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*Wheat dwarf virus*  
**Interaction with Ancestors of  
Wheat**

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# ***Wheat Dwarf Virus*** **Interaction with Ancestors of Wheat**

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## POPULAR SCIENCE:

Around 10,000 years ago, wheat was domesticated in the Near East to benefit human needs. During this process, some of the traits which were present in the wild relatives and ancestors may have been lost. Wheat dwarf disease is a threatening disease to wheat in Sweden as well as other countries in Europe and Asia. It is caused by *Wheat dwarf virus* (WDV). The pathogen belongs to the family *Geminiviridae* and genus *Mastrevirus*. WDV is transmitted by the leafhopper *Psammotettix alienus*. To encounter the virus infection, plants have several defense mechanisms leading to varying levels of resistance or susceptibility. Similarly, wheat and its closest relatives differ in susceptibility to WDV. The experiment was designed to look for resistance or tolerance in the wild ancestors of wheat since it was assumed that during the domestication of wheat, the resistance genes may have been lost if there was no selection for them. Parental lines, which may carry lost resistance genes could be used as the resistance sources for breeding. The outcome would be reduced use of pesticides by farmers whose wheat cultivation is threatened by WDV infection. Virus isolates used for infection tests were analyzed and confirmed to belong to the wheat strain of WDV and they showed a close relationship with previously characterized WDV isolates from Sweden. The result of these assays revealed that the virus transmission rate was low, probably because the source plants used were not infected or had low virus titer. However, it was confirmed that two wheat ancestors *Tritium urartu* and *Aegilops tauschii* are vulnerable to WDV infection.

## ABSTRACT

Among the wheat diseases, wheat dwarf caused by *Wheat dwarf virus* (WDV) has resulted in damage to wheat production in the past years. This virus from the family *Geminiviridae* and genus *Mastrevirus* is transmitted by the leafhopper *Psammotettix alienus*. It has a genome of single stranded DNA (ssDNA) which can be replicated by means of the host replication system. Plants use several mechanisms to confront virus infection, including RNA silencing, hypersensitive response (HR) and DNA methylation. The intention of the study was to identify resistance/tolerance or reduced susceptibility against WDV in wild ancestors of wheat (*Triticum* spp. and *Aegilops* spp.), collected from the Middle East, which are supposed to carry resistance or tolerance against WDV. It was hypothesized that there are differences in susceptibility to WDV among wheat and its ancestors. During the domestication of wheat, the resistance genes may have been lost if there was no selection for them. To start, plants from three species (*T. aestivum*, *T. urartu*, *Ae. tauschii*) were inoculated with WDV using viruliferous leafhoppers (collected from WDV-affected fields close to Uppsala) in two experiments. After the inoculation period, the samples were harvested at different time points. The leaf tissues from the collected samples were tested by Double antibody sandwich ELISA (DAS-ELISA) to determine the virus titer. The result of DAS-ELISA on both source plants and samples revealed that since not all the leafhoppers were viruliferous, the inoculation tests were not successful and the hypothesis could not be tested properly. However, it was confirmed that *T. urartu* and *Ae. tauschii* are susceptible to WDV infection. The source plants were tested for WDV infection by PCR and RCA which did not show WDV infection in all of them. The virus isolate was also sequenced and compared with the GenBank database. The result confirmed that the virus isolates used for virus transmission were typical for the WDV wheat strain, with 99% nucleotide identity to the isolate Enköping 1 (Accession number AJ311031.1, GenBank).

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<b>Phrase</b>	<b>Abbreviation</b>
Movement protein	MP
Coat protein	CP
Double stranded DNA	dsDNA
Single stranded DNA	ssDNA
Maize streak virus	MSV
Wheat dwarf virus	WDV
Open reading frames	ORFs
Intergenic regions	IR
Small interfering RNA	siRNA
Hypersensitive response	HR
Systemic acquired resistance	SAR
Recombination-dependent replication	RDR
Origin of replication	Ori
Large intergenic region	LIR
Short intergenic region	SIR

## Introduction

Wheat as a prominent crop is not only a big portion of human food pyramid, but also a symbol of culture and history (Hovhannisian et al., 2011), but its production has been always invaded by various pathogens such as Wheat Dwarf virus. The cultivation of wheat, a significant crop included in human's diet, is affected by many pathogens, including WDV.

In this experiment, we aimed to characterize the response of two wheat ancestors to infection by WD. The results of this study could be implemented in future breeding study and also it can result in reduced use of pesticides. In order to achieve our goal, the relationship between three organisms was studied in this project: a host plant (wheat), a pathogen (*Wheat dwarf virus*) and a vector (leafhopper). The host plant was confronted by the pathogen via the vector. In this way, the susceptibility of different host plants to pathogen attack was studied.

### Host plant – wheat

Cereals or grasses from the family Poaceae are often considered as the most economically important crops, including maize, rice, sorghum, barley and wheat. Wheat is a staple food all over the world and it was among the first domesticated crops.

Wheat domestication occurred around 10,000 years ago in the Near East in the Fertile Crescent (center of origin), which encompasses the eastern Mediterranean, southeastern Turkey, northern Iraq and western Iran, and the neighboring regions of the Transcaucasus, and northern Iran (Charmet, 2011; Matsuoka, 2011). Naturally, plants are resistant to pathogen invasion by having innate defense mechanisms, unless a pathogen overcomes the plant defenses (Staskawicz, 2001). During domestication plants have been adapted to the agroecosystems by selection for properties, which were present in their wild ancestors. Moreover, some traits and genes may also have been lost that could be found in their wild relatives.

The hexaploid bread wheat *Triticum aestivum* (with the genome of AABBDD) has evolved by crosses of several species with various numbers of chromosomes and by polyploidization events. *Triticum urartu* is assumed as the origin of genome A in bread wheat (Caballero et al., 2009), while diploid *Aegilops tauschii* provided the D chromosome (Matsuoka, 2011). Thus, *Aegilops* and *Triticum* species have been widely applied in wheat breeding as rich genetic

resources, since they are assumed as donors of gene contributing resistance to different pathogens (Hovhannisian et al. 2011).

Widely scattered in Armenia, Iran, Iraq, Lebanon, and Turkey, *T. urartu* is highly susceptible to most of the fungal diseases. On the contrary, through a gene flow from *Ae. tauschii*, leaf rust resistance gene has been added to the wheat gene pool (Gill et al., 2006), so it may be a source of resistance or partial resistance/tolerance to viral infection as well.

## **Pathogen – virus**

Since the plants emerged on earth, they have always encountered biotic and abiotic stresses (Leke, 2010). Among the biotic stresses, viruses (defined by the Dutch microbiologist Martinus Willem Beijerinck in 1889) have been invading many fields and causing various diseases in economically important crops (Ramsell, 2007).

Viruses are obligate parasites, biotrophs, so they live inside the plant without killing their host, since these viruses depend on the plant for multiplication. So far, 90 plant virus genera have been recognized by the International Committee on Taxonomy of Viruses (ICTV) (King et al., 2012).

Most of the plant viruses have RNA genomes, but there are also DNA viruses infecting plants. Plants possess induced mechanisms to confront viruses, including RNA silencing, using small interfering RNA (siRNA) in response to double-stranded RNA (dsRNA) of the virus as well as hypersensitive response (HR)/systemic acquired resistance (SAR) (Leke, 2010).

Cereal cultivation has been facing major yield losses due to infection by various pathogens. Wheat has also encountered devastating diseases caused by different viruses such as *Wheat dwarf virus* (WDV), pathogen of the family *Geminiviridae*, an exceptional plant virus family with DNA genome.

## **Family *Geminiviridae***

Plant viruses can be divided into RNA viruses and DNA viruses and the family *Geminiviridae* belongs to the DNA viruses. Members of family *Geminiviridae* replicate through rolling circle mechanism, however new variants emerge via recombination-

dependent replication (RDR) (Jeske et al., 2001). Geminiviruses encode a few proteins such as the replication associated protein (Rep), although they completely depend on their host for replication (Rojas et al., 2005). Geminiviruses possess a genome of circular single stranded DNA (ssDNA) and are encapsidated by coat protein (CP) forming geminate virions (22 nm by 38 nm) (Brown et al., 2012), two incomplete pairs of twin particles as the result of partial fusion of two quasi-icosahedral halves (Levy and Tzfira, 2010; Boulton, 2002). Geminivirus particles were isolated first in 1974 by Mumford trying to purify *Beet curly top virus*. Geminiviruses are relatively smaller than other viruses (Ramsell, 2007).

Causing devastating disease on many crops, geminiviruses are potentially capable of exchanging their genetic material; hence recombination can widely occur among them which results in changing the infection phenotype (Monci et al., 2002). New recombinant viruses are still emerging through mixed infections (Karkashian et al., 2011), and it has also been detected for WDV (Ramsell et al., 2009). In Sweden mixed infection has been confirmed by detecting two WDV genotypes in wheat and *Psammotettix alienus* (Ramsell et al., 2008).

According to their genome components, they are divided into two groups: monopartite geminiviruses with one ssDNA molecule and bipartite geminiviruses with two ssDNA molecules (Levy and Tzfira, 2010). During evolution, the viral genomic molecule has doubled and specialized to different functions: one molecule for replication and one for movement (Astier et al., 2007).

Various factors should be taken into consideration when dividing geminiviruses into different genera and species. Among these properties the number of DNA components, open reading frames (ORFs) and also intergenic regions (IR) can be pointed out (Ramsell, 2007). Based on their genome composition, vector taxon and host range, more than 200 species of geminiviruses (Fauquet et al., 2008) are classified into four genera: 1) *Begomovirus*, 2) *Curtovirus*, 3) *Mastrevirus*, 4) *Topocuvirus* (Brown et al., 2012).

A monopartite geminivirus, coming from the genus *Mastrevirus*, infecting plants of the Poaceae and transmitted by leafhoppers, has been considered as the progenitor of this family. Later on the geminiviruses have diversified by becoming capable of infecting dicot plants (host specific) and being transmitted by whiteflies (Astier et al., 2007).

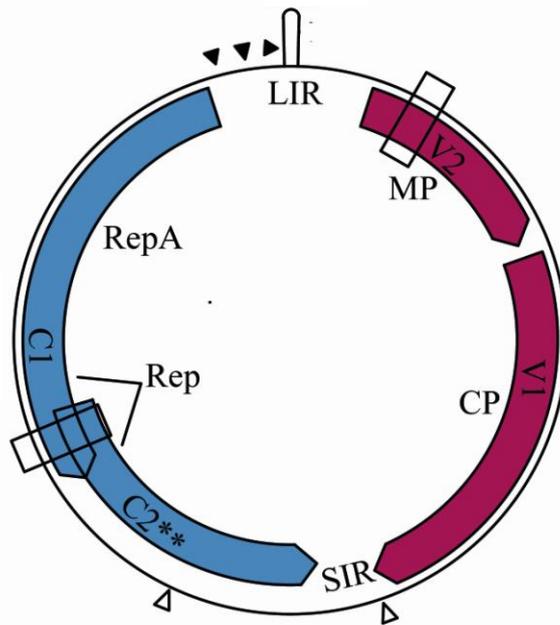
From a biological point of view, some of the viruses in the family *Geminiviridae* are phloem limited while some of them such as mastreviruses can infect various cells (Lazarowitz, 1992).

Infection by geminiviruses can cause various symptoms in the plants. Dramatic reduction in photosynthesis, plant growth and quality of the fruit combined with dwarfism, mosaic pattern, leaf curling and yellowing are among the symptoms geminiviruses can cause in the infected plants (Salimi et al., 2010; Legg et al., 2011).

