf virus-PAV (BYDV-PAV) grouped in a clade together with other BYDV-PAV isolates. Inoculation experiments under greenhouse conditions confirmed that annual ryegrass of various genotypes can be infected with WDV to a very low titre. Moreover, leafhoppers were able to acquire WDV from infected ryegrass plants, despite the low titre, and transmit the virus to wheat, resulting in symptoms. Information from the grass reservoir may contribute to improving strategies for controlling plant virus outbreaks in the field. Knowledge of the likely levels of virus in potential reservoir plants can be used to inform decisions on insect vector control strategies and may help to prevent virus disease outbreaks in the future.

KEYWORDS
geminivirus, luteovirus, mastrevirus, ryegrass, wheat

1 | INTRODUCTION

The host range of plant viruses includes both economically important crops as well as weeds. Grasses (as non-crop plants) can be part of the natural flora or may have been introduced, and in either case, they can influence virus incidence in crop plants (Parry et al., 2012). The presence of viruses has been demonstrated for a range of weeds (Muthukumar et al., 2009; Roossinck et al., 2010), but the role of these hosts as an inoculum source has not been well studied (Duffus, 1971; Roossinck et al., 2010). The presence of virus reservoirs can lead to a higher probability of virus incidence in crop plants (Duffus, 1971). Annual and perennial grasses have been found to constitute

potential reservoirs for cereal-infecting viruses in different parts of the world (Bisnieks et al., 2006; Ramsell et al., 2008). Nevertheless, virus infections in grasses are often symptomless or only mild symptoms are observed when compared to the symptoms in crop plants (Bisnieks et al., 2006; Malmstrom et al., 2005). To control viral diseases, it is essential to study the risk factors influencing virus dissemination and how they influence virus epidemiology. The role of grasses as natural reservoirs can be of great importance and is worth further study (Clarke & Eagling, 1994; Duffus, 1971; Vacke & Cibulka, 1999).

As long as cereals have been cultivated in the world, they have probably been infected by viruses such as wheat dwarf virus (WDV; Ramsell et al., 2008) and barley/cereal yellow dwarf viruses (B/CYDVs; Walls et al., 2019). The first official report of WDV in Sweden appeared in a study in 1970 (Lindsten et al., 1970). However, infection with WDV was probably the cause of severe disease outbreaks in wheat (*Triticum aestivum*) in Sweden as early as 1912, 1915, and 1918 (Lindsten & Lindsten, 1999). Since then, the disease has appeared sporadically until 1997, when there was a larger outbreak causing extensive damage to fields of winter wheat. The reappearance of the disease can be linked with changes in agricultural practices and the presence of alternative hosts in the fields (Lindsten & Lindsten, 1999).

Wheat dwarf virus is a geminivirus belonging to the genus *Mastrevirus* and is transmitted in a persistent manner by the leafhopper *Psammotettix alienus* (Lindsten et al., 1980; Lindsten & Vacke, 1991). The genome of WDV consists of a single molecule of single-stranded (ss) DNA and encodes four proteins: movement protein (MP), coat protein (CP), and two replication-associated proteins (Rep, RepA; Kvarnheden et al., 2002). Infecting plants in the family Poaceae, including grasses, WDV constitutes a potential threat to bread wheat, barley (*Hordeum vulgare*), oat (*Avena sativa*), and triticale (Mehner et al., 2003; Ramsell et al., 2008; Vacke, 1972). While WDV infection in wheat and other grains, such as barley, results in symptoms such as dwarfing, chlorosis, and reduced number of spikes (Ramsell et al., 2008; Vacke, 1972), infected grasses are usually symptomless. However, yellow streaks have been reported for WDV-infected ryegrass (Mehner et al., 2003).

Barley yellow dwarf (BYD) associated viruses (family *Luteoviridae*) constitute an economically important virus complex that is abundant throughout the world, threatening cereal crops (Walls et al., 2019). BYD was found to be caused by virus infection in 1951 by Oswald and Houston (1951), but the BYD-associated viruses are now known to be a viral complex composed of several species in the genera *Luteovirus* and *Polerovirus* (Wu et al., 2011). Being efficiently transmitted by 28 aphid species (Harrington, 2002), these viruses affect a range of grain cereals (Walls et al., 2019). Infection by BYD-associated viruses induces symptoms such as light yellowing, reddish discolouration in the infected crop plants, and hinders normal plant growth and development.

The commonly occurring barley yellow dwarf virus-PAV (BYDV-PAV) has previously been detected in wild and cultivated grasses, including ryegrass (*Lolium* spp.), in different parts of the world (Bisnieks et al., 2004; Delmiglio et al., 2010; Malmstrom et al., 2005; Mastari et al., 1998). Annual ryegrass (*L. multiflorum*) and perennial ryegrass (*L. perenne*) are cool-season grasses that are widely cultivated as forage crops in temperate humid areas. In addition, ryegrass is frequently used as a catch crop or cover crop to reduce nutrient leakage in cereal fields (Aronsson et al., 2016). However, once ryegrass has been introduced to the field, it may remain for a long time in the field borders and is thus of interest because it may act as an important virus reservoir (Lindsten & Lindsten, 1999; Ramsell et al., 2008).

