

Temperature affected Transmission, Symptom Development and Accumulation of *Wheat Dwarf Virus*

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Abstract

Ghodoum Parizipou M.H., Ramazani L., Pakdaman Sardrood B. (2018): Temperature affected transmission, symptom development and accumulation of *Wheat dwarf virus*. Plant Protect. Sci., 54: 222–233.

One of the biotic agents of yellowing and stunting in wheat and barley cultivations is *Wheat dwarf virus* (WDV) which is naturally transmitted by the leafhopper *Psammotettix alienus* (Dahlbom). WDV-Wheat and WDV-Barley isolates of WDV were transmitted to wheat and barley, respectively, using the leafhoppers under four temperature regimes of constant 20, 25, 30, and 35°C. Infection rate, symptom development and virus content of the virus-inoculated plants were determined and the data was statistically analysed. The results showed that the temperature of 25°C was associated with the highest infection rate caused by the viruses. Moreover, *P. alienus* nymphs were found to be more efficient vectors of WDV than adults, highlighting the importance of nymphs in the epidemiology of wheat dwarf disease. WDV-infected plants incubated at 35°C showed less symptoms than those kept at 20, 25, and 30°C. ELISA results showed that these plants had comparatively low virus content. However, there was no significant difference between the infection rate, symptom development and virus content in plants infected by WDV-Wheat or WDV-Barley.

Keywords: geminivirus; *Psammotettix alienus*; symptom development; epidemiology; cereal

Wheat dwarf virus (WDV) is a species of the genus *Mastrevirus*, family *Geminiviridae*, being responsible for considerable crop losses by causing stunting, yellowing or reddening in its economically important hosts including wheat and barley plants cultivated in many parts of the world (TÓBIÁS *et al.* 2011; KVARNHEDEN *et al.* 2016). The twin-shaped quasi-icosahedral virus particles harbour a circular single-stranded (ss) DNA genome which encodes four proteins including movement protein (MP; V1) and coat protein (CP, V2), and replication-associated proteins (RepA, C1 and Rep, C1:C2). V1 and V2 genes are located on the viral-sense strand while C1 and C2

genes are located on the complementary-sense strand (GUTIERREZ *et al.* 2002). Initially, two strains of WDV have been identified: wheat strain and barley strain (referred to as WDV-Wheat – WDV-W and WDV-Barley – WDV-B) which share 83–84% nucleotide identity throughout the genome (KÖKLÜ *et al.* 2007; SCHUBERT *et al.* 2007). A later study suggested that isolates from wheat, barley, and other cereals should be classified as belonging to strains A to E of WDV according to which viruses belonging to strain A of WDV (WDV-A) preferentially infect barley, whereas those from strain C (WDV-C) preferentially infect wheat (MUHIRE *et al.* 2013). The viruses of strains B

Supported by funds from Plant Protection Department, Ramin University of Agriculture and Natural Resources, Mollasani, Iran.

and D that infect barley were exclusively reported from Iran. Recently it was demonstrated that WDV isolates infecting wheat and barley hosts are phylogenetically divided into two super-clades including WDV-W and WDV-B, respectively, and a number of WDV isolates originating from Iran formed a new distinct cluster named strain WDV-F (GHODOUM PARIZIPOUR *et al.* 2017).

The virus is naturally transmitted by leafhoppers of the genus *Psammotettix* (order Hemiptera, family Cicadellidae) in a circulative, non-propagative manner (LINDSTEN & VACKE 1991). *Psammotettix alienus* Dahlbom was reported to transmit WDV in Finland and China (LEMMETTY & HUUSELA-VEISTOLA 2005; WANG *et al.* 2014). However, the species *P. provincialis* Ribaut is responsible for transmission of WDV in Syria (EKZAYEZ & KUMARI 2011). Generally, *P. alienus* is considered to occur mainly in cereal cultivations globally (GREENE 1971; ABT *et al.* 2017). In Iran *P. alienus* is responsible for transmission of WDV among cereals (LOTFIPOUR *et al.* 2013b). WDV can reach the salivary glands via two pathways in the insect body resulting in rapid virus transmission by the vector (WANG *et al.* 2014). Recently, it was found that *P. alienus* individuals originated from Iran are able to transmit WDV-Wheat and WDV-Barley to wheat and barley plants with high transmission efficiency, respectively (GHODOUM PARIZIPOUR *et al.* 2016).

Temperature has been shown to affect the vector transmission of a number of plant viruses significantly (KASSANIS 1957; SYLVESTER & RICHARDSON 1965; BAR-JOSEPH & LOEBENSTEIN 1972; LING & TIONGCO 1975; DAMSTEGT 1984; SMYRNIODIS *et al.* 2001; ANHALT & ALMEIDA 2008). However, there is no greenhouse study showing how temperature can affect the vector transmission of WDV to wheat and barley hosts. In a field study, LINDBLAD and ARENÖ (2002) indicated that cold weather in early summer can adversely affect the lifecycle and population size of *P. alienus*. Moreover, they observed that warm weather in the autumn might lead to a large overwintering population of the leafhoppers. When the average temperatures were < 10°C, only a few *P. alienus* individuals were caught, and as the temperature increased, a larger number of leafhoppers were caught. Another survey on the population dynamics of *P. alienus* was carried out in Germany which identified the temperature as an important factor directly affecting the population size of the leafhopper (MANURUNG *et al.* 2005). It was found that warmer weather of the year 2000 was associ-

ated with a larger population size of the leafhopper in cereal fields compared to the year 1999.