### ***Mastrevirus*; genome organization and life cycle**

The genus *Mastrevirus* includes monopartite geminiviruses, which are transmittable via leafhoppers (Ramsell, 2007). The genus contains so far 11 accepted species and 6 tentative species that mostly infect monocotyledonous plants. There are also some dicotyledon-infecting mastreviruses, such as *Tobacco yellow dwarf virus* (TYDV) and *Bean yellow dwarf virus* (BeYDV) (Kvarnheden et al., 2002), and additional species have also recently been identified (Nahid et al., 2008). *Maize streak virus* (MSV), the type member of the genus *Mastrevirus* and from which it acquired its name, causes a devastating disease of maize in Africa (Efronet al., 1989). Another member of this genus is WDV, which is a pathogen of wheat and barley causing severe yield losses in many countries, including Sweden (Kvarnheden et al., 2002). While many begomoviruses are bipartite and have two genome components, mastreviruses are monopartite, with a single genome component of 2.6 to 2.8 kilo bases (kb) (Gafni et al., 2002; Ramsell, 2007). Mastreviruses replicate by means of an intermediate of double stranded DNA (dsDNA), which is used as a mediator for bidirectional transcription (Liu et al., 2001a). Replication takes place in the host plant cell nucleus, through a rolling circle mechanism (a replication structure through which several copies of the genome can be made) (Rojas et al., 2005).

The genome has two orientations, complementary sense and virion sense, which include four ORFs separated by two non-coding regions (Briddon et al., 2010). The mastrevirus genome can be translated into four viral proteins. Two of the ORFs, V2 and V1, on the virion-sense encode viral movement protein (MP) and coat protein (CP), respectively, while the other two ORFs, C1 and C2, located on complementary-sense, encode the replication-associated proteins Rep and Rep A (Fig. 1) (Dickinson et al., 1996; Kvarnheden et al., 2002; Ramsell, 2007).



**Fig. 1.** Mastrevirus genome composition (Boulton, 2002), LIR: long intergenic region, MP: movement protein, CP: coat protein, SIR: short intergenic region, Rep: replication protein, RepA: replication associated protein A.

The CP is not only an essential element for systemic infection, but it also plays a crucial role in insect transmission, systemic virus movement (Dickinson et al., 1996; Liu et al., 2001a) and encapsidation of viral DNA. The functions of the CP have been mostly studied in detail for MSV (Liu et al., 2001a). In a study by Mullineaux et al. (1988), the V1 product (CP) was detected in infected plant cells. The V2 product (MP) is a 10.9 kD a protein, which is a movement protein involved in cell to cell movement of the virus (Liu et al., 2001b). Moreover, both the V1 and V2 products have been shown to be required for systemic infection while they have no role in virus replication. The CP has been shown *in vitro* to bind ssDNA and dsDNA and its presence is essential in order to accumulate viral ssDNA in infected host cells and protoplast (Kotlizky et al., 2000). In addition, the c-sense genes *rep* and *rep A* have been implicated in the early stages of infection. The Rep protein is required for virus replication, while Rep A affects host cell cycle control in order to assist viral replication (Boulton, 2002). Having a small genome size, geminiviruses are capable of increasing their coding ability and regulating gene expression in different ways (Boulton, 2002).

As illustrated in Fig.1, the genome has also two non-coding regions: the large intergenic region (LIR) from where the transcription commences and the short intergenic region (SIR) where it ends. LIR contains a motif which is highly conserved among geminiviruses (TAATATTAC) (Palmer et al., 1998) and it is a part of a stem loop structure. The motif harbors the origin of replication (Ori) (Boulton, 2002).

### ***Wheat dwarf virus***

WDV infection has been reported from several parts of Europe, e.g. Sweden, the Czech Republic, Hungary, Germany and Finland (Ramsell et al., 2008) and also from Asia and Africa (Schubert et al., 2007) in countries such as Iran and China (Zhang et al., 2010; Behjatnia et al., 2011), where wheat has been cultivated. WDV causes wheat dwarf disease and also affects barley plants. In some cases, the incidence of WDV infections in a wheat field can be quite high and can cause huge yield losses up to 75% (Lindblad and Sigvald, 2004). Outbreaks of wheat dwarf disease have occurred regularly in Sweden for almost 100 years, and only the wheat-infecting strain of WDV has been detected. In surveys, it has been demonstrated that up to 50% of winter wheat in a single field (Lindblad and Sigvald, 2004) and around 0.7% of wild grasses (Ramsell et al., 2008) can be infected by WDV in Sweden during summer.

Two strains of WDV have been identified so far, wheat strain and barley strain (Lindsten and Vacke, 1991; Vacke et al., 2004; Köklü et al., 2007; Schubert et al., 2007). The two strains share 83–84% nucleotide identity (Köklü et al., 2007; Schubert et al., 2007). Although it is not common that isolates of the wheat strain infect barley plants and vice versa, in some rare occasions this has happened in the field (Ramsell, 2007).

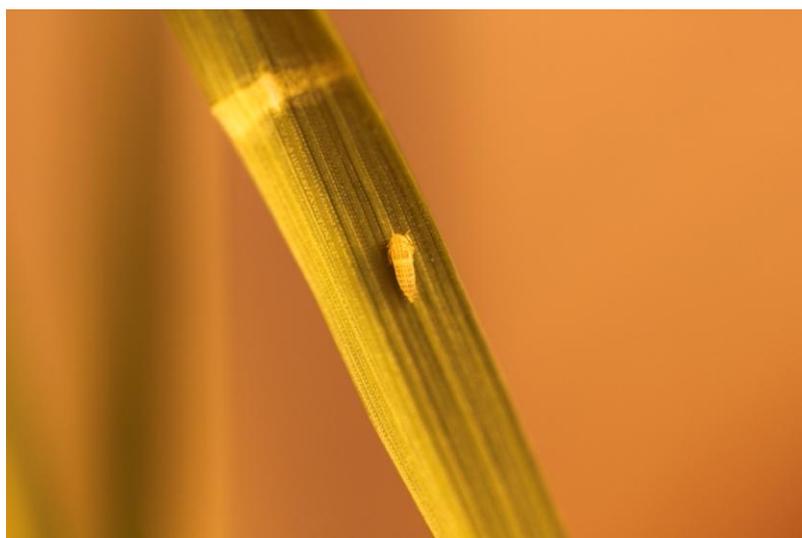
The typical symptoms caused by WDV on infected plants include dwarfing and yellowing, along with reduced headings and infection by WDV may dramatically decrease the yield of wheat and barley (Köklü et al., 2007).

WDV has a wide range of hosts, including agriculturally important crops such as wheat, barley, oat and rye. It is transmitted to its host by the leafhopper *P. alienus* in a circulative, persistent manner (Vacke, 1961), which means that the virus does not multiply within the insect and it is not transmitted to the eggs (Ng et al., 2006).

## Leafhoppers

The vector, *P. alienus*, is a holarctic species that commonly occurs in grasslands and arable fields (Lindblad and Arenö, 2002). *P. alienus*, from the family Cicadellidae, has seven embryonic developmental stages. Hatching in the spring, the nymph goes through five instars to become an imago (adult), which takes roughly 51 days (Manurung et al., 2005; Lindblad and Sigvald, 2004). The leafhopper overwinters as eggs, which have been laid in autumn on young plants of different cereals and weed grasses (Lindblad and Sigvald, 2004; Manurung et al., 2005). Environmental conditions, especially temperature, are crucial factors affecting the leafhopper's life cycle. The primary infection of WDV takes place in autumn after inoculation by adult leafhoppers, while the secondary infection in spring is a result of nymphs feeding on plants (Lindblad and Sigvald, 2004).

It is known that the incidence of leafhoppers can be high in fallows with many self-sown wheat plants, which may serve as a reservoir of WDV while they also support a high leafhopper population (Manurung et al., 2005). The insect population increases significantly when the temperature exceeds 15°C (Lindblad and Sigvald, 2004). In autumn, low temperature can restrict the ability of leafhoppers in transmitting the virus to newly sown plants (Lindblad and Sigvald, 2004). On the contrary in a very mild autumn, leafhoppers become more active, thus the rate of infection will be higher in the following summer.



**Fig. 2.** *Psammotettix alienus* nymph (Jim Nygren, 2010).



**Fig. 3.** Adult *Psammotettix alienus* (Jim Nygren, 2010).

## **Transmission**

How the virus is acquired by the vector and how it is transmitted to the plant are fundamental issues when studying plant-virus interactions. Both nymphs and adults are capable of transmitting the virus (Vacke, 1961). Virus moves from the gut to the salivary gland of the insect vector where it mixes with the saliva and enters the plant tissue when the leafhoppers feed (Harris, 1981).

In a study by Ammar et al. (2009), MSV particles were detected in the cytoplasm of the leafhopper midgut epithelial cells, mainly inside and outside the filter chamber (where the gut is looped). Therefore, it is the most probable site for MSV accumulation. Moreover, accumulation of MSV-like particle enclosed in large membrane-bound vesicles has been shown, which are not detected in non-vectors. A virus in a non-vector insect will not interact properly with the insect's proteins so it cannot enter the vector's body, and the hemolymph, a fluid which circulates in the insect body. The interaction with the insect vector is very specific (Ammer et al., 2009).

According to a study by Reynaud and Peterschmitt (1992), the virus could not cross the gut wall (a trait which is inherited on the sex linked chromosome as a dominant factor; Storey, 1932) in a non-vector. It has been shown that in the case of MSV, after the insect starts feeding on the mesophyll or phloem of infected plants (acquisition period is required time for the vector to acquire the virus from its source) a latency period, which is correlated with

temperature, is required for the vector in order to become capable of transmitting the virus (Storey, 1928).

It has been shown that the insect has a low efficiency in transmitting the virus the first day after acquisition since some of the insects may still be in their latency period, whereas 17 days after acquisition, a transmission efficiency of 90% could be obtained (Reynaud and Peterschmitt, 1992). Moreover, environmental conditions such as temperature also influence transmission efficiency. However, the transmission success depends also on viral virulence and host susceptibility (Reynaud and Peterschmitt, 1992).

After the viruliferous insect vector has started feeding on the plant, the virus will move to the phloem sieve tube or mesophyll cells. Through the phloem, the virus could translocate rapidly to different parts of the plant in less than 2 hours (Peterschmitt et al., 1992). Younger leaves which emerge after inoculation are more likely to be invaded by the virus than older tissue as the viral antigen also seems to be distributed according to the age of the tissue. Likewise, the virus could be detected in the basal meristem of young leaves, since it comes to the leaf through the phloem among the metabolites from the older leaves (Peterschmitt et al., 1992). In the case of *Tobacco mosaic virus* (TMV) it has been demonstrated that as the virus enters the phloem it may rapidly infect the root (Astier et al., 2007). When the virus moves into younger leaves or shoots, it probably comes from infected “sources” of the plant, such as older leaves. The transport of the virus goes from the source of carbohydrate synthesis to sinks. The roots are often sinks, and it is difficult for the virus to move from the roots. Moreover, the stem has a lower virus titer than the leaf sheath (Peterschmitt et al., 1992).

There is a direct relationship between the age of the plant at the time of inoculation and symptom severity. The younger the plant, the more severe the effects will be or it can be said that there is increased resistance/reduced susceptibility with age (Lindblad and Sigvald, 2004). In a study by Vacke (1972), plants at 1<sup>st</sup> leaf stage have been found to be more susceptible compared to other growth stages. Moreover as the plant gets older, the disease symptoms will appear later and will be milder (Peterschmitt et al., 1992), meaning that plants develop weaker or no symptoms if they are infected at an older age which is also true for WDV and wheat. Lindblad and Sigvald (2004) showed that wheat plants become resistant to WDV after pseudo-stem erection stage (Z30) (Zadoks et al., 1974), when the first node can be detected (Z31), This phenomenon is called mature plant resistance. Plants at this stage are less likely to be infected or if infected, they do not show symptoms.

