

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

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Funding information

Swedish Farmers' Foundation for Agricultural Research

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

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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 discoloration 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

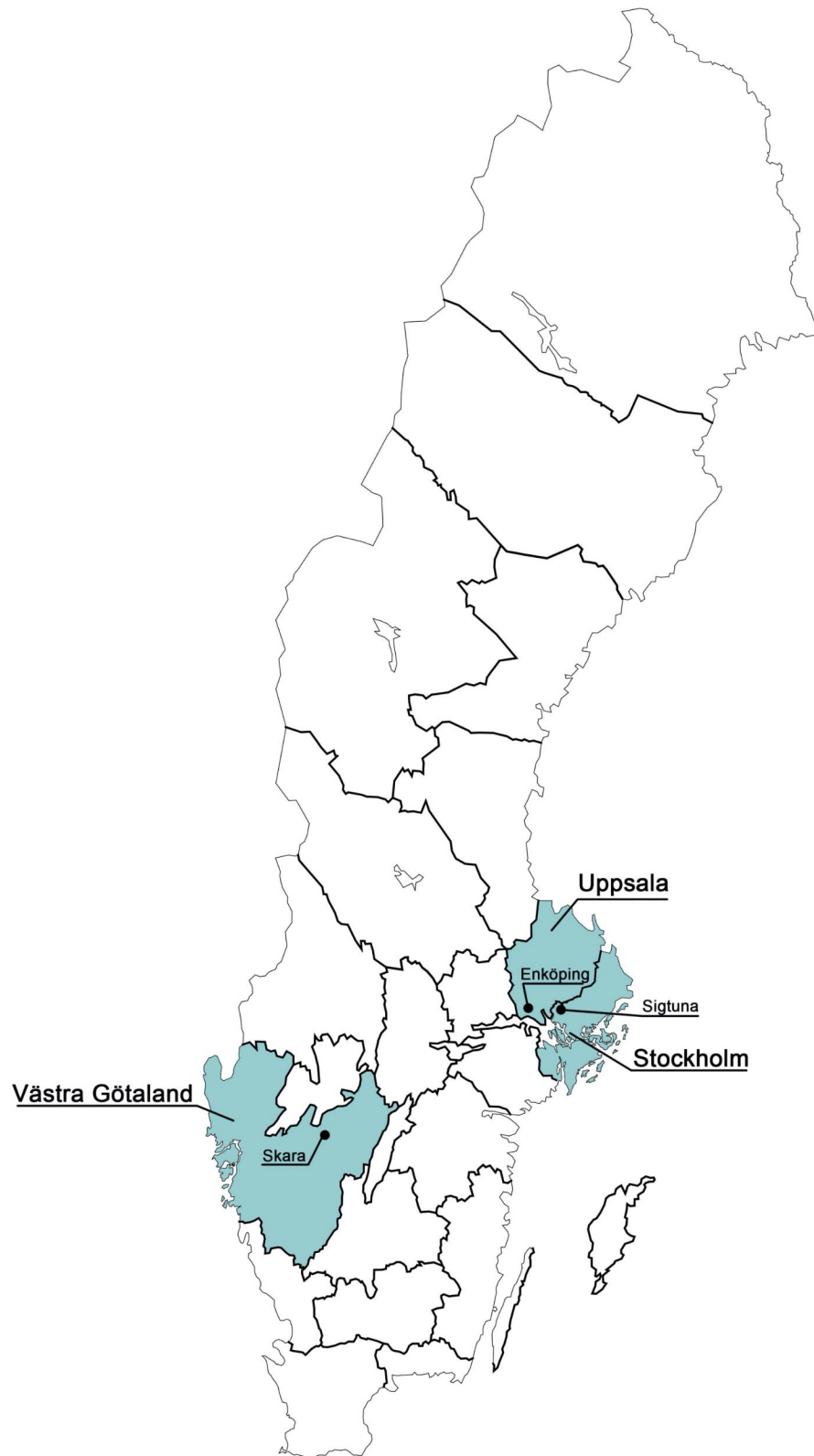


FIGURE 1 Collection sites in Sweden for sampling grasses to be tested for infection by B/CYDVs and wheat dwarf virus (WDV). Counties and the municipalities where the samplings were done are marked on the map: Enköping in Uppsala County, Sigtuna in Stockholm County, and Skara in Västra Götaland County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

Collection site/year	Positive samples/total samples tested			
	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* DH5 α (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).