Generally, it was shown that temperature can also affect the symptom development in several plant–virus interactions (HILLMAN *et al.* 1985; ADAMS *et al.* 1986; SCHUERGER & HAMMER 1995; DAHAL *et al.* 1998, LLAMAS-LLAMAS *et al.* 1998; CHELLAPAN *et al.* 2005; LI *et al.* 2009; SUGIYAMA *et al.* 2009; VELÁZQUEZ *et al.* 2010; GHODOUM PARIZIPOUR *et al.* 2014; GHOSHAL & SANFAÇON 2014; CHUNG *et al.* 2015). In most of the studies it was reported that high temperatures are associated with reduced symptom expression in virus-infected plants (HILLMAN *et al.* 1985; DAHAL *et al.* 1998; LLAMAS-LLAMAS *et al.* 1998; CHELLAPAN *et al.* 2005; VELÁZQUEZ *et al.* 2010; GHODOUM PARIZIPOUR *et al.* 2014; GHOSHAL & SANFAÇON 2014; CHUNG *et al.* 2015). However, there is no evidence whether temperature can affect symptom development on WDV-infected plants.

We examined the effect of different temperature on transmission of WDV-W and WDV-B isolates to wheat and barley plants, respectively, by adult and nymph stages of *P. alienus* and how temperature can affect the mortality rate of the leafhopper vector during the virus transmission. We also investigated the influence of temperature on symptom development and virus content of WDV-infected plants under different temperature regimes.

MATERIAL AND METHODS

Plant materials. Seed of spring wheat (*Triticum aestivum* L. cv. Marvdasht) and spring barley (*Hordeum vulgare* L. cv. Kavir) was sown into Petri dishes with wet filter paper and irrigated daily and kept in a dark chamber at room temperature for three days. The seedlings were then transferred into plastic pots containing peat moss and soil (1 : 1) and grown under greenhouse conditions (20–25°C, 50% RH, 16/8-h photoperiod).

Total DNA extraction. Total DNA was extracted from plant tissues collected from field and greenhouse samples using a silica matrix according to the method described by BOOM *et al.* (1990) with some modifications. Plant tissues (100 mg) were homogenised and mixed with 1 ml of lysis buffer (0.2 M Na acetate pH 5, 25 mM EDTA, 4 M guanidine hydrochloride, 2.5% PVP-40). The suspension was then mixed with 60 µl of 20% lauroylsarcosine. The mixture was incubated at 70°C for 10 min while shaking, then the

<https://doi.org/10.17221/147/2017-PPS>

mixture was centrifuged at 13,000 rpm for 6 min and 700 µl of supernatant was mixed with 50 µl of silica matrix. The absolute ethanol (630 µl) was added to the mixture and it was centrifuged at 8,000 rpm for 20 seconds. The pellet was washed once with 600 µl of washing buffer I (0.2 M Na acetate pH 5, 25 mM EDTA) and twice with 500 µl of washing buffer II (10 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl in 80% ethanol). DNA was eluted using 50 µl of TE buffer (10 mM Tris-HCl pH 8, 0.1 mM EDTA) and stored at –20°C for molecular experiments. DNA was also extracted from whole insects using cetyltrimethylammonium bromide (CTAB) according to the method described by MAIXNER *et al.* (1995).

PCR and sequencing. Polymerase chain reaction (PCR) was performed to detect WDV in both plant tissue and leafhopper vector. Two strain-specific pairs of primers which had been previously shown to amplify a 1 300 and 1 350 bp fragment of the Rep-coding region of WDV-W and WDV-B, respectively (GHODOUM PARIZIPOUR *et al.* 2016) were used in PCR assay (Table 1). The reaction was performed in 25 ml volume containing ~15 ng of total DNA, 1.5 mM MgCl₂, 200 µM of each dNTP, 1 mM of each oligonucleotide, and 2 U of *Taq* DNA polymerase (Thermo Scientific, Darmstadt, Germany) in the reaction buffer provided by the same source. The mixture was heated for 2 min at 96°C and subjected to a 35-cycle PCR program of 30 s at 96°C, 30 s at 62°C, and 2 min at 72°C. The final extension phase was subsequently performed for 10 min at 72°C. Two sets of control samples were used in PCR assay including positive (samples which had been confirmed to be infected with WDV-W and WDV-B) and negative controls (samples from healthy wheat and barley plants). Approximately 5 ml of the

reaction mixture was then loaded directly onto a 1.2% agarose gel previously stained with ethidium bromide and UV-visualised.

Full-length amplification of viral genome. In order to obtain complete genomes of WDV-Wheat and WDV-Barley from the infected wheat and barley plants, four samples including 2 tissue samples from the PCR-positive plants displaying the typical symptoms of WDV infection and 2 leafhopper individuals feeding on them, were then subjected to the second PCR this time using Platinum *Taq* DNA Polymerase with high fidelity amplification (Invitrogen, Carlsbad, USA) and general WDV primer pairs (Table 1). To amplify the complete genome of each virus, three independent PCRs were performed and the resulting PCR products were electrophoresed on 1% agarose gel with TAE buffer [40 mM Tris-acetic acid, 0.1 mM EDTA, pH 8.2–8.4 (at 25°C)] and visualised by ethidium bromide staining. DNA fragments with the expected sizes including 1 300, 1 350, 1 114, and 1 375 bp were excised from the gel and purified using a PCR Clean-up Gel Extraction Kit (Macherey-Nagel, Düren, Germany). The quality and quantity of purified DNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, USA). Purified DNA fragments were sequenced at GATC Biotech (Konstanz, Germany). The Chromas (Technelysium) software was applied for base calling and the sequences determined from each end of the fragments were then assembled using SeqMan Pro software (DNASTAR Lasergene v8) in order to obtain the full length of each virus genome. Then the position of ORFs and intergenic regions of the resulting sequences were determined and the sequences were submitted to GenBank using a BankIt

Table 1. Oligonucleotide primers used in this study

Primer ^a	Sequence [5'-3'] ^b	Target	Genome position ^c	Expected product size (bp)
p2730 ^C	CCRCACACCCDAACASGGCCCA	WDV-Wheat	2 673–2 693	1 300
p1430 ^V	GAAYGAGTAGTTGATGAAYGWCTC	WDV-Wheat	1 403–1 426	
p2720 ^C	CGCGGGACCACCCGTCGCT	WDV-Barley	2 671–2 689	1 350
p1370 ^V	GCGAARAAYGATTCMCCYTCATA	WDV-Barley	1 401–1 423	
p656 ^V	GATAATAATCGGCATACAAATCAGA	WDV	656–680	1 114
p1770 ^C	CTACATCTGCGGACCAAC	WDV	1 770–1 753	
p2539 ^V	CCCTGCGAAAAGGGGAAATGTG	WDV	2 539–2 560	1 375
p1142 ^C	TGCGTATAGGCACATACAACATC	WDV	1 164–1 142	