## **Replication and Movement**

Like most of the geminiviruses, WDV replicates through a rolling circle mechanism via an intermediate dsDNA form for bidirectional transcription (Liu et al., 2001a). Since the replication takes place in the host cell nucleus it requires that the virus passes through barriers such as the nuclear envelope and the plasma membrane to spread the infection (Hehnle et al., 2004). Rep and RepA proteins are produced as results of differential splicing, and they assist virus replication by host factors and deregulate cell cycle control (Ramsell, 2007). Rep protein is required for virus replication, while RepA affects host cell cycle control in order to assist viral replication (Boulton, 2002).

CP is an essential element for virus movement and nuclear transport in monopartite geminiviruses (Astier et al., 2007). Among mastreviruses, the movement of MSV is most studied. Generally the virus moves through plasmodesmata from cell to cell in order to infect the plant, and this process is facilitated by the MP. For viral movement to adjacent cells, MP interacts with plasmodesmata by adjusting their function and structure resulting in higher plasmodesmata size exclusion limit (SEL) (Kotlizky et al., 2000). CP is suggested to have a role in intracellular transport of mastrevirus DNA.

After the virus has entered the plant, it will move rapidly throughout the plant via the phloem (Hehnle et al., 2004). The virus movement depends on the outcome of the interaction with different parts of the cell (e.g. cytoskeleton), plasmodesmata type and virus replication ability in various cells (Astier et al., 2007).

In the mastreviruses, the CP N-terminal domain plays an important role in the interaction with ssDNA and dsDNA which makes it possible to form viral particles as well as to access the nucleus (Liu et al., 1999).

## Possible plant defense mechanisms against WDV

Each living organism has a way to confront pathogen invasions, otherwise an infection can lead it to perish. There are different defense mechanisms implemented by plants to overcome a virus attack including RNA silencing, hypersensitive response (HR) and nucleic acid methylation. RNA silencing is a conserved mechanism used by eukaryotes, including animals, fungi and plants. Using this strategy, cellular and viral mRNA becomes degraded in order to deactivate gene expression. In HR, following infection by a pathogen, the infected cell will commit suicide by releasing signaling compounds. The compounds secreted following HR, broaden the cell wall of infected cells and make a barrier to inhibit spread of the infection; however, HR against WDV has not yet been found. It has been found that plants respond to invasion of DNA viruses by RNA-directed methylation of DNA (Wang et al., 2003). In the case of DNA methylation (used against geminiviruses), the virus genome cannot be transcribed since methylation obstructs the transcription (Leke, 2010). However, nothing is known about the defense against WDV.

## Control

Recently WDV has become more problematic in Europe, and it is predicted that due to climate change the incidence of vector-transmitted viruses will increase globally. Therefore, it is expected that the leafhopper *P. alienus* and the problem with WDV will increase due to warmer autumns in Sweden (Roos et al., 2011).

Chemical control of the vector has been used to control this disease, but it will become less common since the use of pesticides will be restricted by the European Union together with the fact that they have environmental risks. Agricultural practices have been the most important way to control wheat dwarf disease, at least in Sweden. These practices have been very effective and with low cost and low environmental impact (Lindblad and Sigvald, 2004). Chemicals are used when the agricultural practices such as late sowing time and avoiding reduced tillage do not work. Genetic resistance would also be a good complement (Roos et al., 2011), but all wheat cultivars have been susceptible to WDV and only recently, partial resistance (reduced virus titer) has been identified in two Hungarian wheat cultivars: Mv Vekni and Mv Dalma (Benkovic et al., 2010).

## Aims and objectives

The cultivation of wheat, a significant crop included in human's diet, is affected by many pathogens, including WDV.

The experiment was designed to identify WDV resistance or tolerance, which can be determined by several genes. The major objective of this experiment was to study the response of two wheat ancestors to infection by WDV. The results of this study could result in reduced use of pesticides.

It was hypothesized that WDV and the ancestors of wheat have lived together in the Middle East for a long time. In long-term virus-host interactions, there is often some level of resistance/tolerance which can be determined by one or several genes. During the domestication of wheat, the resistance genes may have been lost if there was no selection for them.

The intention of the study was to identify resistance/tolerance or reduced susceptibility against WDV in wild ancestors of wheat (*Triticum* spp. and *Aegilops* spp.), collected from the Middle East, that are supposed to carry resistance or tolerance against WDV. It has been found by Nygren et al. (unpublished) that *Ae. tauschii*, one of the wheat ancestors, is considerably more tolerant or partially resistant to infection by WDV compared to the other wild relative *T. urartu* based on phenotypic evaluation and symptoms. There is no complete resistance known against WDV although different cultivars may vary in their susceptibility to WDV. Following the results from the study by Nygren et al. (unpublished), we aimed to characterize the plant-virus interactions in two wild species, *Ae. tauschii* and *T. urartu*, with different levels of susceptibility to WDV. *T. aestivum* (bread wheat), which is highly susceptible to WDV, was used for comparison. Different leaves and roots were tested for WDV infection to observe any difference.

## **Materials and Methods**

For conducting the experiment, seed material was sown and a culture of leafhoppers was used. Samples were collected and tested for WDV infection by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). A region of the virus genome was amplified using polymerase chain reaction (PCR). Later, for amplifying complete WDV genome, another method called rolling circle amplification (RCA) was used. The virus DNA was sequenced and compared with several sequences from the GenBank database.

### **Plant materials**

Two wild ancestors of wheat (*T. urartu* and *Ae. tauschii*) and bread wheat (*T. aestivum*) cultivar Tarso were used as plant material for this experiment. *T. aestivum* was used for comparison while *T. urartu* and *Ae. tauschii* were tested in order to determine their level of susceptibility to WDV. A study by Nygren et al. (unpublished) has shown that according to the symptoms *T. urartu* is more susceptible to WDV infection whereas *Ae. tauschii* is less susceptible.

The seed material provided by International Center for Agricultural Research in the Dry Areas (Aleppo, Syrian Arab Republic) was sown in pots filled with a composition of soil (20% autoclaved sand, agricultural soil and perlite) and sand.

### **Experimental design**

The plants were grown in a growth chamber at 22°C from 6 am to 10 pm and 20°C from 10 pm to 6 am; with 16 h photoperiod for 10 days. The light source was sodium and metal halide lamps. After being repotted and covered with a net, the plants were placed in a greenhouse (8 hours in light and 16 hours in darkness) in order to be used for inoculation.



**Fig. 4.** Repotted plants, covered with net and ready for WDV inoculation.

To repeat the experiment, a total of two inoculations were conducted and therefore seeds were sown two times. The first time, 11 seeds of each species were sown and for the second experiment 21 seeds of each species were sown.

### **Virus sources and insect vector**

Individuals of *P. alienus* had been collected prior to the experiment from WDV-affected wheat fields outside of Uppsala in 2010. Since then, they had been feeding on source plants (*T. aestivum* plants in pots covered with net, harboring viruliferous leafhoppers) in the greenhouse. The WDV isolates also came from Uppsala region. Due to the increased leafhopper population, prior to the second experiment, 21 pots of source plants were established and the leafhoppers were shifted from old source plants to new ones. This was done to avoid any decrease in the leafhopper population since the old source plants became necrotic and the leafhoppers could not feed on them.



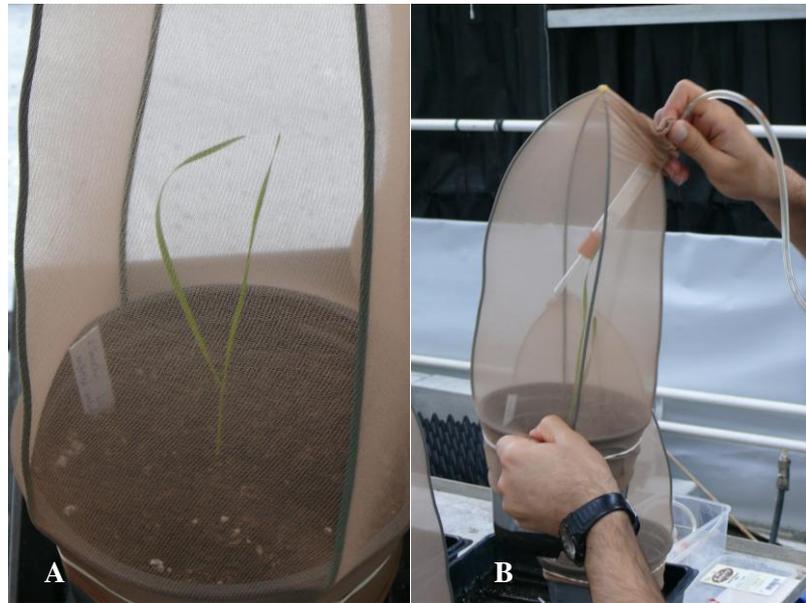
**Fig. 5.** Old source plants for WDV transmission in the greenhouse.

## **Insect transmission**

Plant inoculation was carried out under greenhouse conditions. For both transmission experiments, three leafhoppers were placed on a plant surrounded by net and they were allowed to feed on the plant for three days. These three leafhoppers were chosen from different source plants. In the first study, the plants were in the 2<sup>nd</sup> leaf stage at the time of inoculation, (10 days after sowing). For the second experiment, the plants were at the 3<sup>rd</sup> and 4<sup>th</sup> leaf stage, (23 days after sowing). In the first experiment nine plants from each species were inoculated, while in the second experiment 20 plants per species were inoculated. Moreover, in both experiments, one plant from each species was included as a control (healthy, non-infected plant). After three days of inoculation, the leafhoppers were transferred to new plants. The inoculation period of three days is longer than what was suggested to be enough for the viruliferous leafhoppers to transmit the virus according to Storey (1938). In a study by Peterschmitt et al. (1992) three hours were suggested to be sufficient for successful *Maize streak virus* transmission from insects to plants.

For the first experiment, the source plants were tested for WDV infection by both DAS-ELISA and PCR after the insect transmission. The results obtained from this test showed that

there were non-infected plants among the source plants. Hence, due to this result, prior to the insect transmission in the second study, the source plants were tested for WDV infection by DAS-ELISA. The intention was to use leafhoppers from source plants, which had been confirmed to be infected prior to the study.



**Fig. 6.** Inoculation of *Wheat dwarf virus* in selected wheat species. **A.** Inoculated plant harboring the leafhoppers **B.** Shifting the leafhoppers to the pots via a special apparatus.

## Collection of plant samples

In the first transmission experiment, samples were harvested at three time points after inoculation, and in the second experiment, four harvests took place (Table 1). For the first experiment, each harvest included three replicates of each species, while in the second experiment, five replicates were collected at each harvest time. The whole plant was harvested including roots. In the first experiment, plants were dissected and plant parts (shoot and root) were kept in labeled plastic bags at  $-20^{\circ}\text{C}$ . However, in the second experiment, for the first two harvests, the samples were collected as mentioned above while for the last two harvests the whole plants including shoot and root were kept in the same plastic bag.

## DAS-ELISA

The virus titer was measured in the samples using DAS-ELISA, a technique which uses an antiserum to detect the viral antigen. Subsets of the collected samples from both experiments were analyzed by DAS-ELISA to detect the WDV CP following the Loewe *Wheat Dwarf Virus* kit protocol. Moreover, source plants for both studies were also tested for infection using DAS-ELISA. Five source plants for the first study and 16 plants for the second experiment were tested. For the first study, leaf 1 and leaf 2 along with root were tested for all sampling time-points, while due to the rapid plant growth in the second experiment, one leaf per plant was used for the test. The third leaf was used for the first time-point and the fourth leaf for the second time-point. Since the plants in the 3<sup>rd</sup> and 4<sup>th</sup> harvests had many tillers, the youngest leaf of the main tiller was assumed to be the best leaf for analysis. All the buffers were prepared according to the manufacturer's kit protocol except for the conjugate buffer, which was made without blocking milk. For each sample, 500 mg of the tissue was ground in 400 µl of sample buffer. Positive controls were provided in the Loewe kit and non-infected healthy wheat plants were used as negative controls.