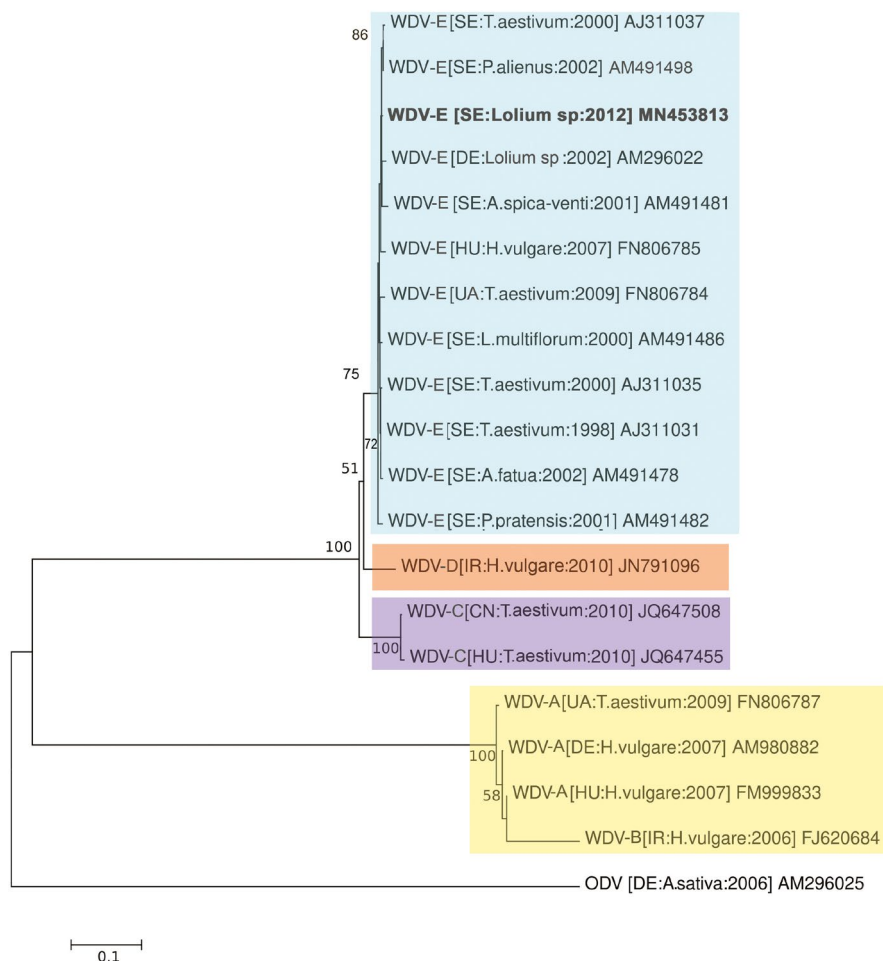


FIGURE 2 Neighbour-joining analysis showing predicted relationships between wheat dwarf virus (WDV) isolates from ryegrass (including isolate SE:Lolium sp.:2012 from this study, marked in bold) and other hosts based on nucleotide sequences (1,162 nucleotides) of the complete long intergenic region and 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]

