NATURAL HOSTS AND EFFICIENCY OF LEAFHOPPER VECTOR IN TRANSMISSION OF *WHEAT DWARF VIRUS*

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SUMMARY

Wheat dwarf virus (WDV) occurs in economic proportions in cereal crops in Iran. Conventionally, it has two strains each adapted to wheat (WDV-W) or barley (WDV-B). A number of gramineous weeds were collected from the main wheat and barley cultivations in Fars province, Iran, in October 2013 and May 2014 and tested for WDV infection by PCR. WDV-W was detected in *Aegilops kotschyi*, *Avena fatua*, *Bromus commutatus*, *Hordeum murinum*, *Lolium persicum*, *Sorghum halepense* and *Cynodon dactylon*. *H. spontaneum* was the only host infected by WDV-B. WDV infection was detected more frequently in fall than in spring. The leafhopper vector, *Psammotettix alienus*, transmitted WDV to wheat and barley seedlings more efficiently after 7-day acquisition than after 1-day acquisition access period. Furthermore, using a different number of leafhoppers per plant led to significantly different ($P<0.01$) infection rates by the two strains in barley and wheat plants. Comparison of WDV-W and WDV-B infection rates in the two hosts indicated that WDV-W was able to infect both hosts with higher level than WDV-B.

Keywords: acquisition access period, barley, infection rate, *Psammotettix alienus*, weed hosts.

INTRODUCTION

Wheat dwarf virus (WDV) is a species of the genus *Mastrevirus* in the family *Geminiviridae* (Brown *et al*., 2012). Mastreviruses mostly infect monocotyledonous plants of the family Poaceae, with the exception of two species which infect dicotyledonous plants (Brown *et al*., 2012). The main natural host crops of WDV include wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and rye (*Secale cereale*) (Schubert *et al*., 2007). Infected wheat and barley plants show yellowing and stunting symptoms. Many wild grasses as well as wild relatives of wheat are reported to be infected by WDV (Mehner, 2005; Nygren *et al*., 2015; Ramsell *et al*., 2008; Ripl and Kundu, 2015).

WDV infection has been reported from several countries of Europe (Bendahmane *et al*., 1995; Kundu *et al*., 2009; Ramsell *et al*., 2008; Schubert *et al*., 2007), Asia (Behjatnia *et al*., 2011; Ekzayez and Kumari, 2011; Xie *et al*., 2007) and Africa (Kapooria and Ndunguru, 2004; Najar *et al*., 2000) where wheat has been cultivated. Based on a conventional classification, there are two strains of WDV: wheat strain (WDV-W) and barley strain (WDV-B) (Schubert *et al*., 2007; Lotfipour *et al*., 2013b). The genome of the two strains share 78-86% nucleotide (nt) identity (Köklü *et al*., 2007; Schubert *et al*., 2007; Lotfipour *et al*., 2013a).

WDV-W is able to infect both wheat and barley naturally while infection of wheat or other species of *Triticum* with WDV-B is not common in nature (Ramsell *et al*., 2009; Behjatnia *et al*., 2011; Lotfipour *et al*., 2013b). The sequence of the genome of European barley and wheat strains of WDV which were recently analyzed (Schubert *et al*., 2013) revealed that their sequences are very stable. WDV-B sequences isolated from two different plants (they were barley and wheat) exhibited remarkable similarity. However, recombination between strains was recorded only for the barley strain (Schubert *et al*., 2013). Recently Wu *et al*. (2015) carried out phylogenetic analyses on the global population of WDV according to which the worldwide isolates of WDV are clustered into two groups based on their specific host, i. e., wheat and barley, and subsequently six strains. A and F strains were mainly from barley and B-E were mainly from wheat.

WDV is transmitted by leafhoppers of the genus *Psammotettix* (family *Cicadellidae*) in a circulative, non-propagative manner (Lindsten and Vacke, 1991). Up to now, three species of this genus including *P. alienus* (Dahlbom), *P. striatus* (Linneaus) and *P. provincialis* have been reported as WDV vectors. Among them *P. alienus* is considered as a dominant species in grasslands and cereal fields all around the world (Greene, 1971). Both *P. alienus* and *P. striatus* were reported to transmit WDV in Finland while in Russia and China *P. striatus* is more frequent (Wang and Guanghe, 2007; Wang *et al*., 2008; Lemmetty and Huusela-Veistola, 2005). The species *P. provincialis* was reported to transmit WDV in Syria (Ekzayez and Kumari, 2011). In Iran *P. alienus* was identified as WDV vector (Lotfipour *et al*., 2013b). It was demonstrated that the coat protein

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Table 1. Wheat dwarf virus oligonucleotide primers used in this study.