To start the assay according to Ramsell et al. (2008), the microtiter plate (for WDV tests of roots, cell culture plates were used) was coated with a specific antibody for WDV CP at 4°C and incubated overnight according to the Loewe kit protocol. During the second step, samples and controls diluted in sample buffer at a ratio of 1:20 were added to the wells, followed by overnight incubation at 4°C in order to let the antigen bind to the fixed antibody. Two technical replicates of each sample were analyzed. The next step was loading the plate with the antibody-alkaline phosphatase (AP)-conjugate and incubation at 37°C for four hours to form the double antibody sandwich containing antibody-antigen complex and AP-labeled antibody. Finally, the wells were filled with substrate solution and kept at room temperature to indicate the presence of the specific antigen by positive enzymatic reactions. The enzymatic reaction between alkaline phosphatase and 4-nitrophenyl-phosphate yielding free 4-nitrophenol was monitored at 405 nm after one and two hours using a Benchmark microplate reader (Microplate Manager, Bio-Rad Laboratories, Inc). Samples with an absorbance value twice of the background (negative controls) were considered as positive.

## **Polymerase chain reaction (PCR)**

Due to the problem with detecting WDV infection in all source plants, symptomatic source plants, which showed necrosis and chlorosis (similar to wheat dwarf disease symptoms), were tested by PCR to amplify part of the virus genome. PCR was run using the primer pair C1/C2 fwd and C1 rev with the sequence as follows: C1 rev 5'- CTA GAG ACC TTG CCC AGG AA-3' and C1/C2 fwd: 5'- ATG GCC TCT TCA TCT GCA CC-3'. This primer pair has been designed to amplify a fragment of 750 bp corresponding to nucleotide 1717 to 2511 of isolate WDV-[Enköping 1] (Kvarnheden et al., 2002) with the accession number AJ311031.1 in GenBank.

The presence of WDV DNA was tested using direct incubation of plant extract and PCR (Wyatt and Brown, 1996; Kvarnheden et al., 2002). Leaf discs of 2 cm size from three source plants were homogenized in ELISA bags using 250 µl Elution buffer (10 mM Tris-HCl, pH 8.5). 50 µl of crude plant extract was added to PCR tubes for incubation at 4°C overnight. The extract was removed by washing the tubes twice with 150 µl Tris-HCl (10 mM; pH 8.0).

PCR was run in a reaction volume of 50 µl containing 5µl of 10X DreamTaq PCR-buffer; 1 µl of dNTP mix (10mM); 0.5 µl of DreamTaq DNA Polymerase (Fermentas); 2.5 µl of primer C1/C2 fwd (10 µM); 2.5 µl of primer C1 rev (10 µM) (Invitrogen) and 38.5 µl of MQ water. The amplification of viral DNA took place in a C1000 Touch™ Thermal Cycler (BIO-RAD) starting with 120 seconds of heating at 94°C, followed by 35 cycles of 30 seconds at 94°C, 60 seconds at 57°C and 2 min at 72°C, and a final extension for 6 min at 72°C (Kvarnheden et al., 2002). The PCR result was analyzed using a 1% agarose gel.

## **Rolling circle amplification (RCA)**

Amplification of viral circular DNA was carried out through a method called RCA, during which the circular DNA was amplified to a large number of copies at a constant temperature using the bacteriophage Φ29 DNA polymerase (Inoue-Nagata et al., 2004).

To run RCA, leaf discs of 10 mg from 2 source plants (2<sup>nd</sup> experiment), which had been proven to be WDV-infected by ELISA, were homogenized in PCR tubes, followed by two quick methods to isolate plant DNA. First, Extraction buffer (Extract-N-Amp<sup>™</sup> Plant PCR Kit, Sigma) was used by adding 50 µl of the buffer to each plant homogenate, followed by 10 minutes of incubation at 95°C. Another protocol was also tried for preparing plant samples by grinding 10 mg of plant tissue with 100 µl of 0.5 N NaOH. Subsequently, 20 µl of the ground samples were added to new tubes containing 485 µl of 100 mM Tris-HCl pH 8.0 followed by mixing. Finally, one µl of this aliquot was transferred to PCR tubes (Shepherd et al., 2008).

Circular DNA was amplified via RCA using Illustra TempliPhi 100 Amplification Kit (GE Health Care). One µl of template DNA was mixed with five µl of sample buffer and incubated at 95°C for three minutes. Five µl of Master Mix was added to the mixture and the reaction was incubated at 30°C for 18 hours. The reaction was inactivated by incubation at 65°C for 10 minutes. The amplified products were visualized in a 1% agarose gel.

## **Cloning and sequencing**

In order to verify the virus strain and to confirm that it is a typical isolate of the WDV wheat strain, the virus genome was sequenced.

The RCA concatamer products of the two plant samples were separately digested with *SacI*, *EcoRI* or *HindIII*. *SacI* and *HindIII* have one unique restriction site in the WDV genome and restriction yields a 2.7 kb product representing the complete genome. For *EcoRI*, there are at least two sites in the WDV genome. The restrictions were done using two µl of RCA product according to the protocol. Each restriction digest was prepared in two replicates to increase the yield. The restricted DNA was analyzed in a 0.8% agarose gel run at 80 V. The digested DNA (full genome) was purified using GeneJET<sup>™</sup> Gel Extraction Kit (Fermentas). The purified fragments were ligated into pBluescript KS+ (Stratagene), which had been restricted with the same restriction enzyme, dephosphorylated using CIAP (Calf intestine alkaline phosphatase) and purified using the GeneJET<sup>™</sup> PCR Purification Kit (Fermentas). Following the purification, the DNA concentration of each fragment and the vector was measured using a NanoDropND-1000 spectrophotometer (NanoDrop Technologies). The ligation reaction was setup in a total volume of 10 µl based on the manufacturer protocol (T4 DNA ligase,

Fermentas) using insert and the vector (pBluescript KS+) at a molar ratio of 3:1. The ligations were incubated at 16°C overnight. The reaction was stopped by incubation at 65°C for 10 minutes. The plasmids were transformed into *Escherichia coli* DH5α competent cells; mixing 100 µl of the bacterial cells with five µl of ligation product. The mixture was chilled on ice for 30 minutes. Following one minute of heat shock at 42°C in a water bath, the mixture was incubated on ice for two minutes. Under sterile conditions, 900 µl of SOC media was added and the cells were incubated for one hour at 37°C with regular shaking at 225 rpm. 100 µl of the bacterial solutions were spread on LB plates containing 100 µg/ml ampicillin and 80 µl from 20 mg/ml X-gal stock as the substrate. The remaining solution was centrifuged for three minutes at 5000 rpm to pellet the bacteria. The pellet was dissolved in 100 µl of LB medium and spread on plates. To let the bacterial cells grow, the plates were incubated at 37°C overnight. The white colonies were picked and transferred to culture tubes containing four ml LB media and ampicillin. The culture tubes were incubated at 37°C overnight with regular shaking at 225 rpm. Using the GeneJET™ Plasmid Miniprep Kit (Fermentas), the plasmid DNA was purified. To confirm that the plasmids contained the correct insert, fast digest *SacI* and *HindIII* were used for digestion following the manufacturer's protocol (Fermentas). Then, PCR was also run as before to confirm the successful cloning. Three positive plasmids for each isolate (two isolates) containing an insert of 2.7 kb were sent for sequencing in the forward and reverse directions to Macrogen Inc. (South Korea). Following the receiving of sequence data from Macrogen, new primers were designed two times in both directions. Consequently, full length sequences were assembled from three overlapping sequences in each direction using DNASTAR software (Lasergene).

## Sequence analysis

To verify the identity of the WDV isolate, which had been used for inoculation, the cloned DNA sequences were analyzed. Using nucleotide Blast, the sequences were compared with those present in the GenBank nucleotide database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Phylogenetic and bootstrap analyses were done with neighbor-joining method, using MEGA5 software (Tamura et al., 2011). For calculating the distances between sequences, maximum likelihood method was used.

## RESULTS

### First Experiment

In the first trial, five source plants were tested for WDV infection by ELISA after the transmission experiment (Table 1). The source plants for the first study were old and bushy inside the cages; they showed some chlorosis and necrosis.



**Fig. 7.**Source plants harboring leafhoppers **A.** Old source plant tested with DAS-ELISA **B.** Infected source plant showing necrosis and chlorosis due to WDV infection.

**Table 1.** Detection of WDV in old source plants (first experiment) by double antibody sandwich ELISA.

Sample	A 405 nm*	Symptom	WDV detect.
Buffer	0.154	-	-
Negative control	0.136	Non-infected wheat	Negative
Positive Control	1.014	Loewe kit control	Positive
Source plant A	0.381	Necrotic leaves	Positive
Source plant B	0.705	Necrotic leaves	Positive
Source plant C	0.413	Necrotic leaves	Positive
Source plant D	0.125	Necrotic leaves	Negative
Source plant E	0.122	Necrotic leaves	Negative

\* Mean calculated using two replicates of each sample, A: absorbance, at 405 nm: wavelength.

According to the DAS-ELISA values, three source plants for the first transmission experiment were infected by WDV (Table 1), hence, the leafhoppers feeding on these plants were most likely to be viruliferous and they could be used as the source of virus to set up new source plants for the second experiment. The other two source plants were not found to have the virus although they showed some symptoms of the wheat dwarf disease, such as necrosis and chlorosis. These symptoms can be the result of aging, other biotic stresses or abiotic stresses such as nutrient deficiency.

Test plants were harvested three times, and each harvest consisted of three replicates of each species. Harvest I was carried out at 0 days post-inoculation (dpi), harvest II at 4dpi and harvest III at 10 dpi. The virus tests were done on leaf 1 and leaf 2, which had already emerged at the time of inoculation, likewise leafhoppers were more likely to have been feeding on these leaves and transmitting the virus. Occasionally, it was observed that the leafhoppers were sitting at the base of the second leaf and sometimes on the stem, suggesting that they were feeding and transmitting the virus to the plant.

**Symptoms:** Three plants of *Ae. tauschii* showed typical symptoms of WDV infection at 4 dpi (Fig. 9) and leaf 1 from *Ae. tauschii* plant 2 at 10 dpi, while the other plants looked healthy with no visible symptoms. At 0 dpi, most of the plants were at the second leaf stage, while at 4 dpi they had three leaves, and four leaves at 10 dpi. In general, in both test plants and control, *T. urartu* plants were comparatively smaller and thinner in size than *T. aestivum* and

*Ae. tauschii*, respectively. Except two test plants, the other test plants were inoculated by leafhoppers, which came from infected source plants. When analyzing samples from 0 dpi, no WDV infection was detected, while at 4 dpi two plants of *T. aestivum* were strongly positive, 14 additional test plants were suspected to be positive and only one plant of *Ae. tauschii* was clearly negative. At 10 dpi again no clear WDV infection was detected. The data from the DAS-ELISA infection test is presented in Table 2.

**Table 2.** Detection of WDV in crude sap from the leaves of inoculated plants (first experiment) by double antibody sandwich ELISA.