^av – virion-sense strand, c – complementary-sense strand; ^bR – puRine (A or G), Y – pYrimidine (C or T/U), S – Strong (C or G), W – Weak (A or T), D – not C (A or G or T/U), M – aMino (A or C); ^cwith reference to WDV-Wheat [MRDTA24-1] and WDV-Barley [MRDHV02-4]

Table 2. Details of full-length genome sequences of *Wheat dwarf virus* (WDV) and *Oat dwarf virus* (ODV) isolates

Virus	Accession number	Collection			Reference	
		country	source	year		
ODV-[SxA25]	AM296025	Germany	<i>Avena sativa</i>	2005	SCHUBERT <i>et al.</i> (2007)	
WDV-Barley [BaW1]	AM411651	Germany	<i>Hordeum vulgare</i>	2005		
WDV- Barley [SxA18]	AM296018	Germany	<i>A. sativa</i>	2003		
WDV- Barley [Cz19]	AM296019	Czech Rep	<i>H. vulgare</i>	2006		
WDV-Wheat [SxA22]	AM296022	Germany	<i>Lolium perenne</i>	2002	WU <i>et al.</i> (2015)	
WDV-Wheat [Winter wheat 110]	KJ473700	Germany	<i>Triticum aestivum</i>	2008		
WDV- Wheat [Spelt9]	KJ473696	Germany	<i>T. spelta</i>	2008		
WDV- Wheat [Winter rye 102]	KJ473699	Germany	<i>Secale cereale</i>	2008		
WDV- Wheat [Triticale 117]	KJ473702	Germany	triticale	2008		
WDV- Wheat [Rye4-6]	KJ473706	Germany	<i>S. cereale</i>	2008		
WDV- Wheat [07HBWH2]	KJ536126	China	<i>A. sativa</i>	2007		
WDV- Wheat 08YNKM10]	KJ536129	China	<i>T. aestivum</i>	2008		
WDV- Wheat [08YNKM13]	KJ536141	China	<i>H. vulgare</i>	2008		
WDV-Wheat [HU-B]	AM040732	Hungary	<i>Psammotettix alienus</i>	2005		TÓBIÁS <i>et al.</i> (2006)
WDV- Wheat [HU-F]	AM040733	Hungary	<i>P. alienus</i>	2005		
WDV-Barley [D01]	FM999832	Hungary	<i>H. vulgare</i>	2007		
WDV-Wheat [HU-KP10-1]	JQ647455	Hungary	<i>T. aestivum</i>	2010	LIU <i>et al.</i> (2012)	
WDV- Wheat [SYZSH03]	KT958235	Iran	<i>Sorghum halepense</i>	2015	GHODOUM PARIZIPOUR <i>et al.</i> (2016)	
WDV- Wheat [SYZAF12]	KT958237	Iran	<i>A. fatua</i>	2015		
WDV- Wheat [SYZBC14]	KT958238	Iran	<i>Bromus commutatus</i>	2015		
WDV- Wheat [SYZEP26]	KT958240	Iran	<i>Eremopoa persica</i>	2015		
WDV- Wheat [SYZHM32]	KT958241	Iran	<i>H. murinum</i>	2015		
WDV- Wheat [SYZLP33]	KT958242	Iran	<i>L. persicum</i>	2015		
WDV- Wheat [SYZCD35]	KT958243	Iran	<i>Cynodon dactylon</i>	2015		
WDV- Wheat [SYZAK54]	KT958244	Iran	<i>Aegilops kotschyi</i>	2015		
WDV-Barley [SYZHS06]	KT958236	Iran	<i>H. spontaneum</i>	2015		
WDV-Wheat [MRTTA56]	KT958245	Iran	<i>T. aestivum</i>	2015		
WDV-Barley [KLTHV03]	KT958251	Iran	<i>H. vulgare</i>	2009		
WDV-Barley [MRDTA22-1]	KY679016	Iran	<i>H. vulgare</i>	2016		this study
WDV-Wheat [MRDTA24-1]	KY793552	Iran	<i>T. aestivum</i>	2016		
WDV-Barley [MRDHV02-4]	KY679017	Iran	<i>P. alienus</i>	2016		
WDV-Wheat [MRDHV04-2]	KY793553	Iran	<i>P. alienus</i>	2016		
WDV-Barley [Bg17]	AM989927	Bulgaria	<i>H. vulgare</i>	2006	TÓBIÁS <i>et al.</i> (2009)	
WDV-Wheat [Enkopping]	AJ311031	Sweden	<i>T. aestivum</i>	2001	KVARNHEDEN <i>et al.</i> (2002)	
WDV-Wheat [FR]	X82104	France	<i>T. aestivum</i>	1994	BENDAHDANE <i>et al.</i> (1995)	
WDV-Wheat [UK193WDV]	HF968634	UK	<i>T. aestivum</i>	2012	SCHUBERT <i>et al.</i> (2013)	
WDV- Wheat [UK193WDV1]	HF968633	UK	<i>T. aestivum</i>	2012		
WDV- Barley [ES1BDV_1]	HF968639	Spain	<i>H. vulgare</i>	2012		
WDV-Barley [ES1BDV]	HF968640	Spain	<i>H. vulgare</i>	2012		
WDV- Barley [AU196BDV1]	HF968646	Austria	<i>H. vulgare</i>	2012		
WDV-Barley [TR2]	AJ783960	Turkey	<i>H. vulgare</i>	2004	KÖKLÜ <i>et al.</i> (2007)	
WDV-Wheat [Uk-Miron]	FN806784	Ukraine	<i>T. aestivum</i>	2009	TÓBIÁS <i>et al.</i> (2011)	
WDV-Wheat [Sanxi Yangling]	EF536879	China	<i>T. aestivum</i>	2009	WU <i>et al.</i> (2008)	
WDV-Wheat [07YNKM29]	EU541489	China	<i>H. vulgare</i>	2007		
WDV-Wheat [SXTY04]	DQ868525	China	<i>T. aestivum</i>	2004		
WDV-Barley [CZ11229]	FJ546181	Czech Rep	<i>H. vulgare</i>	2008	KUNDU <i>et al.</i> (2009)	
WDV- Barley [CZ6482]	FJ546178	Czech Rep	<i>H. vulgare</i>	2008		
WDV-Wheat [CZ1841]	FJ546191	Czech Rep	<i>T. aestivum</i>	2008		
WDV- Barley [Poland: Lagiewniki]	KM079155	Poland: Lagiewniki	<i>H. vulgare</i>	2007	TRZIMEL & JEZEWSKA (unpubl.)	
WDV- Wheat [Bavanat]	JN791096	Iran	<i>H. vulgare</i>	2010	LOTFIPOUR <i>et al.</i> (2013a)	
WDV- Wheat [Shahrekord]	JN791095	Iran	<i>T. aestivum</i>	2010		