Primer	Sense[a]	Position[b]	Sequence [5'-3'][c]	Target	Expected product size (bp)
WDV-2730	C	degenerate	CCRCACACCDAACASGGCCCA	WDV-W	1300
WDV-1430	V	degenerate	GAAYGAGTAGTTGATGAAYGWCTC	WDV-W	
WDV-2720	C	degenerate	CGCGGGACCACCCGTCGCT	WDV-B	1350
WDV-1370	V	degenerate	GCGAARAAYGATTCMCCYTCATA	WDV-B	
WDV-655	V	655-679	GATAATAATCGCCATACAAATCAGA	Both strains	1115
WDV-1770	C	1753-1770	CTACATCTCCGGACCAAC	Both strains	
WDV-2526	V	2526-2547	CTTAGCGAAAAAACGGGGTGTG	Both strains	1384
WDV-1162	C	1139-1162	TGCGTATAGGCACATACAACTTC	Both strains	

[a] V, virion-sense strand; C, complementary-sense stran.
[b] Nucleotide position of WDV as in the Genbank database under accession number KX212082.
[c] R, 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).

of WDV has a key role in transmission of the virus by *P. alienus* (Wang *et al*., 2014). Based on the results achieved from studying the localization and distribution of WDV in its vector leafhopper, it was suggested that WDV not only is able to enter the salivary glands from the fore- and midgut but also can spread rapidly through the filter chamber and subsequently reach the salivary glands via the hemocoel. It was concluded that rapid transmission of WDV by its vector might be due to the direct transmission of virions through the filter chamber to the salivary glands before entering the epithelial cells of the gut (Wang *et al*., 2014).

It has been shown that the leafhopper vector of *Maize streak virus* (MSV), *Cicadulina mbila*, has a low efficiency in transmitting the virus in the first day after the virus acquisition from MSV-infected plants 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). Environmental conditions such as temperature may also influence on vector transmission efficiency of MSV. The transmission success also depends on viral virulence and host susceptibility (Reynaud and Peterschmitt, 1992). It has been shown that individuals of *P. alienus* transmitted WDV in all stages of their development. However, transmission mode of leafhopper individuals was reported to have very broad fluctuations depending on different development stages (Mehner *et al*., 2003). There is little information on the transmission efficiency of WDV strains by the leafhopper vectors. The aim of this study was to identify the potential natural hosts of WDV in wheat and barley cultivations areas in Iran and to investigate the transmission efficiency of both WDV-W and WDV-B by *P. alienus* in wheat and barley plants, the most economically important hosts of WDV in Iran.

MATERIALS AND METHODS

Sampling. In order to detect potential hosts of WDV-W and WDV-B, sampling was carried out in October 2013 and May 2014, in wheat and barley fields in Fars province, Iran. The following weeds were sampled regardless of their symptoms: *Aegilops kotschyi* Boiss, *Agropyron repens* (L.) P. Beauv., *Avena fatua* L., *Bromus commutatus* Schrad, *Eremopoa persica* (Trin.) Roshev., *Hordeum murinum* Bioss, *Lolium persicum* B. and H. ex Bioss, *Sorghum halepense* L. (Pers.), *Cynodon dactylon* L. (Pers.), *Phalaris minor* Retz. and *Hordeum spontaneum* K. Koch.

DNA extraction. In order to extract DNA from plant tissues, cethyl-trimethyl-ammonium bromide (CTAB) solution was used as described by Gawel and Jarret (1991). Briefly, the plant tissues (200 mg) were homogenized in 3.5 volumes of extraction buffer (1.4 M NaCl, 20 mM EDTA, 2.5% CTAB, 2% 2-mercaptoethanol, 10 mM Tris-HCl, pH 8) and incubated at 65°C for 30 min. The DNA was extracted with an equal volume of chloroform/isoamylalcohol (24 : 1), precipitated with one volume of isopropanol, washed with 70% ethanol and re-suspended in distilled water at a rate of 250 ml for each g of tissue extracted. The resulting DNAs were subjected to polymerase chain reaction (PCR) using strain specific primers (Table 1) to detect the WDV-W and WDV-B (see below).