Sample <sup>1</sup>	Harvest I (0 dpi)			Harvest II (4 dpi)			Harvest III (10 dpi)			Virus Source <sup>5</sup>
	Symptom <sup>2</sup>	A 405 nm <sup>3</sup>	WDV detect. <sup>4</sup>	Symptom	A 405 nm	WDV detect.	Symptom	A 405 nm	WDV detect.	
Buffer	-	0.783	-	-	0.425	-	-	0.425	-	-
Negative control	-	0.589	-	-	0.786	-	-	0.786	-	-
Positive control	-	3.1885	P	-	3.667	P	-	3.667	P	-
<i>Ae. tauschii</i> 1- L1	AS	0.183	N	RD	1.274	LP	AS	0.378	PP	Mix <sup>6</sup>
<i>Ae. tauschii</i> 1- L2	AS	0.228	N	RD	1.324	LP	AS	0.444	PP	Mix
<i>Ae. tauschii</i> 2- L1	AS	0.239	N	RD	1.327	LP	AS	0.709	PP	P
<i>Ae. tauschii</i> 2- L2	AS	0.668	PP	RD	1.475	LP	AS	0.459	PP	P
<i>Ae. tauschii</i> 3- L1	AS	0.116	N	NS	0.379	N	AS	0.203	N	Unknown
<i>Ae. tauschii</i> 3- L2	AS	0.140	N	RD	0.510	PP	AS	0.280	N	Unknown
<i>T. aestivum</i> 1- L1	AS	0.549	PP	AS	1.377	LP	AS	0.466	PP	P
<i>T. aestivum</i> 1- L2	AS	0.724	LP	AS	1.479	LP	AS	0.588	PP	P
<i>T. aestivum</i> 2- L1	AS	0.717	LP	AS	1.752	P	AS	0.666	PP	P
<i>T. aestivum</i> 2- L2	AS	0.808	LP	AS	2.411	P	AS	0.833	PP	P
<i>T. aestivum</i> 3- L1	AS	0.107	N	AS	0.612	PP	AS	0.163	N	P
<i>T. aestivum</i> 3- L2	AS	0.156	N	AS	0.863	PP	AS	0.276	N	P

<i>T. urartu</i> 1- L1	AS	0.253	N	AS	1.063	LP	AS	0.331	N	P
<i>T. urartu</i> 1- L2	AS	0.433	PP	AS	1.331	LP	AS	0.471	PP	P
<i>T. urartu</i> 2- L 1	AS	0.409	PP	AS	1.439	LP	NC	0.557	PP	P
<i>T. urartu</i> 2- L 2	AS	0.648	PP	AS	1.529	LP	NC	0.660	PP	P
<i>T. urartu</i> 3- L 1	AS	0.098	N	AS	0.402	PP	NC	0.084	N	P
<i>T. urartu</i> 3- L 2	AS	0.119	N	AS	0.921	PP	NC	0.250	N	P

<sup>1</sup> L1: Leaf 1, L2: Leaf 2

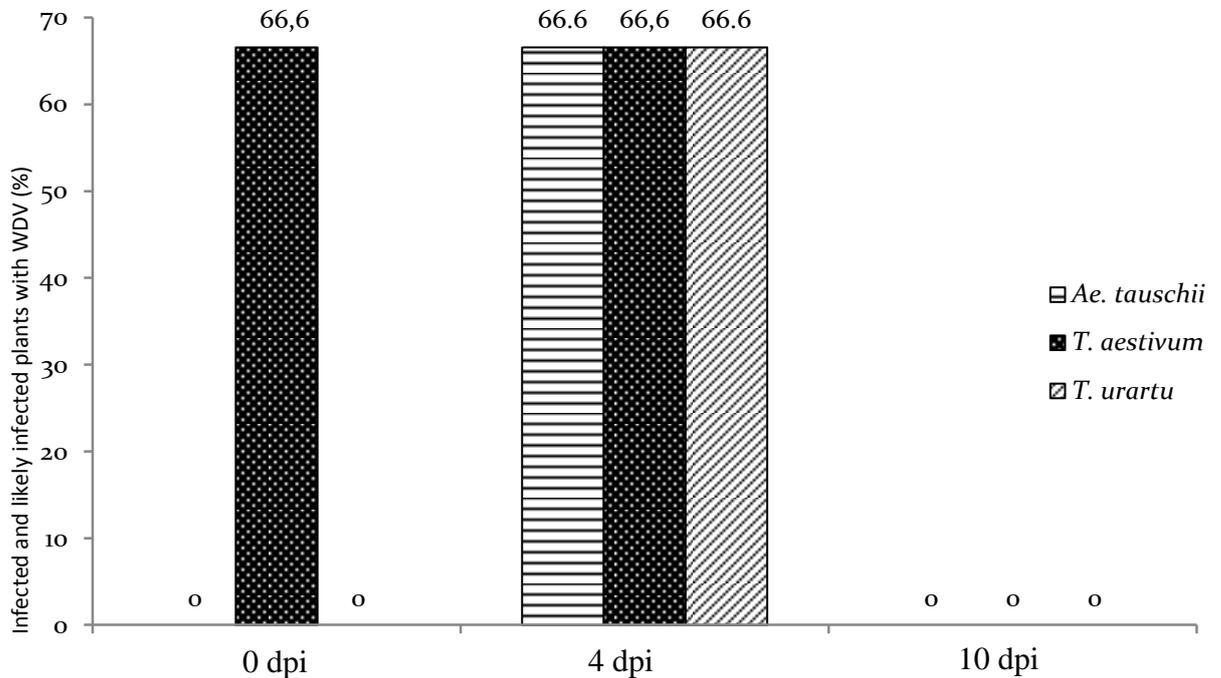
<sup>2</sup> RD: Red-to purple discoloration, NS: No symptom, AS: Asymptomatic, NC: Necrosis

<sup>3</sup> Mean calculated using two replicates of each sample, A: absorbance, at 405 nm: wavelength

<sup>4</sup> P=positive; more than twice the value of the background, N=negative, PP=potentially positive; slightly more than background, LP=likely positive; close to positive (since the background was high some samples were assumed to be potentially positive or likely positive).

<sup>5</sup> Source plant harboring leafhoppers

<sup>6</sup> Leafhoppers came from both infected and non-infected source plants



**Fig. 8.** The percentage of plants infected with WDV in experiment 1.

It is clear that the virus transmission was successful in the second harvest with 66.6% of infection in all species (including clearly positive and likely positive plants, but not

potentially positive), while among the samples from the first harvest just *T. aestivum* plants were positive for WDV and no WDV infection was detected by DAS-ELISA at 10 dpi (Fig. 8 and Fig. 11). This could be due to the problem with the source plants which were not infected. Unfortunately, separation between the gained results from delayed response, multiplication of the virus or movement was not possible, since the leafhoppers feeding point was not observed in this experiment.

**Table 3.** Detection of WDV in the crude sap from the roots of inoculated plants (first experiment) by double antibody sandwich ELISA.

Sample	0 dpi		4 dpi		10 dpi		Virus source <sup>3</sup>
	A 405 nm <sup>1</sup>	WDV detect. <sup>2</sup>	A 405 nm	WDV detect.	A 405 nm	WDV detect.	
Buffer	0.189		0.186		0.171		-
Negative control	0.348	-	0.362	-	0.360	-	-
Positive Control	0.602	-	0.742	-	0.784	-	-
<i>Ae. tauschii</i> 1	0.297	N	0.304	N	0.351	N	Mix <sup>4</sup>
<i>Ae. tauschii</i> 2	0.277	N	0.315	N	0.221	N	P
<i>Ae. tauschii</i> 3	0.379	PP	0.298	N	0.255	N	Unknown
<i>T. aestivum</i> 1	-	-	0.475	PP	2.416	P	P
<i>T. aestivum</i> 2	-	-	0.296	N	0.340	PP	P
<i>T. aestivum</i> 3	-	-	0.369	PP	0.260	N	P
<i>T. urartu</i> 1	-	-	0.191	N	0.298	N	P
<i>T. urartu</i> 2	-	-	0.187	N	0.284	N	P
<i>T. urartu</i> 3	-	-	0.230	N	0.293	N	P

<sup>1</sup> Mean calculated using two replicates of each sample, A: absorbance, at 405 nm: wavelength.

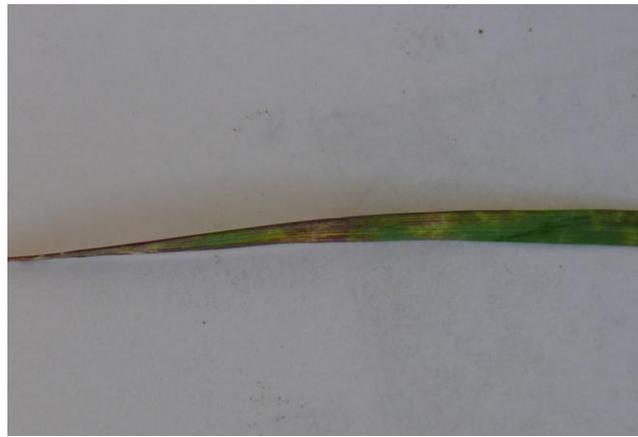
<sup>2</sup>P=positive; more than twice the value of the background, N=negative, PP=potentially positive; slightly more than background; (since the background was high some samples were assumed to be potentially positive).

<sup>3</sup>Source plant harboring leafhoppers

<sup>4</sup>Leafhoppers came from both infected and non-infected source plants

Virus movement is usually from the source of carbohydrate synthesis to the sinks (Astier et al., 2007). Mostly, the root is a sink, so at 0 dpi, root samples from *Ae. tauschii* were also selected for WDV testing. For the two other time-points, all the root samples were tested

(Table 3). In correlation with the ELISA results of leaves, all the root samples from *Ae. tauschii* (0 dpi) were found to be negative except one that was suspected to be potentially positive. For the root samples from 4 dpi and 10 dpi it is difficult to interpret the data since the background is high and the positive control is not as high as it should be. However, they were mostly negative except for *T. aestivum* plant 1 at 10 dpi that showed a high value, even higher than the positive control. The tested leaf samples from this plant had been found to be infected by WDV.



**Fig. 9.** Reddish discoloration, a typical symptom of infection by *Wheat dwarf virus*, on a leaf of *Ae. tauschii* at 4 dpi, first study.

DAS-ELISA absorbance values showed that a few samples were clearly positive while others could be potentially or likely positive. Since the background was high, the interpretation of the DAS-ELISA results was difficult. Looking back at the inoculation, the positive plants had been harboring leafhoppers merely from infected source plants, so it is more probable that they would be infected.

Two randomly selected samples from the first experiment were also tested with PCR along with one of the infected source plants (Fig. 10).



**Fig. 10.** Result of PCR amplification of the *Wheat dwarf virus* repA gene of selected samples. Each PCR amplification was loaded on the gel in two replicates. M is GeneRuler™ 1kb DNA Ladder marker, N is a negative control for PCR, lanes 1 and 2 are source plant A, lanes 3 and 4 are inoculated *Ae. tauschii* at 4 dpi, lanes 5 and 6 are inoculated *T. urartu* plant sample from 4 dpi, lanes 7 and 8 are *T. aestivum* (new established source plant).

Although PCR confirmed WDV infection of the selected old source plant A in one of the replicates, by yielding a PCR product with the size of 750 bp (Lane 1 in Fig. 10), no band was obtained for the other samples including inoculated plants of each *Ae. tauschii* and *T. urartu* (although they were found out to be clearly positive by ELISA) together with a new source plant (*T. aestivum*).

## Second Experiment

For the second study a new set of *T. aestivum* plants were established as source plants prior to the experiment. These new plants were at the 2<sup>nd</sup> leaf stage at the time of insect transmission and WDV infection tests were done on 15 source plants, which were more likely to be infected based on their appearance, since they showed some symptoms similar to wheat dwarf disease (Table 4).

**Table 4.** Detection of WDV in leaves of new established source plants (Second experiment) by double antibody sandwich ELISA.