Although wild grasses and weeds can serve as inoculum sources for viruses (Ramsell et al., 2008), interaction between plant viruses, potential reservoirs, and insect vectors is poorly studied (Chen et al., 2013). Looking more into this underestimated aspect of epidemiology can shed light on controlling plant virus disease outbreaks, due to the epidemiology of a virus being significantly affected by the weed flora and insect community of the area (Duffus, 1971). To understand the complex epidemiology of cereal-infecting viruses, where many alternative hosts often play an important role, we investigated the potential role of ryegrass, a perennial crop, as an alternative reservoir for WDV and BYD-associated viruses. The ability of ryegrass to act as a virus host and source was tested by surveying the occurrence of virus infections of ryegrass in the field and subsequent analyses of viral nucleotide sequences as well as by transmission experiments with WDV using a viruliferous vector.

2 | MATERIALS AND METHODS

2.1 | Field survey

To test the potential of ryegrass to act as a virus reservoir, a plant survey was carried out in different parts of Sweden to investigate the occurrence of WDV and BYD-associated viruses (Figure 1; Table 1). In 2012, 423 samples of ryegrass were collected from land adjacent to two cereal fields close to Skara, Västra Götaland County, western Sweden. In 2013, 400 samples of ryegrass were collected from a field trial with ryegrass close to Enköping, Uppsala County, eastern Sweden, and 20 samples from a cereal field close to Sigtuna, Stockholm County, eastern Sweden. In addition, in the latter location, 61 wheat plants were sampled based on possible symptoms of wheat dwarf disease (such as dwarfing and yellowing) as well as eight random samples of timothy (*Phleum pratense*) and five random samples of couch-grass (*Elymus repens*). All these locations were chosen based on previous reports of WDV infection in the area (Kvarnheden et al., 2002; Ramsell et al., 2008). The collected leaf material was kept at −20 °C for subsequent analyses.

2.2 | Greenhouse experiment

In order to test the response of different ryegrass species to WDV infection, virus inoculation tests were carried out under greenhouse

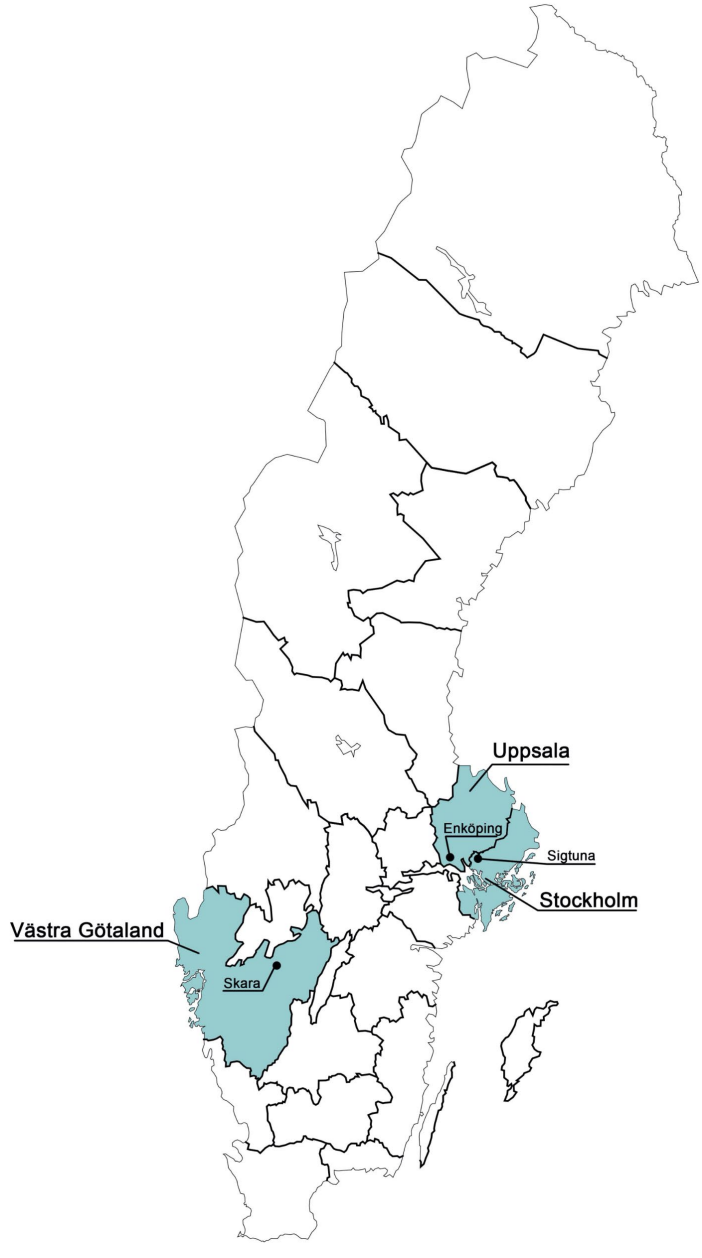

FIGURE 1 Collection sites in Sweden for sampling grasses to be tested for infection by B/CYDVs and wheat dwarf virus (WDV). Counties and the municipalities where the samplings were done are marked on the map: Enköping in Uppsala County, Sigtuna in Stockholm County, and Skara in Västra Götaland County [Colour figure can be viewed at wileyonlinelibrary.com]