WDV detection and sequence analysis. Two degenerate primer pairs were used to detect WDV in the tested plants (Table 1). The first primer pair (WDV-2730/WDV-1430) amplifies a 1300 bp fragment of Rep-coding region of WDV-W. The second primer pair (WDV-2720/WDV-1370) amplifies a 1350 bp of the same region in WDV-B. PCR was carried out in 25 ml reaction mixture containing 10-20 ng of DNA template, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 mM of each primer and 2 U of *Taq* DNA polymerase (Cinagen, Iran) in the reaction buffer provided by the same source. The mixture was heated for 2 min at 96°C and subjected to a 32 cycle-PCR program of 30 s at 96°C, 30 s at 62°C and 2 min at 72°C. The final cycle was followed by 10 min incubation at 72°C. A part of the reaction mixture was then loaded directly onto a 1.2% agarose gel, stained with ethidium bromide and visualized by UV light.

The rest of the PCR-generated products were purified (Macherey-Nagel, Germany) and sequenced (GATC Biotech, Germany). The resulting DNA sequences were

Table 2. Mastreviruses used for sequence comparisons with Iranian isolates of WDV.

Virus	Abbreviation	GenBank Accession number	Country	Source	Year	Reference
Barley dwarf virus-[Cz19]	Cz19	AM296019	Czech Republic	*Hordeum vulgare*	2002	Schubert *et al.* (2007)
Maize streak virus-[South Africa]	MSV-ZA	NC_001346	South Africa	*Zea mays*	1988	Lazarowitz (1988)
Oat dwarf virus-[SxA25]	SxA25	AM296025	Germany	*Avena sativa*	2005	Schubert *et al.* (2007)
Wheat dwarf virus -[HU-KP10-1]	HU-KP10-1	JQ647455	Hungary	*Triticum aestivum*	2010	Liu *et al.* (2012)
Wheat dwarf virus-Barley-[TR]	WDV-Bar-[TR]	AJ783960	Turkey	*H. vulgare*	2004	Köklü *et al.* (2007)
Wheat dwarf virus-[HU]	WDV-[HU-F]	AM040733	Hungary	*Psammotettix alienus*	2005	Tóbiás *et al.* (2006)
Wheat dwarf virus-[SxTY05-3]	SxTY05-3	EF536874	China	*T. aestivum*	2008	Wu *et al.* (2008)
Wheat dwarf virus-[Taiyuan]	WDV-[TA]	DQ868525	China	*T. aestivum*	2006	Xie *et al.* (2007)
Wheat dwarf virus-[IRAN]	WDV-IR[Bar]	FJ620684	Iran	*H. vulgare*	2009	Behjatnia *et al.* (2011)
Wheat dwarf virus -[Bavanat]	Whe [Iran:Ba:Bar]	JN791096	Iran-Bavanat	*H. vulgare*	2010	Lotfipour *et al.* (2013a)
Wheat dwarf virus -[Shahrekord]	Whe[Iran:Sh:Whe]	JN791095	Iran-Shahrekord	*T. aestivum*	2010	Lotfipour *et al.* (2013a)
Wheat dwarf virus –[SYZSH03]	SYZSH03	KT958235	Iran	*Sorgum halepense*	2015	This study
Wheat dwarf virus –[SYZHS06]	SYZHS06	KT958236	Iran	*H. spontaneum*	2015	This study
Wheat dwarf virus –[SYZAF12]	SYZAF12	KT958237	Iran	*A. fatua*	2015	This study
Wheat dwarf virus –[SYZBC14]	SYZBC14	KT958238	Iran	*Bromus commutatus*	2015	This study
Wheat dwarf virus –[SYZEP26]	SYZEP26	KT958240	Iran	*Eremopoa persica*	2015	This study
Wheat dwarf virus –[SYZHM32]	SYZHM32	KT958241	Iran	*H. murinum*	2015	This study
Wheat dwarf virus –[SYZLP33]	SYZLP33	KT958242	Iran	*Lolium persicum*	2015	This study
Wheat dwarf virus –[SYZCD35]	SYZCD35	KT958243	Iran	*Cynodon dactylon*	2015	This study
Wheat dwarf virus –[SYZAK54]	SYZAK54	KT958244	Iran	*Aegilops kotschyi*	2015	This study
Wheat dwarf virus-[Enkoping1]	WDV-[SE]	AJ311031	Sweden	*T. aestivum*	2002	Kvarnheden *et al.* (2002)
Wheat dwarf virus-[BB21]	BB21	AM296021	Germany	*Secale cereale*	2004	Schubert *et al.* (2007)
Wheat dwarf virus-[France]	WDV-[FR]	X82104	France	*T. aestivum*	1994	Bendahmane *et al.* (1995)
Wheat dwarf virus-[HE]	WDV-[HE]	FM999833	Hungary	*H. vulgare*	2007	Tóbiás *et al.* (2011)
Wheat dwarf virus-[SxA22]	SxA22	AM296022	Germany	*Lolium perenne*	2002	Schubert *et al.* (2007)
Wheat dwarf virus-[SxA23]	SxA23	AM296023	Germany	*T. aestivum*	2004	Schubert *et al.* (2007)
Wheat dwarf virus-[HU-Pula]	WDV-[HU-P]	FN806783	Ukraine	*T. aestivum*	2008	Tóbiás *et al.* (2011)
Wheat dwarf virus-[Bg17]	Bg17	AM989927	Bulgaria	*H. vulgare*	2006	Tóbiás *et al.* (2009)