<https://doi.org/10.17221/147/2017-PPS>

submission tool [online available at www.ncbi.nlm.nih.gov/WebSub/?form=history&tool=genbank]. Table 2 shows the details of sequences which were deposited in the GenBank under the corresponding accession numbers.

Sequence analysis of WDV isolates. A total number of 53 WDV complete sequences including 4 sequences obtained in the present study and 49 sequences from different hosts and various geographical locations previously published at GenBank were used to perform a phylogenetic analysis (Table 2). The sequences were aligned by CLC Main Workbench software (version 7.6.2) and the maximum likelihood method using the general time-reversible model test (TAVARÉ 1986) with 1000 bootstrap replicates was applied to construct a phylogenetic tree. One isolate of *Oat dwarf virus* (ODV) was included as an outgroup.

Leafhopper. A D.VAC vacuum insect collector (Rincon-Vitova Insectaries, Ventura, USA) was used to collect *P. alienus* individuals from wheat and barley fields with plants showing typical symptoms of WDV infection in Marvdasht, Fars province, Iran, in June 2016. Two sampling processes were carried out separately in order to collect the leafhoppers on cultivated wheat and barley plants. Adult individuals of *P. alienus* were primarily characterised by the ground creamy colour of their head, pronotum and scutellum, of 2.2–2.8 mm in length (GREENE 1971) and were isolated using an aspirator for further experiments. In order to rear the insects, they were caged on healthy barley seedlings and kept at greenhouse conditions. Morphological identification of leafhoppers was carried out as described by GHODOUM PARIZIPOUR *et al.* (2016). The leafhopper species was identified using morphological descriptions (NIELSON 1968). In order to propagate virus-free colonies of leafhoppers, one male and one female insect were transferred to a healthy barley seedling. The progenies of each mate were then transferred to new healthy barley seedlings for three generations. Barley plants with leafhoppers were inspected daily to observe any symptoms of the viral infection. To ensure that resulting leafhoppers are virus-free, PCR assay using a WDV-specific primer pair (p656^V/p1770^C; Table 1) was performed, as described above, to detect WDV in leafhoppers and barley plants on which they had been caged. The virus-free leafhoppers were then used for the vector transmission experiments.

Infection sources of WDV. Two infection sources of WDV-Wheat and WDV-Barley isolates were prepared on wheat and barley plants, respectively. To

this end, two groups (3–5 individuals) of leafhoppers collected from fields with WDV-infected wheat and barley plants were immediately caged on wheat and barley seedlings, respectively, for 7 days. The insects were then removed and the inoculated plants were kept in the greenhouse with the conditions described below. Visual inspections were carried out to find the typical symptoms of WDV infection on the inoculated plants. PCR assay using strain-specific primer pairs of WDV-W and WDV-B (Table 1) was performed at 30 days post inoculation (dpi) as described above, to confirm the respective viral infections in wheat and barley plants. Complete genomes of WDV-W and WDV-B isolates were obtained from the respective plants as described above. These plants were then used as infection sources of WDV-Wheat and WDV-Barley for vector transmission experiments.

Virus transmission. The virus-free leafhoppers were allowed to feed on the infection sources for 7 days. Three combinations of viruliferous leafhoppers (2 insects per plant) including 2 adults, 1 adult & 1 nymph, and 2 nymphs were used to inoculate plants. For each vector combination, 14 single-plant pots were used. The leafhoppers were caged on one-week-old seedlings of wheat and barley for six days. Then leafhoppers were removed and inoculated plants were kept at different conditions (see below) for the monitoring of symptom development. The inoculation of wheat and barley seedlings with WDV-W and WDV-B isolates, respectively, was conducted in 4 growth chambers with constant temperatures of 20, 25, 30, and 35°C, 40% RH and 16/8-h photoperiod to investigate the effect of temperature on vector transmission, symptom development and insect mortality. At the end of each inoculation, the number of viruliferous leafhoppers was counted to calculate the mortality rate of insects for each temperature regime. Inoculated plants were then inspected for the typical symptoms of WDV infection and scored based on their symptoms [0 = no symptom, 1 = mild chlorosis without stunting, 2 = chlorosis and stunting, 3 = severe chlorosis and stunting, 4 = dead] at 34 dpi. Fourteen plants per temperature regime were used. The experiment was repeated two times.