conditions. Commercially available ryegrass seeds were obtained from Lantmännen SW Seed (Sweden): perennial ryegrass (*L. perenne*, 2n or 4n), Italian ryegrass (*L. multiflorum.* subsp. *italicum*, 4n), and Westervold ryegrass (*L. multiflorum* var. *westerwoldicum*, 2n or 4n). Ten seeds of each cultivar were sown in pots (10 × 10 × 10 cm) containing commercial potting compost (Hasselfors garden) and grown

TABLE 1 Detection of wheat dwarf virus (WDV), BYDV-PAV, BYDV-MAV, and CYDV-RPV by ELISA in field samples of ryegrass from Sweden

	Positive samples/total samples tested			
Collection site/year	WDV	PAV	MAV	RPV
Västra Götaland/2012	1/423	2/423	17/423	16/423
Stockholm/2013	0/20	0/20	0/20	0/20
Uppsala/2013	4/400	0/400	0/400	0/400

under greenhouse conditions (16 hr light, 22 °C during the day and 18 °C during the night). Simultaneously, wheat plants of cultivar Tarso were grown from seeds and were used as controls.

2.3 | Enzyme-linked immunosorbent assay

In order to detect infection with WDV or the BYD-associated viruses BYDV-PAV, BYDV-MAV, and cereal yellow dwarf virus-RPV (CYDV-RPV) in samples of wheat or grasses, double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) was performed using commercially available antisera (Loewe Biochemica and Bioreba). Plant leaves were homogenized in sample extraction phosphate buffer (pH 7.4), and ELISA was carried out according to Ramsell et al. (2008). The absorbance was measured at 405 nm using a Benchmark Microplate reader (Bio-Rad Laboratories). One positive (WDV-infected wheat leaf) and two negative (noninfected wheat leaves) controls were included in each 96-well microtitre plate. Samples were considered positive if the absorbance measured was at least two times higher than the value obtained for healthy controls. All positive samples underwent further analyses with PCR or immunocapture reverse transcription PCR (IC-RT-PCR) for confirmation.

2.4 | Detection of WDV infection by PCR and IC-PCR

Detection of WDV in plant extracts was done by performing PCR using total plant DNA extracted by GenElute Plant Genomic DNA Miniprep Kit (Sigma Aldrich) according to the manufacturer's protocol. For amplification, Phusion High-Fidelity DNA polymerase (Thermo Scientific) was used with PCR conditions according to the manufacturer's protocol and the primer pair 1877–1896/328–309 amplifying the long intergenic region (LIR) and 5′ ends of the *Rep* and *MP* genes of WDV (Kvarnheden et al., 2002). All the amplifications were carried out using a C1000 thermal cycler (Bio-Rad).

For detection of WDV in leafhoppers from transmission experiments, an immunocapture (IC) PCR method was used. PCR tubes were coated with WDV polyclonal antibodies, the same as those used in ELISA (1:50 vol/vol in ELISA coating buffer overnight at 4 °C). The tubes were then filled with extract of homogenized leafhoppers (ground in Tris.HCl buffer, pH 8.0) and incubated at 4 °C overnight. Tubes were washed with Tris.HCl (pH 7.5), filled with PCR master mix, and the template was used for amplification with DreamTaq Green DNA polymerase (Thermo Scientific), the primer pair 1877–1896/328–309, and PCR conditions as described by Kvarnheden et al. (2002).

2.5 | Detection of BYDV-PAV by IC-RT-PCR

To confirm the results of DAS-ELISA, positive samples from the ELISA were used for IC-RT-PCR (Bisnieks et al., 2004). For IC-RT-PCR, the same polyclonal antibodies were used for coating of tubes as for ELISA (1:150 vol/vol in ELISA coating buffer overnight at 4 °C). Plant material homogenized in phosphate-buffered saline (PBS) containing Tween was incubated overnight at 4 °C in antibody-coated tubes followed by reverse transcription using the primer Yan-R (Malmstrom & Shu, 2004) and Superscript III (Invitrogen). PCR was carried out in order to amplify the *CP* gene in a C1000 thermal cycler using Phusion High-Fidelity DNA polymerase (Thermo Scientific), the primers Yan-R and Shu-F, and the PCR protocol according to Malmstrom and Shu (2004).

2.6 | Cloning and sequencing

In order to clone the amplification products from PCR and RT-PCR, they were ligated into CloneJET cloning vector (Thermo Scientific) and transformed into *Escherichia coli* DH5α (Invitrogen) according to the manufacturer's recommendations. For each fragment, three clones were sequenced on both strands by Macrogen Inc.