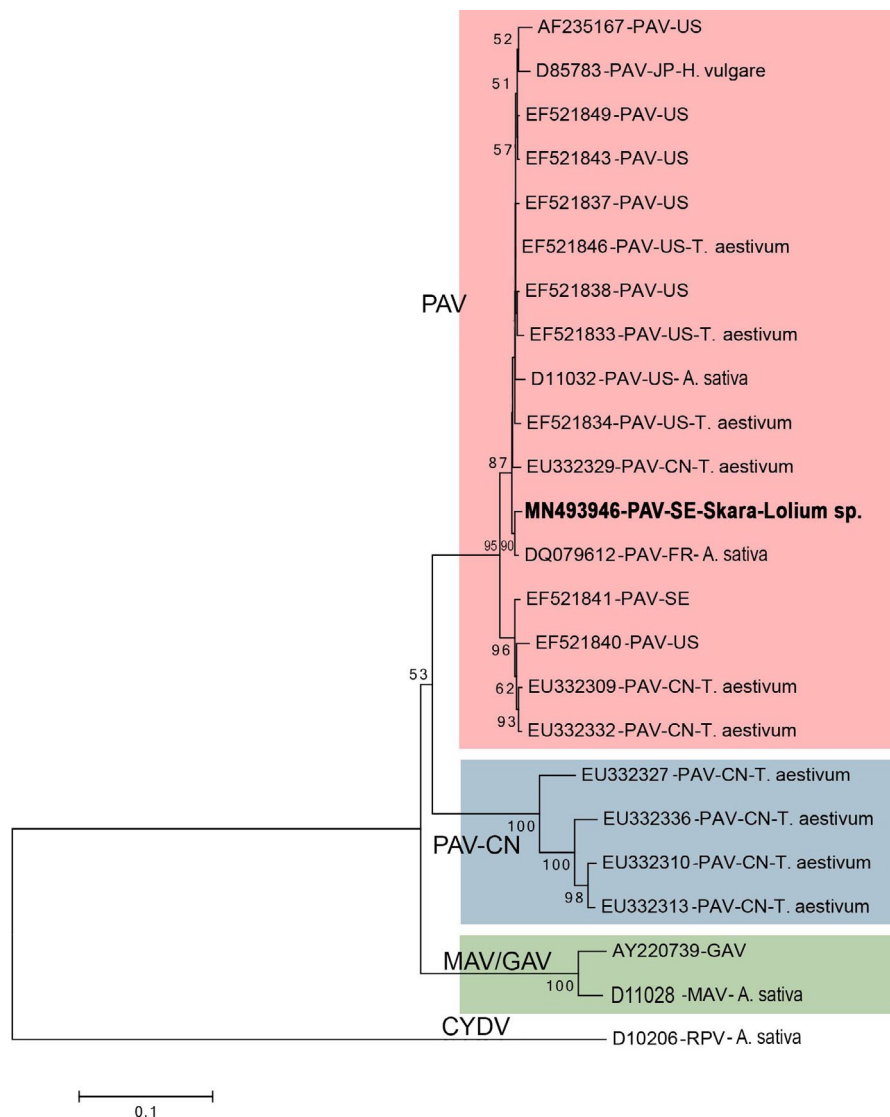


FIGURE 3 Neighbour-joining analysis showing predicted relationships between isolates of barley yellow dwarf virus (BYDV) from ryegrass (including isolate BYDV-PAV-SE-Skara-Lolium sp. from this study, marked in bold) and other hosts based on nucleotide sequences (828 nucleotides) of the CP gene. An isolate 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
<i>Lolium perenne</i> (2n)	0.183	12 (2.7)
<i>L. perenne</i> (4n)	0.154	1 (0.3)
<i>Lolium multiflorum</i>	0.181	128 (9.0)
<i>L. multiflorum</i> var. <i>westerwoldicum</i> (2n)	0.195	13,682 (0.0)
<i>L. multiflorum</i> var. <i>westerwoldicum</i> (4n)	0.185	10,226 (10.2)
<i>Triticum aestivum</i>	1.931	59,064 (2.5)

^aAbsorbance value at 405 nm.

^bValue in relation to that of the WDV-inoculated plants with the highest C_t value (*L. perenne*, 4n).

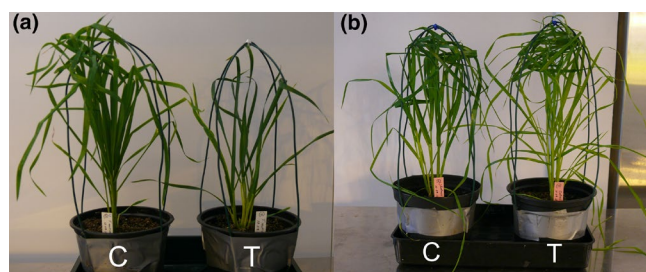


FIGURE 4 Response of wheat (*Triticum aestivum*) (a) and ryegrass (*Lolium multiflorum* var. *westerwoldicum*, 4n) (b) plants to inoculation by wheat dwarf virus (WDV) using viruliferous leafhoppers. T, plant inoculated with WDV; C, noninoculated plant. Pictures were taken 26 days postinoculation [Colour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

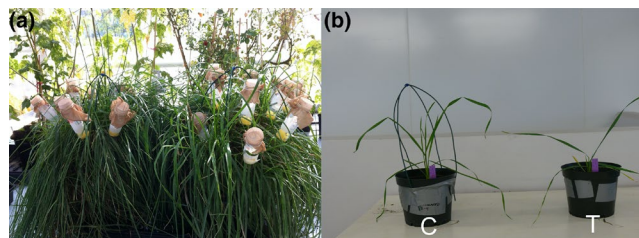


FIGURE 5 Transmission of wheat dwarf virus (WDV) from ryegrass to wheat using viruliferous leafhoppers confirming that ryegrass can serve as a reservoir for WDV. (a) Two ryegrass plants (*Lolium perenne*, *L. multiflorum*) used as a source for WDV and the method used for WDV acquisition by leafhoppers 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.

ACKNOWLEDGEMENTS

We gratefully acknowledge Ingrid Eriksson, Louise Lidemalm, Lotta Gashi, and Ludivine Castan for technical assistance, as well as Dr Jim Nygren for help with maintenance of the leafhopper culture. The authors also thank the Swedish Board of Agriculture for assistance with sample collection and Lantmännen SW Seed for providing ryegrass seeds. This study was supported by a grant from the Swedish Farmers' Foundation for Agricultural Research.

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.

How to cite this article: Yazdkhasti, E., Hopkins R. J. & Kvarnheden A. (2021) Reservoirs of plant virus disease: Occurrence of wheat dwarf virus and barley/cereal yellow dwarf viruses in Sweden. *Plant Pathology*, *70*, 1552–1561. <https://doi.org/10.1111/ppa.13414>