ELISA. WDV was detected in plants by indirect ELISA using the polyclonal antibody of WDV (DSMZ, Braunschweig, Germany) which has been shown to be able to detect both wheat and barley strains of Iranian isolates of WDV (LOTFIPOUR *et al.* 2013b). Indirect ELISA was performed using the method described by CONVERSE and MARTIN (1990). A plant

sample which had been previously confirmed to be WDV-infected was used as positive control. A tissue sample from healthy plants was also used as negative control. The plates were analysed using a Thermo Labsystems microplate reader (Thermo Scientific, Schwerte, Germany) and ELISA extinction values measured at a wavelength of 405 nm were recorded in order to quantify the virus. The samples with absorbance values double those of buffer controls were considered as positive. The plant samples showing positive results in ELISA were then subjected to PCR as described above.

Statistical analysis. Due to the lack of normal distribution of data, a non-parametric statistical approach was applied to analyse the data collected from the experiments about the effect of temperature on vector transmission and symptom development. To this end, the Kruskal-Wallis H test was applied using SPSS v22 software (IBM, Greenville, USA). In case of data obtained from indirect ELISA and mortality rate, however, parametric statistical indices were determined. The mean value of each treatment was subjected to the analysis of variance (ANOVA) using SPSS v22 software. Then Duncan's multiple range test was applied to find any significant difference at two levels of $P = 0.01$ and 0.05 .

RESULTS

WDV detection. The typical symptoms of WDV infection including yellowing and stunting were observed in a wheat field in Marvdasht, Fars province, Iran (Figure 1A). The leafhopper vector (*P. alienus*) was also collected in the affected field and primarily characterised by the ground creamy colour of their head, pronotum and scutellum, of 2.2–2.8 mm in length and transparent wings with cleared veins in the hind wing (Figure 1B). Further characterisation of the leafhopper individuals revealed a depression in the expanded apex of the aedeagus (data not shown), which is a distinguishing character of *P. alienus* (GREENE 1971). The leafhoppers were allowed to feed on wheat and barley seedlings which resulted in development of the typical symptoms in wheat and barley plants at 20 dpi (data not shown). These plants were subjected to PCR with strain-specific primer pairs (p2730^C/p1430^V and p2720^C/p1370^V) leading to partial amplification of the WDV-Wheat and WDV-Barley genome sequence, respectively (Figure 1C). The leafhoppers were also tested by the

same PCR assay and similar DNA fragments were obtained (Figure 1C).

Sequence analysis. A total of 4 complete genome sequences of WDV were generated consisting of one isolate from wheat (WDV-W [MRDTA24-1]), one isolate from barley (WDV-B [MRDTA22-1]) and two isolates from *P. alienus* (WDV-B [MRDHFV02-4] and (WDV-W [MRDHFV04-2]) and deposited in GenBank under the accession numbers presented in Table 2. Multiple alignments of complete genome sequences were performed and a maximum likelihood phylogenetic tree was constructed according to which WDV strains were generally divided into two super-clades of WDV-W and WDV-B. The WDV-B super-clade includes strains A and B, while strains D, C, E, and F are included in the WDV-W super-clade. Interestingly, two WDV sequences isolated from wheat-derived sample and leafhopper (KY793552 and KY793553,

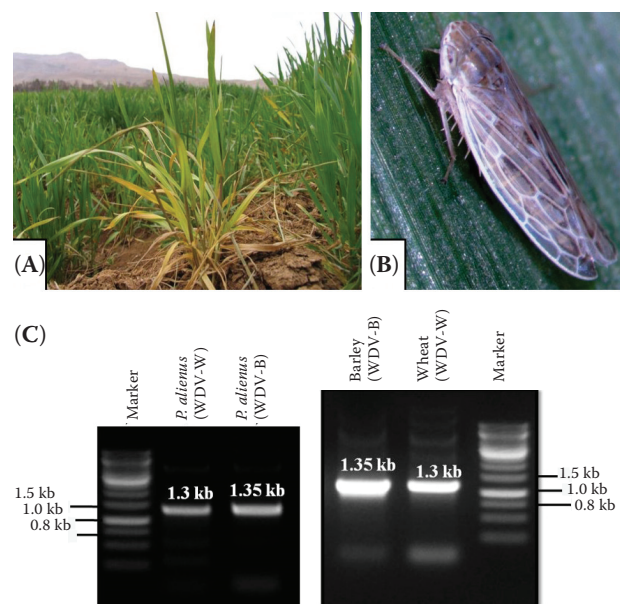


Figure 1. Detection of WDV infection and its leafhopper vector in a wheat field in Marvdasht, Fars province, Iran: (A) typical symptoms of WDV infection including yellowing and stunting observed on a wheat plant; (B) an adult individual of *Psammotettix alienus* as the main vector of WDV collected from the disease-affected fields; (C) gel electrophoresis pattern of PCR products from WDV-positive individuals of *P. alienus* (left) and plant samples (right) using strain-specific primer pairs of WDV-Wheat and WDV-Barley isolates (WDV-2730^C/WDV-1430^V and WDV-2720^C/WDV-1370^V, respectively)

Marker: 100 bp (left) and 1 Kb (right) GeneRuler™ DNA ladder (Thermo Scientific, USA); the details of primer characteristics are shown in Table 1

<https://doi.org/10.17221/147/2017-PPS>

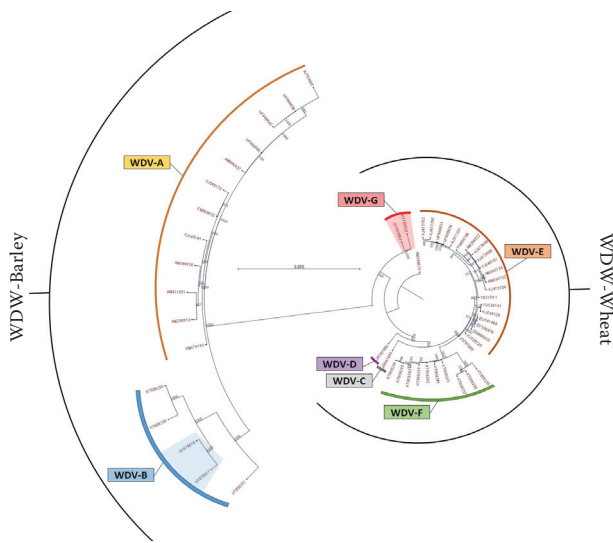


Figure 2. Phylogenetic tree obtained from multiple alignments of complete genomes from different isolates of WDV. One isolate of ODV was used as outgroup reference. The phylogenetic tree was constructed using maximum likelihood and bootstrap (1000 replicates) by CLC Main Workbench 7.6.2. The bar represents the estimated nucleotide substitutions per site. Different strains of WDV isolates, two super-clades of WDV strains (WDV-Wheat and WDV-Barley, respectively) and one isolate of ODV (outgroup) are indicated. WDV isolates presented in this study are highlighted