2.7 | Sequence and phylogenetic analysis

The 1,162 nucleotide (nt) sequence of the WDV isolate from ryegrass (accession number MN453813), together with available WDV sequences in GenBank, were aligned using ClustalW in MEGA 6 (Tamura et al., 2013). A phylogenetic tree was constructed using the neighbour-joining method. Bootstrap analysis with 1,000 replicates was performed to test the robustness of the internal branches. Phylogenetic analyses were carried out in the same way for the BYDV-PAV isolate from ryegrass (accession number MN493946). One tree was constructed based on the complete determined sequence of 828 nt, and another was based on 502 nt, to enable other available partial sequences to be included.

2.8 | Insect material

The initial culture of *P. alienus* had been collected from wheat fields around Uppsala (Nygren et al., 2015). Cultures of viruliferous and nonviruliferous leafhoppers (*P. alienus*) were established in the greenhouse prior to the experiments. Viruliferous individuals of *P. alienus* were reared on wheat while a nonviruliferous culture was established by feeding on barley, a nonhost for wheat-infecting isolates of WDV.

2.9 | Inoculation test of ryegrass with viruliferous leafhoppers

To confirm that ryegrass could serve as a reservoir for WDV, 10 healthy wheat plants (positive control) and 10 ryegrass plants of each species (*L. perenne* [2n], *L. perenne* [4n], *L. multiflorum*, *L. multiflorum* var. *westerwoldicum* [2n], *L. multiflorum* var. *westerwoldicum* [4n]) were inoculated with WDV using viruliferous *P. alienus* leafhoppers. The leafhoppers, which had been kept on WDV-infected wheat plants (*T. aestivum*), were transferred to ryegrass test plants of different species or to wheat plants (three leafhoppers/plant) (Nygren et al., 2015), for an inoculation access period (IAP) of 7 days. In addition, two healthy plants of each species were used as negative controls, which were not exposed to leafhoppers. Plants were monitored weekly for symptoms. At 3 weeks postinoculation (WPI), test plants were checked for typical WDV symptoms and the youngest leaf of each plant was collected to be analysed by ELISA and PCR, as well as quantitative real-time PCR (qPCR).

2.10 | Detection of WDV by qPCR

In order to compare the virus titre in different ryegrass species and wheat, two WDV-inoculated plants of each species were tested by qPCR. Total DNA was extracted using GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) and analysed by qPCR using Biorad MyiQ Real-Time PCR detection system and the SYBR Green PCR Master Mix (Thermo Scientific). qPCR conditions and primers were the same as described by Benkovics et al. (2010). In addition, primers for the sequence of a second internal reference, *FT3* gene for flowering time of *L. perenne* (GenBank accession no. DQ309592), were designed and used (Ft3for 5′-CAGGAGGTGATGTGCTACGA-3′ and Ft3rev 5′-GTTGTAGAGCTCGGCGAAGT-3′). The reactions were carried out with 500 ng of total plant DNA in a final volume of 20 µl and three technical replicates of each sample. One common sample was used as a bridge between all plates analysed. For the negative controls, water or DNA from healthy plants of each genotype were added to the reaction mixtures. The data were analysed using the Pfaffl equation (Pfaffl et al., 2002). The results are presented as the relative level of the WDV *Rep* gene in test samples compared to the average C_t values of the WDV-inoculated plant (*L. perenne*; 4n) with highest C_t value (as a calibrator). In addition, series of 10-fold dilutions of mixed DNA extracts were used to generate standard curves in order to determine assay efficiency.

2.11 | Leafhopper-mediated virus transmission from WDV-infected ryegrass to healthy wheat plants

To confirm the role of ryegrass as a reservoir plant, a plant-to-plant transmission analysis was performed. For the transmission experiment, WDV-free leafhoppers were obtained by rearing them on barley. An acquisition access period (AAP) of 7 days was given to virus-free leafhoppers to feed on two WDV-infected ryegrass plants (one plant each of *L. perenne* [2n] and *L. multiflorum* with 15 leafhoppers/plant), which had been inoculated by viruliferous leafhoppers two years before. After the AAP, the leafhoppers were transferred from the ryegrass plants to eight healthy wheat plants where they were kept for a week (four leafhoppers/plant). As a control, virus-free leafhoppers were transferred to two healthy wheat plants (four leafhoppers/plant). The plants were observed for the appearance of WDV symptoms for 3 weeks. At 3 WPI, wheat plants were analysed for WDV infection by DAS-ELISA and PCR, as described above.