Sample	A 405 nm <sup>1</sup>	Symptom	WDV detect. <sup>5</sup>
Buffer	0.120	-	-
Negative control	0.394	Non-infected wheat	N
Positive Control	4.595	Loewe kit control	P
Source plant 1	0.521	Asymptomatic	PP
Source plant 4	0.450	Asymptomatic	PP
Source plant 7	0.195	Symptomatic <sup>2</sup>	N
Source plant 8	0.236	Symptomatic	N
Source plant 8-1	0.211	Symptomatic	N
Source plant 8-2	0.199	Symptomatic	N
Source plant 9	0.241	Symptomatic	N
Source plant 9-1	0.215	Symptomatic	N
Source plant 10	0.346	<i>Ae. tauschii</i> <sup>4</sup> , Symptomatic	N
Source plant 11	0.338	Symptomatic	N
Source plant 12	0.384	<i>Ae. tauschii</i> , Symptomatic	N
Source plant 12-1	0.179	Symptomatic	N
Source plant 13	4.589	Symptomatic	P
Source plant 14	0.282	Asymptomatic	N
Source plant 15	4.390	Symptomatic	P
Source plant 15-1	4.110	Asymptomatic	P
Source plant 16 <sup>3</sup>	4.631	Symptomatic; old plant	P
Source plant 16 -1 <sup>3</sup>	0.437	Asymptomatic; young plant	PP
Source plant 17	0.322	Symptomatic	N
Source plant 18	0.209	Symptomatic	N
Source plant 19	0.274	Symptomatic	N

<sup>1</sup> Mean calculated using two replicates of each sample, A: absorbance, at 405 nm: wavelength.

<sup>2</sup> Typical symptoms of WDV such as chlorosis and necrosis.

<sup>3</sup> Two plants in the same pot, one younger and one older.

<sup>4</sup> P=positive; more than twice the value of the background, N=negative, PP=potentially positive; slightly more than background (since the background was high some samples were assumed to be potentially positive or likely positive).

<sup>5</sup> All the source plants are *T. aestivum* unless something else is indicated.

Based on the ELISA value, it was found that three source plants (13, 15, and 16) had high titers of WDV. In pot 16, two plants were planted as source plants, one younger plant and another older one. According to the WDV test, the older plant was infected while the young plant only showed a slightly increased absorbance value and was considered as potentially positive. In total, three samples were found to be potentially positive. Several factors could have affected the results that many plants were negative for WDV, such as presence of non-viruliferous leafhoppers among the population used for virus transmission.

In this experiment, plants were at different developmental stages. At harvest I (0 dpi), all five replicates of *T. aestivum* had four leaves while plants of *Ae. tauschii* together with *T. urartu* had three leaves. Second harvest (5 dpi) consisted of plants at four-leaf stage. In the last two harvests (10 dpi and 14 dpi), the plants had grown fast and they had several tillers at the time of sampling. These four batches of samples were analyzed by DAS-ELISA. For 0 dpi, the third leaf of all plants was tested, while for 5 dpi, leaf four, which was not present at the time of inoculation and assumed to have a higher virus titer (since the virus enters the phloem and there is a rapid flow of phloem to younger leaves), was analyzed. At 10 dpi and 14 dpi, it was difficult to identify the different leaves, since they had several tillers and it was difficult finding the first and second leaves. Therefore, the youngest leaf of the main tiller was selected. In addition, during storage of the samples from 10 dpi, the leaves by accident became damaged and fragmented, so it was almost impossible to take the youngest leaf of the main tiller. Therefore, several leaf tips from different tillers were analyzed together for each individual.

In DAS-ELISA, the blocking milk powder used to reduce unspecific binding was suspected to be the cause of slow signal regeneration. To test if the problem was the milk blocking powder, positive controls were tested with and without blocking milk powder (2%) added to the conjugate buffer following the Loewe assay protocol. In the analyses including blocking milk powder only weak absorbance signals were obtained, while the controls without the powder had a very strong signal, as expected from a normal positive kit control (Table 5). It is concluded that the powder was interfering with the enzymatic reaction. Hence, conjugate buffer without blocking milk powder was used.

**Table 5.** Detection of WDV in the crude sap from inoculated plants (second experiment) by double antibody sandwich ELISA.

Sample	Harvest I (0 dpi)			Harvest II (5 dpi)			Harvest III (10 dpi)			Harvest IV (14 dpi)		
	Symptom <sup>2</sup>	A405 nm <sup>3</sup>	WDV detect. <sup>4</sup>	Symptom	A405 nm	WDV detect.	Symptom	A405 nm	WDV detect.	Symptom	A405 nm	WDV detect.
Buffer <sup>1</sup>	-	-	-	-	-	-	-	0.171	-	-	0.171	-
Negative control	-	0.228	-	-	0.228	-	-	0.168	-	-	0.168	-
Positive control	-	3.818	-	-	3.818	-	-	2.824	-	-	2.824	-
<i>Ae. tauschii</i> 1	AS	0.115	N	AS	0.119	N	AS	0.153	N	AS	0.391	LP
<i>Ae. tauschii</i> 2	AS	0.143	N	AS	0.142	N	AS	0.209	N	AS	2.419	P
<i>Ae. tauschii</i> 3	AS	0.144	N	AS	0.138	N	AS	0.163	N	AS	0.138	N
<i>Ae. tauschii</i> 4	AS	0.123	N	AS	0.123	N	AS	0.176	N	-	-	-
<i>Ae. tauschii</i> 5	AS	0.127	N	AS	0.108	N	AS	0.237	N	-	-	-
<i>T. aestivum</i> 1	AS	0.196	N	AS	0.128	N	AS	0.211	N	AS	0.148	N
<i>T. aestivum</i> 2	AS	0.236	N	AS	0.126	N	AS	0.287	N	AS	0.227	N
<i>T. aestivum</i> 3	AS	0.136	N	AS	0.150	N	AS	0.144	N	AS	0.189	N
<i>T. aestivum</i> 4	AS	0.232	N	AS	0.145	N	AS	0.256	N	AS	0.188	N
<i>T. aestivum</i> 5	AS	0.123	N	AS	0.142	N	AS	0.255	N	AS	0.699	P
<i>T. urartu</i> 1 <sup>2</sup>	AS	0.122	N	AS	0.176	N	AS	0.338	LP	NC	0.201	N
<i>T. urartu</i> 2 <sup>2</sup>	AS	0.127	N	AS	0.122	N	AS	0.188	N	NC	0.220	N
<i>T. urartu</i> 3 <sup>2</sup>	AS	0.185	N	AS	0.419	LP	AS	0.173	N	NC	3.414	P
<i>T. urartu</i> 4 <sup>2</sup>	AS	0.126	N	AS	0.111	N	AS	0.172	N	NC	3.193	P
<i>T. urartu</i> 5 <sup>2</sup>	AS	0.119	N	AS	0.103	N	AS	0.254	N	NC	0.175	N

<sup>1</sup> For the first two harvests, no buffer was tested without blocking milk powder.

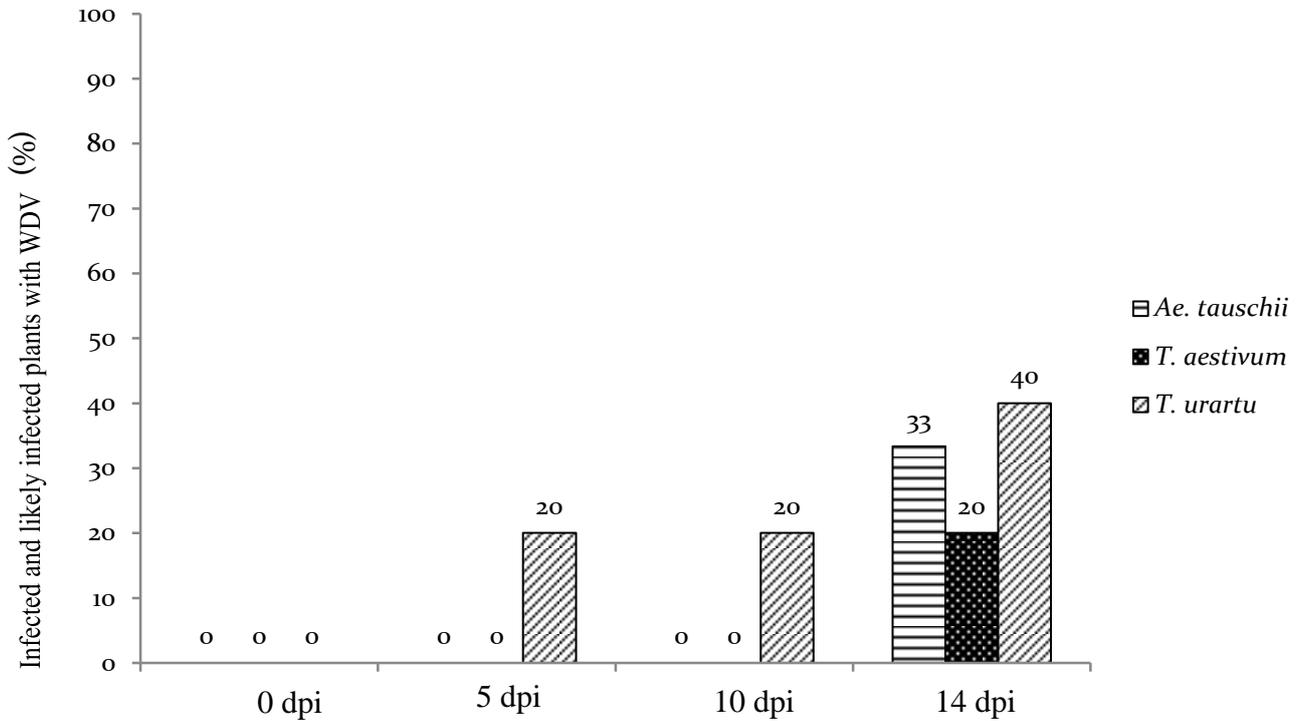
<sup>2</sup> All *T. urartu* plants had at least one wilted or necrotic leaf at the end, AS= Asymptomatic, NC= Necrosis.

<sup>3</sup> Mean calculated using two replicates of each sample, A: absorbance, at 405 nm: wavelength.

<sup>4</sup> P=positive; more than twice the value of the background, N= negative, LP= likely positive; close to positive.

Based on the ELISA value, no WDV infection could be detected in samples from 0 dpi, while at 5 dpi one *T. urartu* plant was likely to be infected. At 10 dpi, there was just one sample which could be likely positive for WDV infection which may not be trusted, due to the damage to leaves that occurred during storage, it was impossible to separate the different leaves. At 14 dpi, one plant out of five *T. aestivum* plants, one plant out of three *Ae. tauschii* plants, and two out of five *T. urartu* plants were clearly positive (Table 5). Thus, at 14 dpi 33% of the *Ae. tauschii* plants and 40% of the *T. urartu* plants along with 20% of the *T.*

*aestivum* plants were clearly positive for WDV (Fig. 11). Eventually, not so many plants were detected positive for WDV.

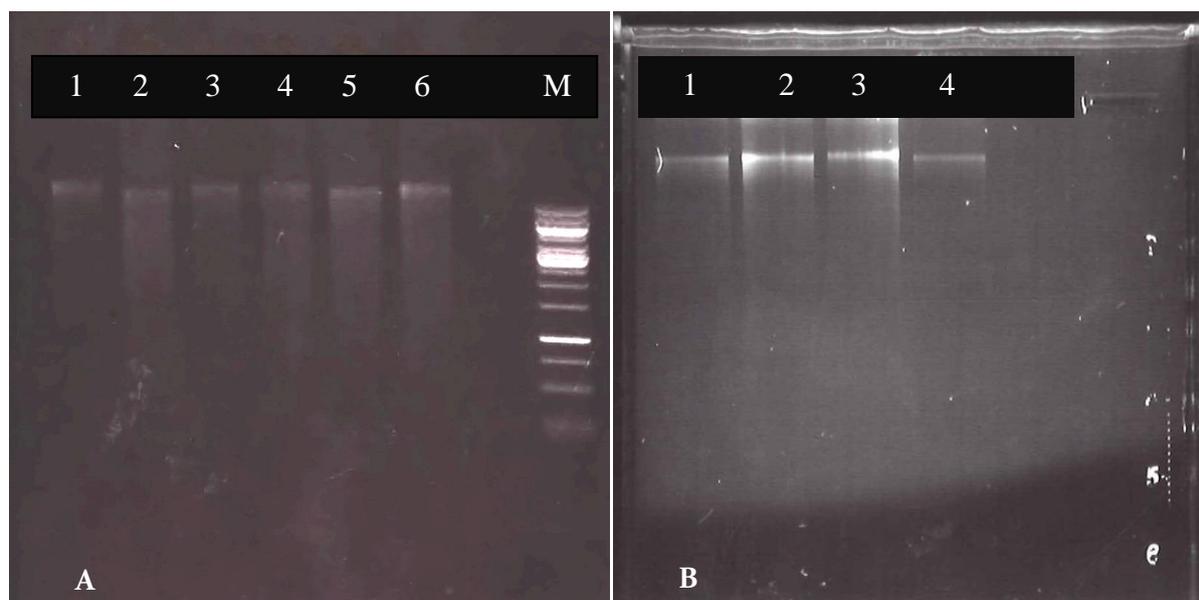


**Fig. 11.** Percentage of plants infected with WDV in the second experiment.

Although there was no WDV infection detected at 0 dpi, it cannot be said that the inoculation was not successful, since the plant material (wild species) used was not clonal and there might be genetic variation for WDV susceptibility in the plant population or the virus titer was too low to be detected by DAS-ELISA at this time-point. 20% of the *T. urartu* plants at the second and third time-points tested positive.