respectively) formed a separate cluster which is classified into a novel strain preliminarily named WDV-G (Figure 2). WDV isolates from barley plant and viruliferous leafhopper (KY679016 and KY679017, respectively) obtained in this study clustered with other WDV-B isolates which had been previously isolated from barley (Figure 2).

Effect of temperature on vector transmission.

The virus-carrying leafhoppers either as nymphs or adults could transmit both WDV-W and WDV-B isolates to wheat and barley seedlings, respectively, in all temperature regimes. However, there was a

significant difference between the infection rates of the viruses in these temperature regimes (Figure 3). It was shown that the highest infection rate using all vector combinations including adult, adult and nymph and nymph occurred at 25°C. Moreover, the efficiency of vector transmission by leafhoppers at nymph stage was significantly higher than that of adult leafhoppers (Figure 3). No statistically significant difference was found between the infection rates of WDV-W and WDV-B isolates in wheat and barley plants, respectively (Figure 3). The mortality rate of leafhoppers was determined in each temperature regime and it was found that there was no significant correlation between the mortality rate of the vector and transmission efficiency of the virus (Table 3), although the number of dead leafhoppers increased at a constant temperature of 35°C. When only the nymphs were used as the virus vector, the number of dead leafhoppers was smaller compared to other transmission experiments in which only adults or a combination of adults and nymphs were used. These results indicate that temperature significantly affects the vector transmission of WDV-W and WDV-B isolates to wheat and barley plants.

Effect of temperature on symptom development.

The virus-infected plants incubated at various temperatures developed the typical symptoms of the viral infection including yellowing and stunting (Figure 4). When the virus-positive plants were scored according to their symptoms and the data was analysed statistically, it was found that there is a significant difference between them. Based on the symptom indices determined for each plant, the virus-infected plants which had been incubated at 35°C showed the least development of symptoms compared to those incubated at 20, 25, and 30°C (Figure 5). Also, the developmental stage of the vector could not significantly affect the symptom development in any temperature regime. There was no significant difference between

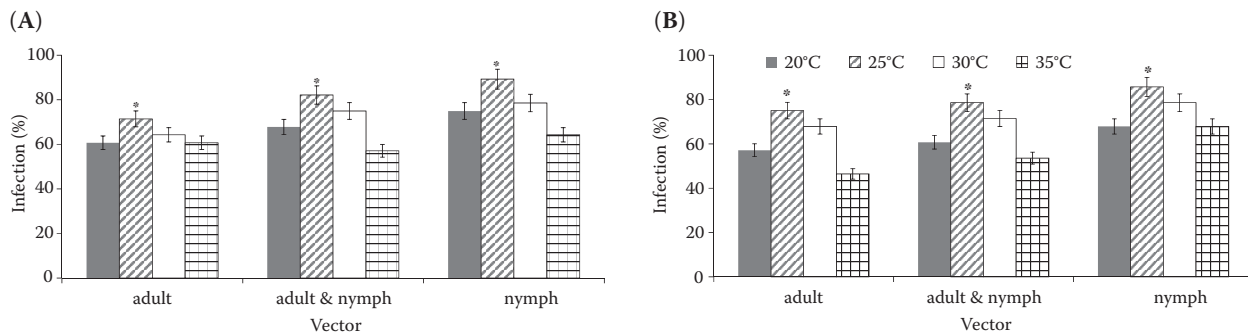


Figure 3. The effects of the vector (*Psammotettix alienus*) developmental stage and temperature regimes on the infection rate (%) of WDV-Wheat (A) and WDV-Barley (B) isolates in wheat and barley plants, respectively (* $P = 0.05$)

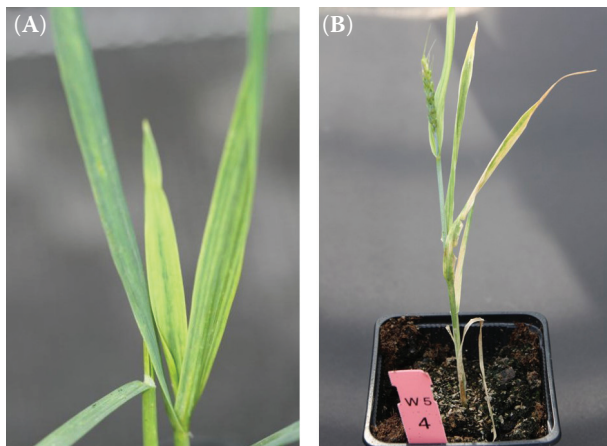


Figure 4. Symptoms of the vector-transmitted WDV-Wheat isolate in wheat plants including leaf yellowing (A) and dwarfing (B) at 34 dpi

the symptom indices for virus-infected plants incubated at 20, 25, and 30°C (Figure 5). Similarly, there was no significant difference in symptom severity between wheat and barley infected by WDV-W and WDV-B isolates, respectively. The results showed that temperature is able to affect symptom development induced by WDV-W and WDV-B isolates in wheat and barley hosts, respectively.