3 | RESULTS

3.1 | Detection of WDV and B/CYDVs in field samples using DAS-ELISA and PCR

Of 843 randomly collected ryegrass samples from three counties in Sweden (Figure 1), a total of five plants were found to be infected by WDV when tested with DAS-ELISA (Table 1): one out of 423 ryegrass samples from the county of Västra Götaland (0.2%), together with four out of 400 ryegrass samples from the county of Uppsala (1.0%). Confirming the positive results from ELISA, PCR amplification yielded a band of 1.2 kb when WDV-specific primers were used. None of the 20 ryegrass samples from the county of Stockholm were found to be WDV positive. Four out of 61 wheat samples with symptoms from the county of Stockholm were clearly positive for WDV (6.5%), confirming the presence of WDV in this region. For the WDV-negative wheat samples, the symptoms were most likely caused by the dry weather conditions. The ELISA result was negative for WDV-infection in the tested timothy (eight plants) and couch-grass plants (five plants) that were sampled from the same field as the wheat plants. The grass samples did not show any evident symptoms suggesting virus infection, and thus were not scored for symptoms in a systematic way.

BYDV-PAV infection was detected by DAS-ELISA in two out of 423 ryegrass samples from the county of Västra Götaland (Table 1), which was confirmed by IC-RT-PCR. In addition, 17 out of 423 tested ryegrass samples from the county of Västra Götaland were found to be positive for infection by BYDV-MAV and 16 samples for CYDV-RPV (Table 1). No samples from Uppsala County (400 samples) or Stockholm County (20 samples) tested positive for BYDV-PAV, BYDV-MAV, or CYDV-RPV.

3.2 | Sequence and phylogenetic analyses of WDV and BYDV-PAV

The partial sequence of one WDV isolate from ryegrass originating from the county of Västra Götaland was determined. The 1.2 kb PCR fragment displayed 98%–99% identity to previously sequenced wheat-infecting isolates of the WDV-E strain. The WDV isolate from ryegrass displayed the highest nucleotide

identity (99%) to three Swedish WDV isolates of different origin (AM491489, *P. alienus*; AJ311037, *T. aestivum*; AM491481, *Apera spica-venti*).

A phylogenetic analysis was carried out, including the partial nucleotide sequence (5′ ends of *Rep*/*RepA* and *MP*, as well as the complete LIR) of the ryegrass isolate (WDV-E[SE:ryegrass:2012]) and available sequences of WDV isolates (Figure 2). The isolates belonging to the strains WDV-A and WDV-E formed two well-supported clades (bootstrap value 100%) with the ryegrass isolate grouping in WDV-E. The diversity among isolates within the WDV-E strain was confirmed to be very low and no grouping based on geographic origin or host species was formed. The close relationship between WDV isolates of different hosts indicate that WDV can be transmitted between wheat and grasses.

The *CP* gene of one BYDV-PAV isolate from an infected ryegrass sample (BYDV-PAV-Skara) was partially sequenced (828 nt) and analysed. The sequence was 99% identical to BYDV-PAV isolates in GenBank, and showed 95.6% identity to the BYDV-PAV isolates FL3-PAV (AJ223587) and Priekuli2 derived from ryegrass (AJ563414). In the phylogenetic analysis, BYDV-PAV-Skara clustered closely with BYDV-PAV isolates from different hosts and geographic origin (Figure 3). The same grouping was observed when the sequence was analysed together with available shorter BYDV-PAV sequences (502 nt) derived from different hosts, including ryegrass (Figure S1). According to these analyses, no significant correlation between the isolates, their host plant, and their geographic origin was observed (Figure 3; Figure S1).

3.3 | WDV detection in inoculated ryegrass by DAS-ELISA, PCR, and qPCR

Plants of five species of ryegrass exposed to viruliferous leafhoppers in the WDV-inoculation experiment tested negative by ELISA (Table 2; Table S1). The mean absorbance values of these plants were similar to nonexposed plants of the same species (negative controls).