## PCR, RCA and cloning

While there were difficulties in amplifying viral DNA with PCR, tests with RCA were successful. The RCA method accomplished in amplifying the complete genome of WDV from the three tested source plants 13, 15 and 16, confirming the presence of circular virus DNA within the source plants (Fig. 12).



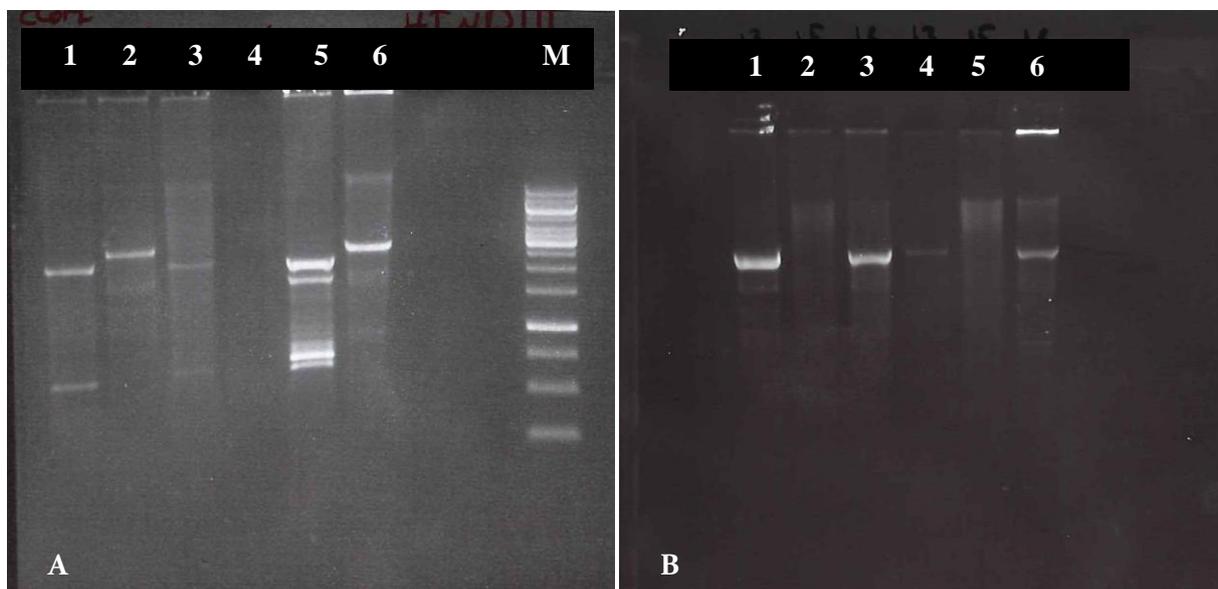
**Fig. 12.** Amplified *Wheat dwarf virus* DNA by RCA **A.** Amplified *Wheat dwarf virus* DNA by RCA, from source plants 13 (lanes 1 and 2), 15 (lanes 3 and 4) and 16 (lanes 5 and 6), M is GeneRuler™ 1kb DNA Ladder marker, **B.** Amplified *Wheat dwarf virus* DNA by RCA, from source plants 13 (lanes 1 and 2) and 16 (lanes 3 and 4).

The RCA products were digested by *SacI*, which is predicted to have one restriction site within the WDV genome. The RCA product from source plants 13 and 16 was cut once, while no digestion was seen in the RCA product from source plant 15, suggesting that there is no *SacI* restriction site present in this product (Fig. 13). The RCA products for all samples were also digested by two other restriction enzymes: *EcoRI* and *HindIII* (Fig. 13, Fig. 14).

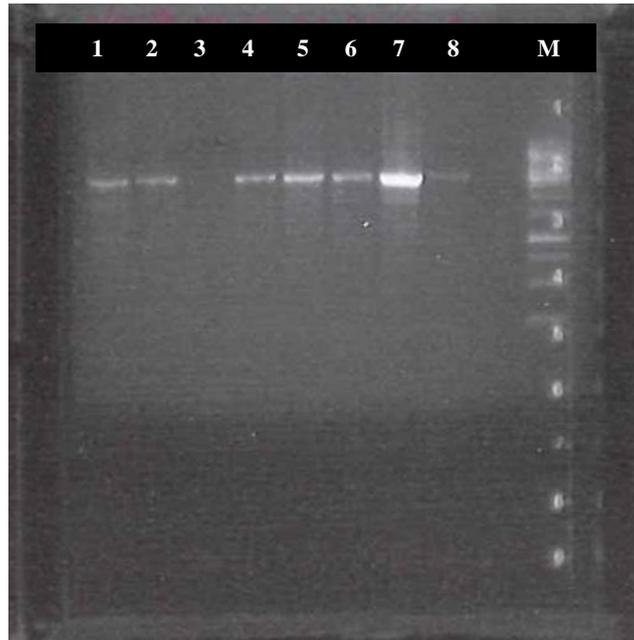
The results showed that there was more than one restriction site for *EcoRI* in the RCA products from all tested plants while *HindIII* cut the DNA from source plants 13 and 16 once (Fig. 13). Hence, the procedure continued using samples from source plants 13 and 16 and

*HindIII*, which has one restriction site in the WDV genome, for digestion. Four digests of the same RCA from each sample were done to increase the yield.

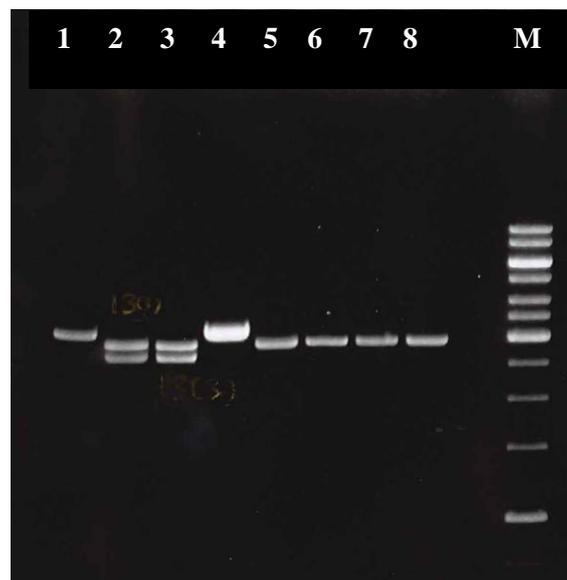
To determine the WDV genome sequences, the restricted genomes were purified, ligated into pBluescript KS+ and transformed into *E. coli*. Restriction enzyme digest of the obtained plasmid clones showed that 14 of the tested clones contained an insert of the expected size, approximately 2.7 kb (Fig. 15, Fig. 16). The successful cloning was also confirmed by PCR subsequently (Fig. 16). In total, six clones (three clones per plant sample) were sequenced.



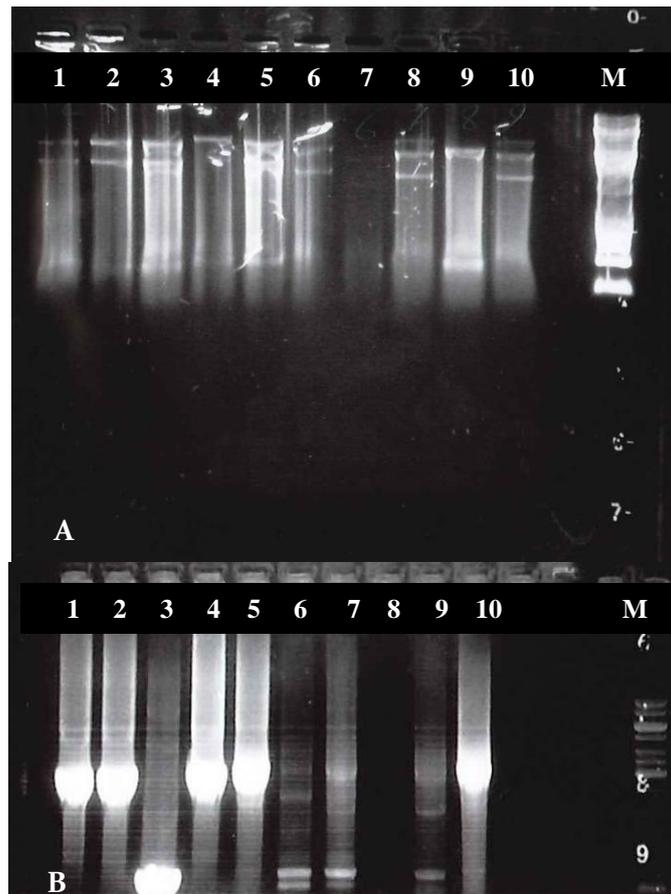
**Fig. 13.** Restriction of RCA product by different restriction enzymes **A.** Restriction of RCA products by *EcoRI* and *HindIII*; lane 1 is sample 13, lane 3 sample 15 and lane 5 sample 16 cut by *EcoRI*; Lane 2 is sample 13, lane 4 sample 15 and lane 6 sample 16 digested by *HindIII*, M is GeneRuler™ 1kb DNA Ladder marker, **B.** Digestion of RCA product by *SacI* which has one restriction site present in WDV genome. Lanes 1 and 4 are from source plant 13, lanes 2 and 5 from 15 and lanes 3 and 6 from source plant 16.



**Fig. 14.** Digestion of the RCA products by *Hind*III with one restriction site present in the WDV genome. Lanes 1-4 are four RCA reactions for source plant 13, lanes 5-8 are four RCA reactions for source plant 16. M is GeneRuler™ 1kb DNA Ladder marker.



**Fig. 15.** Restriction enzyme analysis by *Hind*III of plasmids for cloned WDV genome A. The lanes represent different clones. Lanes 2 and 3 show the digested plasmid by *Hind*III, the upper bands is the vector with the size of 3 kb and the lower band is the inserted WDV DNA from sample 13 with the size of 2.7 kb. Other lanes show plasmids without insert.



**Fig. 16.** Restriction enzyme analysis by *Hind*III of plasmids for cloned WDV genome from source plant 16 **A**. The upper band in lanes 2, 3, 5, 6, 8 and 10 are the digested vector and the lower band is an insert of DNA with the expected size of 2.7 kb **B**. The samples were also tested by PCR to confirm cloning of the correct insert (lanes 1, 2, 4, 5, 10).