Effect of temperature on virus content. ELISA extinction values from WDV-infected wheat and barley plants which reflect the *in vivo* virus content were compared resulting in a significant difference between them (Figure 6). Thus infected plants under the temperature regime of constant 35°C exhibited the lowest ELISA values. However, the infected plants incubated at 20, 25, and 30°C did not show any significantly different ELISA extinction values. Moreover, there was no significant difference between the ELISA extinction values from plants infected by

WDV-W or WDV-B isolates under any temperature regime. Similarly, the developmental stage of the vector did not affect the ELISA extinction values at any temperature (Figure 6). The results showed that temperature is able to affect the virus content in WDV-infected plants.

DISCUSSION

Genetic diversity among WDV isolates reported from various regions of the world has been shown for many years using a phylogenetic analysis (KÖKLÜ *et al.* 2007; SCHUBERT *et al.* 2007; KUNDU *et al.* 2009; TÓBIÁS *et al.* 2011; GHODOUM PARIZIPOUR *et al.* 2017). According to these data, it is expected that novel strains of WDV exist in the Fertile Crescent where agriculture originated (GHODOUM PARIZIPOUR *et al.* 2017). Since Iran is located in the region of the Fertile Crescent (HARLAN 1971), it is likely that a significant variation occurs among WDV isolates originated from this region. Accordingly, GHODOUM PARIZIPOUR *et al.* (2017) found a novel phylogenetically distinct cluster of WDV isolates from Iran which was introduced as WDV-F. Similarly, here we reported two sequences of WDV (KY793552 and KY793553) which interestingly formed a new cluster in the phylogenetic analysis that was named WDV-G (Figure 2). This relatively high level of variability among WDV isolates in this region might be the result of a long-term co-evolution of WDV and its hosts. Additionally, the phylogenetic analysis showed that WDV isolates generally formed two super-clades, WDV-W and WDV-B, on the tree which was consistent with the results reported by GHODOUM PARIZIPOUR *et al.* (2017). A further phylogenetic analysis with more isolates is required to

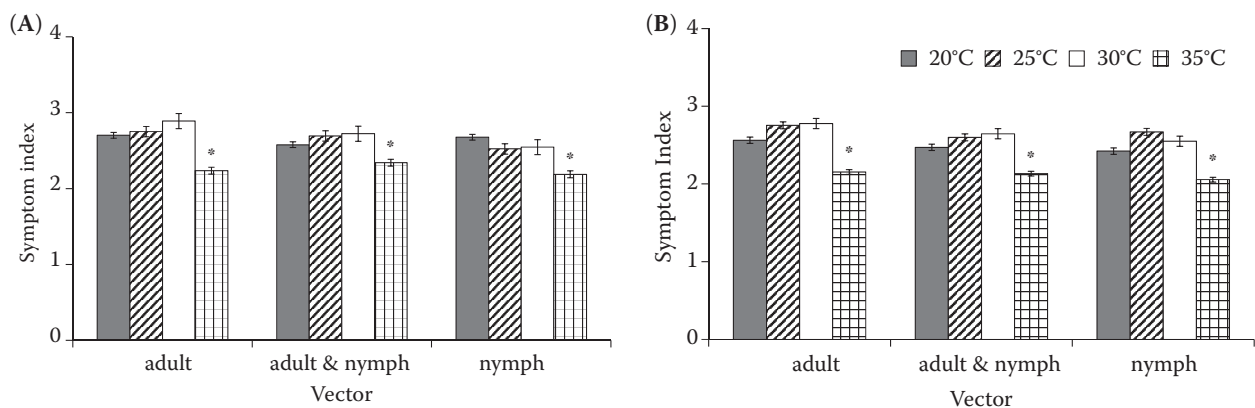


Figure 5. The effects of vector (*Psammotettix alienus*) developmental stage and temperature regimes on symptom development caused by the viral infection of WDV-Wheat (A) and WDV-Barley (B) isolates in wheat and barley plants, respectively (* $P = 0.05$)

https://doi.org/10.17221/147/2017-PPS

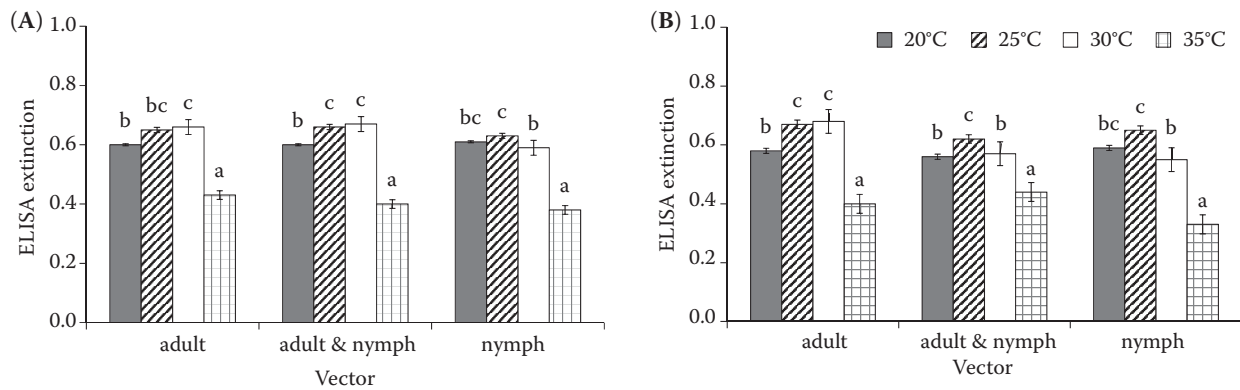


Figure 6. The mean of ELISA extinction values from wheat and barley plants infected by WDV-Wheat (A) and WDV-Barley (B) isolates, respectively, through vector transmission under different temperature regimes (letters on bars show the significant difference between the treatments according to the results of Duncan’s multiple range test)

better understand the genomic variation between WDV populations from different regions of the world.