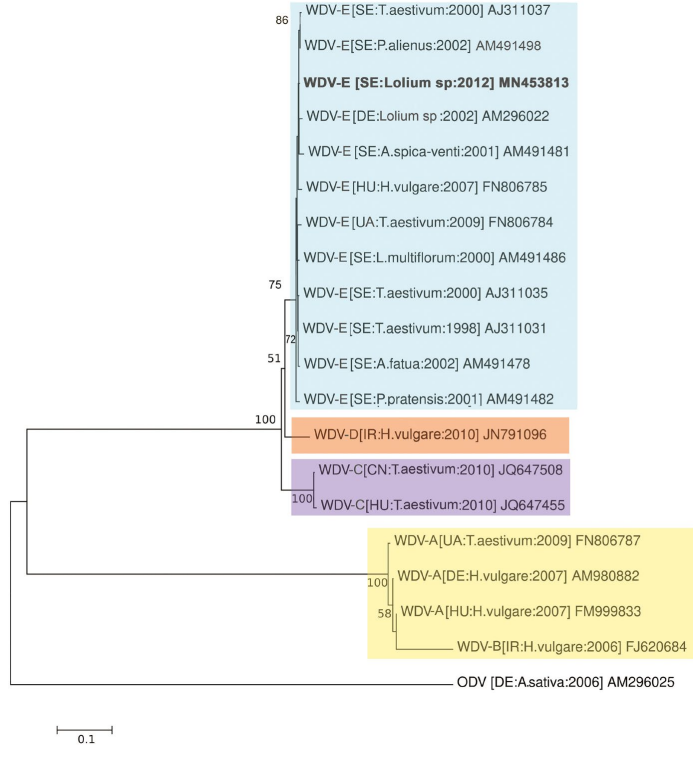

FIGURE 2 Neighbour-joining analysis showing predicted relationships between wheat dwarf virus (WDV) isolates from ryegrass (including isolate SE:Lolium sp.:2012 from this study, marked in bold) and other hosts based on nucleotide sequences (1,162 nucleotides) of the complete long intergenic region and partial *Rep* and *MP* genes. An isolate of oat dwarf virus (ODV-[DE:A.sativa:2006]) was used as an out-group. Numbers represent the percentages of bootstrap replicates that support each node (1,000 replicates). Only bootstrap values >50% are shown. The scale shows nucleotide substitutions per site [Colour figure can be viewed at wileyonlinelibrary.com]

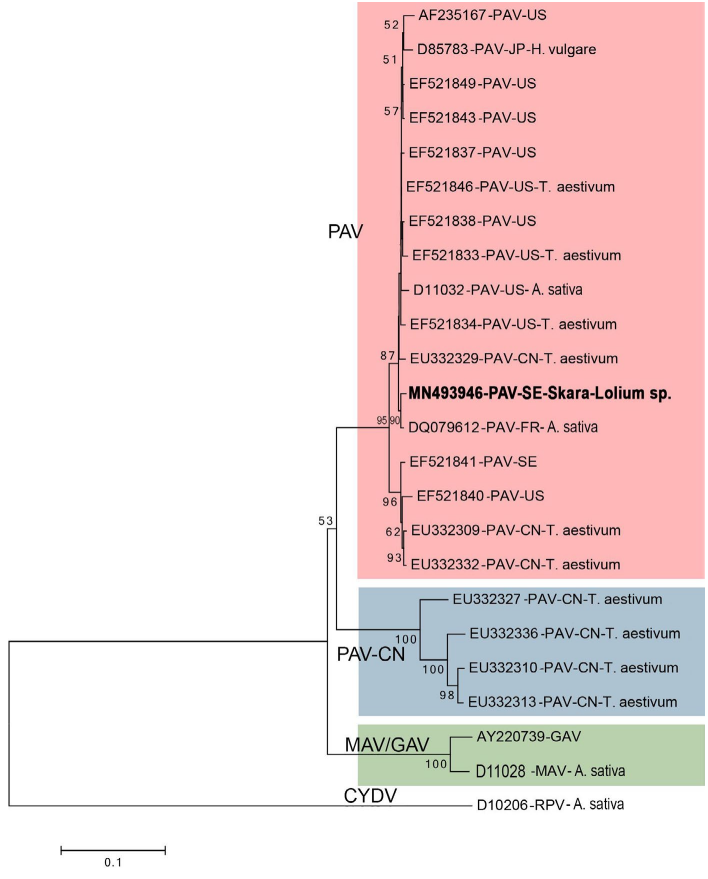

FIGURE 3 Neighbour-joining analysis showing predicted relationships between isolates of barley yellow dwarf virus (BYDV) from ryegrass (including isolate BYDV-PAV-SE-Skara-Lolium sp. from this study, marked in bold) and other hosts based on nucleotide sequences (828 nucleotides) of the *CP* gene. An isolate of cereal yellow dwarf virus-RPV (CYDV-RPV-A.sativa) was used as an out-group. Numbers represent the percentages of bootstrap replicates that support each node (1,000 replicates). Only bootstrap values >50% are shown. The scale shows nucleotide substitutions per site [Colour figure can be viewed at wileyonlinelibrary.com]

These plants showed no typical wheat dwarf disease symptoms (Figure 4), but three individual plants were found to be positive by PCR (one each of *L. multiflorum*, *L. multiflorum* var. *westerwoldicum* [2n], and *L. multiflorum var. westerwoldicum* [4n]). Negative controls from each species were confirmed to be virus-free by both DAS-ELISA and PCR. Wheat plants used as positive controls for the experiment showed high absorbance values by DAS-ELISA and infection was also confirmed by PCR. The inoculation efficiency of the wheat plants was quite high (80%).

To compare the DNA levels of WDV in plants from the inoculation experiment, qPCR assays were carried out. Two inoculated plants of each species were used in the tests. Analyses of the results from the relative quantification of the *Rep* gene in WDV-inoculated wheat samples showed much higher virus titres compared to ryegrass (Table 2). Importantly, this analysis also confirmed that WDV accumulated in some ryegrass species, including *L. multiflorum*, *L. multiflorum* var. *westerwoldicum* (2n), and *L. multiflorum* var. *westerwoldicum* (4n), but to much lower levels compared to wheat. However, variation in WDV titre was observed among the ryegrass species, with samples of Westervold ryegrass 2n showing comparatively higher titre of the virus (Table 2). The measured relative level of WDV DNA in plants of *L. perenne* (2n) and *L. perenne* (4n) was very close to that of the healthy control, suggesting that they were not infected (Table 2; Table S2). For the qPCR analyses, similar results were obtained when the *FT3* gene was used as an internal control instead of 25S rDNA (data not shown).