## Sequence analysis

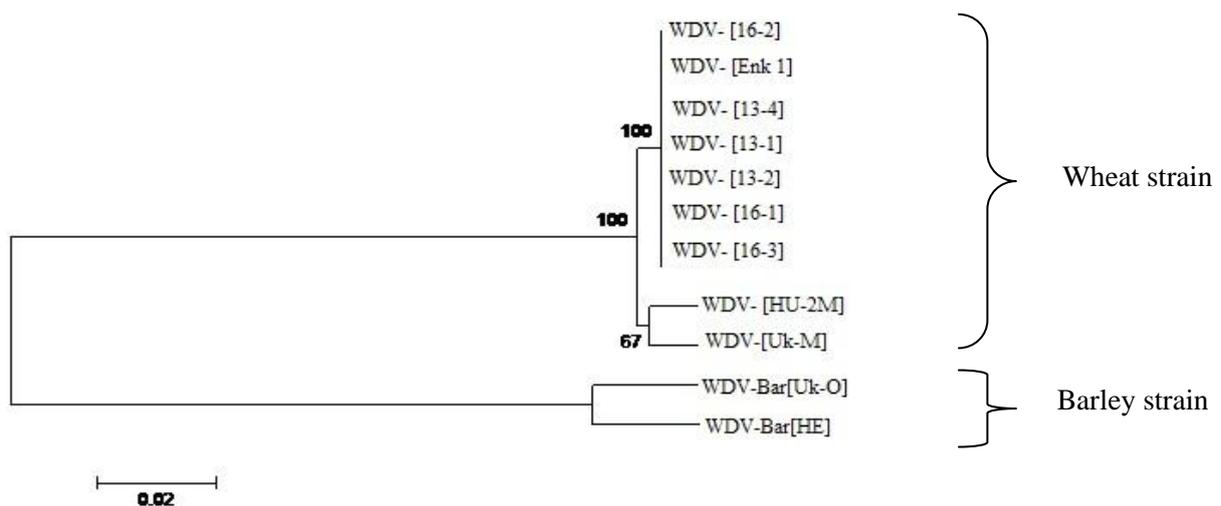
The sequences of 2746 to 2755 bp were compared with the complete WDV genome sequences available in the GenBank database. It was found that the complete nucleotide sequences of the six virus clones were 99% identical to the WDV isolate Enköping 1 (Accession number AJ311031). Of 2750 bp, around 27 nucleotides were different (1% difference) between the analyzed clones and WDV-[Enk1]. The sequencing results confirm that the virus isolates used for inoculation belonged to the wheat strain of WDV.A phylogenetic analysis was carried out to show the relationships among the determined sequences (Fig. 17).

**Table 6.** Description of WDV isolates used for comparisons

Virus isolate	Acronym	Accession no	Reference
<i>Wheat dwarf virus</i> isolate Enköping <sup>1</sup>	WDV-[Enk1]	AJ311031.1	Kvarnheden et al., 2002
<i>Wheat dwarf virus</i> isolate WDV-HU-2Marton <sup>1</sup>	WDV-[HU-2M]	FN806785.1	Tóbiás et al., 2011
<i>Wheat dwarf virus</i> isolate WDV-Uk-Miron <sup>2</sup>	WDV-[Uk-M]	FN806784.1	Tóbiás et al., 2011
<i>Wheat dwarf virus</i> isolate WDV-Uk-Odessa	WDV-Bar[Uk-O]	FN806787.1	Tóbiás et al., 2011
<i>Wheat dwarf virus</i> barley strain	WDV-Bar[HE]	FM999833.1	Tóbiás et al., 2011

<sup>1</sup> HU= Hungary

<sup>2</sup> UK= Ukraine



**Fig. 17.** Neighbor-joining analysis showing predicted relationships between 11 isolates of *Wheat dwarf virus* (WDV) based on complete genomic nucleotide sequences. Horizontal lines are in proportion to the number of nucleotide differences between nodes. Numbers represent bootstrap values. For abbreviations of virus names and accession numbers, see Table 6.

The phylogenetic analysis confirmed that the six analyzed isolates belonged to the wheat strain of WDV (Fig. 17). All the analyzed clones showed a close relationship with WDV-[Enk1] (bootstrap value 100%), and were clearly separated from the barley strain. The isolates from barley and wheat formed two well-supported clades (bootstrap value 100%).

## Discussion

To compare the differences in WDV susceptibility of different species, two experiments were designed. In the first study, after WDV inoculation, three different species, including *T. aestivum*, *Ae. tauschii* and *T. urartu*, were tested by DAS-ELISA to measure the virus titer in different parts of the inoculated plants. The result of this test revealed that the virus transmission was not successful for all plants. According to a recent study by Nygren et al. (unpublished) these three species display different levels of susceptibility to WDV infection according to phenotypic evaluation. The seed material for the wild species that were used for this experiment was not clonal, so the plants could vary in their response to infection by WDV since they are genetically different. After testing the source plants, it was found that not all of them were infected by WDV (Table 1), hence it could be assumed that some of the leafhoppers, which were used to transmit the virus to the plants, were not viruliferous and just those leafhoppers feeding on the infected source plants harbored the virus and could inoculate the plants. Still, in the second harvest at 4 dpi plants of all the species got infected, which means they are vulnerable to WDV infection.

Since the result of harvest III (10 dpi) in the first experiment showed that the plants were not infected by WDV, it is more likely that the plants were harboring non-viruliferous leafhoppers (leafhoppers from source plants D and E, Table 1). Otherwise, a higher virus titer was expected in harvest III because the virus would have more time to multiply and move throughout the plant tissue.

In the first experiment, root tissues were also tested by DAS-ELISA. In the case of *Tobacco mosaic virus*, virus is expected to move to the roots where the sink is (Astier et al., 2007), but in our case, only one sample was clearly positive and a few samples showed potential WDV infection in the root. Some of the inoculated *T. aestivum* plants showed potential infection in their leaves and one clear infection in the roots was detected as well, but due to the high background and the low value of the positive control (maybe as a result of using cell culture plate instead of ELISA plate), the results could not be interpreted very well. The clear positive result for *T. aestivum* 1 at 10 dpi could be the result of a larger amount of virus inoculated by leafhoppers to this plant. There can be a correlation between the virus titer detected in the analyzed tissues and the feeding points of leafhoppers. A higher virus titer is

expected (in susceptible species) in the leaves which have emerged after inoculation compared to the old leaves. However, the leafhoppers' feeding points were not monitored.

In the second study, prior to setting up the inoculation, the source plants were tested by ELISA to avoid using non-viruliferous leafhoppers. The result of DAS-ELISA revealed that just three source plants were clearly positive although more plants appeared to have WDV symptoms. For instance, source plant 17 was found not to be infected with WDV when tested by ELISA, but it was strongly suspected to be infected because it showed symptoms of WDV infection and leafhoppers from infected old source plants had been transferred to it. It is believed that the virus was not detected in the ELISA analysis because leafhoppers had recently been transmitted to this cage (latency period) or the virus titer was too low to be detected by ELISA. Some plants showed symptoms similar to wheat dwarf disease, but these symptoms may have appeared as the result of other biotic or abiotic stresses such as nutrient deficiency.

Having the experience from the first study, the second experiment was designed with four sampling time-points in order to get a better overview of how the virus level varies over time in inoculated plants (Table 6). Based on the ELISA values, there was no detectable infection at 0 dpi, while at 5 dpi one *T. urartu* plant was likely to be infected. At 10 dpi, there was no clear infection, maybe due to the damage that happened to the leaves during storage at – 20°C. At 14 dpi, 1 out of 5 *T. aestivum* plants, two out of three *Ae. tauschii* plants, and two out of five *T. urartu* plants were clearly positive. Hence, again it could be concluded that at 0 dpi the virus titer was too low to be detected by ELISA, whereas at 5 dpi the low virus titer could be due to using non-viruliferous leafhoppers.

Virus acquisition by leafhoppers and transmission to plants are key issues in studying plant-virus interactions and an infected source plants is a major requirement for studying virus movement. According to Storey (1928), although the acquisition time can be short, a latency period of at least one day is required (Reynaud and Peterschmitt, 1992). It can be also speculated that the new environment may affect the leafhoppers and their feeding behaviors when they are moved between cages. It may take time to adapt to the new environment and to start feeding on the plant. The success of virus transmission is also correlated with viral virulence and host susceptibility (Reynaud and Peterschmitt, 1992), which was mainly studied in this experiment. However, maybe the right leaf samples were not tested, because it

is presumed that leaves that emerge after inoculation should have a higher virus level (Peterschmitt et al., 1992). In the second trial, due to the rapid growth of the plant at 10 dpi and 14 dpi, it was difficult to find the youngest leaf.

It is clear that the transmission probably did not completely work because of problems with the source plants, so the main objective of the experiment could not be answered. However, it was confirmed by this study that WDV is able to infect *Ae. tauschii* and *T. urartu*.

There are many notable factors affecting virus transmission from the leafhoppers to the plant and development of infection in the plants. These factors should be taken into consideration for future studies: (1) Duration of the inoculation period could be extended to increase the efficiency, even if the three days used in these two studies are assumed to be enough. Moreover, in the study by Reynaud and Peterschmitt (1992), a positive correlation between transmission efficiency and virus acquisition period was also observed, especially for non-propagative viruses. (2) Individual differences among the leafhoppers could affect virus transmission since they are not identical and they could differ in their genotype and virus transmission ability. (3) There could be differences between the sexes among the leafhoppers as well, maybe the females would be more efficient in transmitting the virus (Idris et al., 2001). (4) Differences in age among the leafhoppers, since the nymphs are more capable of transmitting the virus (Manurung et al., 2005). (5) Age of the plant at the time of inoculation (gradually increasing resistance by age; mature plant resistance): In the second experiment, plants were inoculated at third and fourth leaf stage, but they would be more susceptible to WDV at the first leaf stage. Inoculating the plant at a more developed stage could have effects on how fast the virus could spread. Inoculated mature plants show reduced virus titer, therefore milder symptoms appear. Inoculating plants at 1<sup>st</sup> leaf stage would be beneficial since the site of the virus inoculation will be known (the first emerged leaf) and it would mark the beginning of systemic spread of the virus. (6) Infection will be more severe with a high virus inoculation dose, so in this study different test plants might vary in virus load. It has been shown that transmission success is significantly correlated with the virus dose within the insect vector *Agalliopsis nooella* (Granados et al., 1967), so using additional viruliferous leafhoppers for inoculation might be beneficial. (7) The temperature has effects on both leafhoppers and plants. As the temperature increases the rate of infection may raise; however plants will also develop faster at higher temperature. (8) Sometimes low virus titer or its absence is due to the lack of virus replication (Reynaud and Peterschmitt, 1992).

Sequence analyses of two WDV isolates from the source plants revealed a close relationship with previously characterized studied WDV isolates from wheat (Kvarnheden et al., 2002). These results confirmed that the experiments had been carried out using the normal wheat infecting strain of WDV.

However, not only the problem with non-infected source plants, which was the main limitation of this study, but also the high background in the ELISA absorbance values for the first experiment made the result interpretation difficult. The high background could be the result of un-specific binding of the antibody, which could be the outcome of using old buffers or antibodies, pipetting errors, contamination of the negative control which could mean that the negative control was not really negative. Anyhow, still it could be concluded that the virus titer was higher in the second harvest compared to the first one which showed no infection. Likewise the negative result in detection of virus CP in the first harvest (0 dpi) could be due to low virus titer below the detection level of ELISA.

In order to amplify the virus DNA for subsequent sequencing, PCR was performed on two infected samples; one old source plant and one new established source plant (Fig. 9). Although just one plant was confirmed to be infected by PCR, it is assumed that there are better primer pairs (Kvarnheden et al., 2002), which have been demonstrated to work for a broad range of WDV isolates and can be used for detecting WDV by PCR in the future.

To conclude, considering the results obtained from two trials, the best way for conducting additional studies on this subject and to test the hypothesis, it is crucial to be more careful with the plant age at the time of inoculation, extension of inoculation period and using a sufficient number of viruliferous leafhoppers to have a successful inoculation. However, most important will be to have source plants with a high virus titer. Also more studies are required with exact monitoring of leafhopper feeding point. Then it will be possible to continue with further molecular studies.

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