Environmental factors are able to affect virus infections among which temperature is an important factor due to its key role in the ecology of vector-transmitted plant viruses. Our results showed that the constant temperature of 25°C is associated with the highest infection rate caused by WDV-W and WDV-B isolates in wheat and barley, respectively, through the vector transmission (Figure 3) which was similar to the results of a study according to which the constant temperature of 25°C is optimum for the vector transmission of *Maize streak virus* (MSV) to maize plants (DAMSTEGT 1984). It seems that this high rate of infection through the vector transmission is a result of the temperature effect on the vector insect rather than on the host plant as the biology of leafhoppers is temperature-dependent (SCHIEMENZ 1969) and the insect translocation was believed to be a result of the change in environmental factors (e.g. temperature) leading to ascending or descending flights of the insect vectors (MARAMOROSCH *et al.* 2006). Similarly, we observed that at high temperatures (> 30°C) leafhoppers tend to descend on the ground, which reduces the insect feeding on them leading to a decreased rate of WDV transmission to wheat and barley plants. Moreover, our data showed that the high temperature (35°C) was associated with an increased mortality rate of leafhoppers (Table 3) compared to other temperature regimes, although we could not find any statistically significant correlation between WDV infection and mortality rate of *P. alienus* individuals. Additionally, in our study the average infection rate of WDV using the virus-carrying leafhopper was approximately 70% while it has been previously shown that viruliferous leafhoppers originated from Iran are able to cause the average infection

rate of approximately 100% (GHODOUM PARIZIPOUR *et al.* 2016). This relatively low infection rate might be due to application of different conditions for the vector transmission of WDV as greenhouse conditions with alternating temperatures of 20–25°C were used in the previous study by GHODOUM PARIZIPOUR *et al.* (2016). Similarly, it has been shown that various parameters such as environmental factors (temperature and humidity), host susceptibility, and the level of virus virulence may affect the rate of transmission (REYNAUD & PETERSCHMITT 1992).

Also, it was found that WDV-carrying leafhoppers at nymph stage can cause the highest infection rate in wheat and barley plants (Figure 3). This might be due to the fact that the nymph stage of *P. alienus* is much longer than the imago (adult) stage (MANURUNG *et al.* 2005). Furthermore, the nymphs of *P. alienus* as the vector of WDV, although wingless, are more active than adult individuals jumping from

Table 3. The mortality rate of *P. alienus* individuals in different developmental stages determined after transmission of WDV-Wheat and WDV-Barley isolates to wheat and barley hosts, respectively, under various temperature regimes

Temperature (°C)	Virus	Vector mortality rate (%)		
		adult	adult & nymph	nymph
20	WDV-Wheat	16.07	12.50	10.71
	WDV-Barley	16.07	12.50	8.93
25	WDV-Wheat	14.29	10.71	8.93
	WDV-Barley	12.50	8.93	5.36
30	WDV-Wheat	16.07	12.50	10.71
	WDV-Barley	14.29	10.71	7.14
35	WDV-Wheat	19.64	16.07	16.07
	WDV-Barley	23.21	17.86	16.07

one plant to another and feeding on them, which increases the likelihood of virus transmission within the fields (LINDBLAD & ARENÖ 2002). Also, the key role of nymphs in the early transmission of WDV to the cultivated cereals has been indicated (ABT & JACQUOT 2015). The second-instar nymphs of *P. alienus* can transmit WDV after a very short acquisition access period (WANG *et al.* 2014). However, due to the role of adult leafhoppers in early transmission of WDV among the fields (MANURUNG *et al.* 2005) and the slight difference observed between infection rates caused by viruliferous nymphs and adults of *P. alienus* (Figure 3), it can be concluded that both nymph and adult stages of the leafhopper vector are important components in the epidemiology of WDV. A field study is required to investigate the population dynamics of the leafhopper vector in the local WDV-affected sites.

Our data showed that the symptom development of WDV-W and WDV-B isolates on wheat and barley plants, respectively, is also affected by temperature (Figure 5). High temperature (35°C) was associated with symptom remission on the virus-infected plants as reported in other virus-plant pathosystems (HILLMAN *et al.* 1985; DAHAL *et al.* 1998; LLAMAS-LLAMAS *et al.* 1998; CHELLAPAN *et al.* 2005; VELÁZQUEZ *et al.* 2010; GHODOUM PARIZIPOUR *et al.* 2014; GHOSHAL & SANFAÇON 2014; CHUNG *et al.* 2015). Moreover, WDV-infected plants incubated at 35°C exhibited the lower virus content than those kept under temperature regimes of constant 20, 25, and 30°C (Figure 6). These results were similar to those obtained by CHELLAPAN *et al.* (2005) and GHODOUM PARIZIPOUR *et al.* (2014) based on which geminivirus-infected plants incubated at a high temperature (30°C) had the reduced virus titre compared to those incubated at cool temperatures (< 30°C). Similarly, LLAMAS-LLAMAS *et al.* (1998) showed that the virion accumulation of *Tomato spotted wilt virus* is higher in virus-inoculated *Datura stramonium* L., *Nicotiana tabacum* L., and *Physalis ixocarpa* Brot. plants incubated at a low temperature (~24°C) compared to those kept at a high temperature (~30°C). These results suggest that higher temperature has a negative effect on the virus content of virus-infected plants.

The RNA silencing machinery as an antiviral defence system was shown to be one of the reasons for reduced virus content in virus-infected plants with symptom remission (CARRILLO-TRIPP *et al.* 2007), although the RNA silencing is not always associated with reduced virus concentration (JOVEL *et al.* 2007). Further experiments are required to find the mechanism(s) responsible

for the low virus content in WDV-infected plants which showed symptom remission at 35°C.

It can be concluded that temperature, as an important environmental factor, is able to affect vector transmission, symptom development and viral content of WDV-W and WDV-B isolates in wheat and barley plants, respectively. The results of this study might be used for the more effective control of wheat dwarf disease, thus choosing a planting date that makes young seedlings face high temperatures (> 30°C) is likely to reduce both incidence and damage of the viral infection as these temperatures were found to be associated with low vector transmission, reduced virus content and symptom remission.

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Received: 2017–11–26

Accepted after corrections: 2017–02–03

Published online: 2018–03–01