TABLE 2 Wheat dwarf virus (WDV) titre in plants assessed by ELISA and relative level of the WDV *Rep* gene determined by quantitative PCR

Plant sample	ELISA value[a]	Relative WDV value (*SD*)[b]
Lolium perenne (2n)	0.183	12 (2.7)
L. perenne (4n)	0.154	1 (0.3)
Lolium multiflorum	0.181	128 (9.0)
L. multiflorum var. *westerwoldicum* (2n)	0.195	13,682 (0.0)
L. multiflorum var. *westerwoldicum* (4n)	0.185	10,226 (10.2)
Triticum aestivum	1.931	59,064 (2.5)

[a]Absorbance value at 405 nm.

[b]Value in relation to that of the WDV-inoculated plants with the highest C_t value (*L. perenne*, 4n).

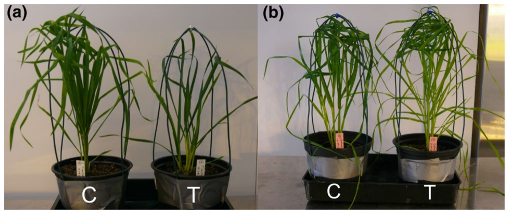

FIGURE 4 Response of wheat (*Triticum aestivum*) (a) and ryegrass (*Lolium multiflorum* var. *westerwoldicum*, 4n) (b) plants to inoculation by wheat dwarf virus (WDV) using viruliferous leafhoppers. T, plant inoculated with WDV; C, noninoculated plant. Pictures were taken 26 days postinoculation [Colour figure can be viewed at wileyonlinelibrary.com]

3.4 | Vector ability to transmit WDV from ryegrass to wheat

In order to assess the ability of *P. alienus* to transmit WDV from ryegrass to wheat, a transmission experiment was conducted. Two wheat plants showed typical WDV symptoms while the rest (six plants) were symptomless even at 3 WPI (Figure 5). Analysis of the inoculated wheat plants by PCR and ELISA confirmed WDV infection in the plants with symptoms, while symptomless plants were negative. Moreover, analyses of the leafhoppers used in this experiment by PCR showed weak bands of the expected fragment size in two out of five pooled samples.

4 | DISCUSSION

The observations in this study demonstrate that WDV, BYDV-PAV, BYDV-MAV, and CYDV-RPV can be found among ryegrass plants

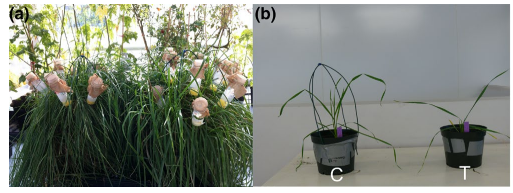

FIGURE 5 Transmission of wheat dwarf virus (WDV) from ryegrass to wheat using viruliferous leafhoppers confirming that ryegrass can serve as a reservoir for WDV. (a) Two ryegrass plants (*Lolium perenne*, *L. multiflorum*) used as a source for WDV and the method used for WDV acquisition by leafhoppers from ryegrass; (b) wheat plants at 23 days after exposure to leafhoppers. C, control plant exposed to WDV-free leafhoppers; T, test plant exposed to leafhoppers carrying WDV [Colour figure can be viewed at wileyonlinelibrary.com]

growing in and around winter wheat fields. These findings are consistent with previous reports on the detection of WDV in some grass species, such as *Poa annua*, *P. pratensis*, and *Avena fatua* (Lindsten & Lindsten, 1999; Ramsell et al., 2008). Finding virus-infected plants to be present in ryegrass, which is a very common agricultural break crop over an extensive area, is very important for the dynamics of cereal viruses. Although the number of infected plants and the titre are both relatively modest in this study, the area of ryegrass under cultivation is large. Thus, it indicates that ryegrass being grown as a crop, or present in field margins and as volunteers in the crop, may well be a significant source of outbreaks in cereal crops. This study also identified the virus present to be a common type from crop outbreaks, further serving to reinforce this point. The small number of couch-grass plants tested in this study were not found to be infected by WDV, which supports the result from a study by Lindsten and Lindsten (1999) where they suggested that couch-grass is not a host for WDV. Similarly, in one previous study, none of the samples of timothy carried WDV (Ramsell et al., 2008), which is consistent with our results.

In this study, BYDV-PAV, BYDV-MAV, and CYDV-RPV were all detected in field samples of ryegrass from Västra Götaland County, which could be expected, as B/CYDVs have been shown to be common in grasses in different parts of the world (Bisnieks et al., 2006; Clarke & Eagling, 1994; Delmiglio et al., 2010). Infections by BYD-associated viruses were not detected at the locations in the counties of Uppsala and Stockholm, which could be due to factors such as low virus titre in the grass samples, resistance to these viruses in the grasses present (Bisnieks et al., 2006), or absence of viruliferous aphids.

A close relationship of virus isolates from ryegrass and wheat was found by phylogenetic analyses, where the sequences of the two ryegrass isolates from this study, WDV-E[SE:ryegrass:2012] and BYDV-PAV-Skara, grouped closely with sequences of isolates from other hosts, including wheat. Previously, we have also identified the same genotype of WDV in ryegrass and wheat in the field (Ramsell

et al., 2008), clearly indicating that the same virus isolates may infect both hosts.

The inoculation experiments under greenhouse conditions confirmed that annual ryegrass of various genotypes can be infected with WDV. Often, infections of grasses with WDV or B/CYDVs do not induce clear symptoms (Mehner et al., 2003; Parry et al., 2012), but the virus infection may still result in reduced fitness (Alexander et al., 2017). In ryegrass, WDV infection did not induce any typical disease symptoms, possibly due to a very low titre of the virus compared to infected wheat. Nevertheless, ryegrass remained infected with WDV for two years and could act as a source for infection of wheat after leafhopper transmission. The observed reduced rate of WDV transmission from ryegrass to wheat (2/6 plants) was probably a result of the low concentration of virus inoculum in ryegrass plants resulting in the vector not acquiring the virus. These results demonstrate that ryegrass is a likely reservoir host for the virus and that leafhoppers can feed on the ryegrass and then later transfer the virus to adjacent cereal fields.

The failure to detect the virus by ELISA in inoculated ryegrass plants suggests that this serological method is not sufficiently sensitive to react to the low virus concentration in grasses. In such circumstances, PCR and qPCR are more reliable methods (Ingwell & Bosque-Pérez, 2015). Accordingly, relative quantification of the WDV titre in inoculated wheat and ryegrass plants evidently confirmed that the WDV titre is much lower in inoculated grass compared to infected cereal. A low titre of virus inoculum in grasses compared to cereals has been reported previously in the case of BYDV-PAV (Delmiglio et al., 2010). However, the transmission experiments show that even with a low virus titre, grass may still play a role in virus dissemination.

Taken together, the results of this study reveal the role of grasses as a reservoir for viruses within the arable landscape, through weeds or undersown cereal crops, although the virus infection in grasses may not affect the crop production directly. These grasses are the only host left in the field after harvest, which means they become the primary feeding source for the insect vectors and a reservoir for the virus (Duffus, 1971). According to the results obtained in this study, the potential role of ryegrass in the epidemiology of WDV (Lindsten & Lindsten, 1999) as a symptomless reservoir has been proven. Additionally, the presented results emphasize the broad host range of these viruses, that on the one hand may contribute to their wide distribution and on the other hand enables them to stay in the field between growing seasons (Duffus, 1971). Serving as a reservoir, grasses can act as a green bridge and cause subsequent infection of the crop (Clarke & Eagling, 1994), making them a key component in plant-virus ecology (Duffus, 1971). Recently, this was demonstrated for the role of several grass species, including *L. multiflorum*, as a grass reservoir for wheat streak mosaic virus (WSMV; Chalupnikova et al., 2017). In the future, WDV and BYD-associated virus infections could become more widespread compared to the results presented in this study. Predicted climate changes, particularly a prolonged autumn (Roos et al., 2011), will favour the reproduction of aphids (Fargette et al., 1982) and leafhoppers, which may result in severe outbreaks (Lindsten & Lindsten, 1999). It is suggested that climate change can also have an impact on the pattern of insect movements between grasses and cereals (Fargette et al., 1982). An improved understanding of the ecology of cereal virus transmission will be of great value in predicting the occurrence and severity of these crop diseases.

The major unique finding of this study was the demonstration of the importance of ryegrass as a cereal virus reservoir. The results suggest that *P. alienus* is able to acquire WDV from WDV-infected ryegrass plants and transmit it to wheat plants, proving the capacity of ryegrass plants to act as a reservoir for WDV, although many questions still remain. This information can be used to develop strategies to control virus-induced diseases and may help to understand and prevent disease outbreaks (Ingwell & Bosque-Pérez, 2015). Further studies might be useful to identify other reservoirs of WDV and BYDV, growing close to cereal fields, to control virus outbreaks. Our results suggest that removing grasses acting as reservoirs can impair their role and help eradicate the cereal viruses. Moreover, it may be worth studying resistance to WDV and B/CYDVs in cultivated ryegrass to select or breed for cultivars with high levels of resistance. This could result in a great reduction of viral infection in cereal fields.

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DATA AVAILABILITY STATEMENT

The sequences determined in this study were deposited in GenBank under the accession numbers MN453813 and MN493946. Additional data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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In Sweden and many parts of Europe, wheat dwarf virus (WDV) causes disease in cereals. The virus is transmitted by leafhoppers. Here we looked at the role of ryegrass as a reservoir for WDV. We found variation in symptoms development in WDV-infected bread wheat and its ancestors. A relationship was demonstrated between leafhopper populations, disease incidence and temperature in autumn. These results will help to get a deeper understanding of WDV epidemiology and management of the disease.

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