Table 3. Wild grass species analyzed for WDV-W and WDV-B infection in two sampling dates (October 2013 and May 2014).

Host plant		Number infected/number tested (incidence rate)		Detected virus	
Common name	Scientific name	October 2013	May 2014	WDV-W	WDV-B
Goatgrass	*Aegilops kotschki* Bioss	8/12 (67%)	2/9 (23%)	+	-
Dog tooth	*Cynodon dactylon* (L.) Pers.	4/11 (36%)	2/12 (17%)	+	-
Hairy brome	*Bromus commutatus* Schrad	12/14 (86%)	3/10 (30%)	+	-
-	*Eremopoa persicsa* (Trin.) Roshev.	9/14 (64%)	3/12 (25%)	+	-
Johnson grass	*Sorgum halepense* L. (Pers.)	13/15 (87%)	6/12 (50%)	+	-
Mouse barley	*Hordeum murinum* Bioss	16/18 (89%)	5/13 (38%)	+	-
Ryegrass	*Lolium persicum* B. and H. ex Bioss	3/8 (37%)	1/9 (11%)	+	-
Spring wild oat	*Avena fatua* L.	11/13 (85%)	4/9 (44%)	+	-
Wild barl	*Hordeum spontaneum* Koch	15/18 (84%)	12/16 (75%)	-	+
Wheatgrass	*Agropyron repens* (L.) P. Beauv.	0/10 (0%)	0/7 (0%)	-	-
Small canary grass	*Phalaris minor* Retz.	0/16 (0%)	0/8 (0%)	-	-
Total		91/149 (61.07%)	38/117 (32.47%)		

compared to the corresponding parts of the genomes of WDV isolates and other mastreviruses for which complete genome information was available in the GenBank database (Table 2). Sequence alignment was performed using CLC Main Workbench software (ver. 7.6.2) and the phylogenetic tree was drawn.

Natural host ranges. Gramineous weed samples from 11 plant species were individually tested by PCR for infection by WDV strains. Sample size for each plant species is shown in Table 3. PCR positive plants were regarded as the natural hosts of the virus.

Vector transmission. Highly susceptible winter wheat (*Triticum aestivum* cv. Marvdasht) and winter barley (*Hordeum vulgare* cv. Kavir) were used to investigate the transmission efficiency of WDV strains by *P. alienus.* In addition, the effect of the leafhopper numbers per plant and the length of acquisition access period on transmission efficiency were investigated. Wheat and barley seeds

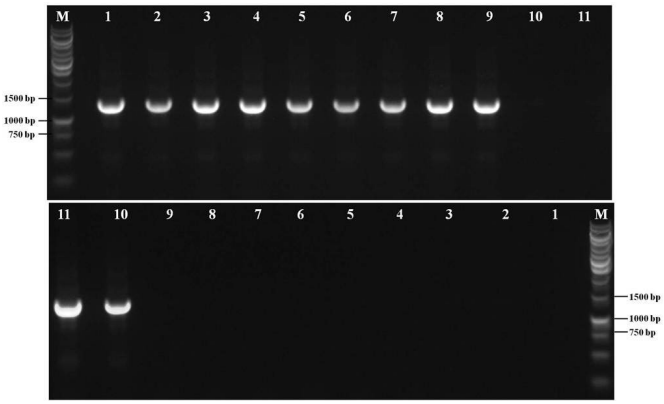

Fig. 1. WDV-W (top) and WDV-B (bottom) detection by PCR using WDV-2730/WDV-1430 (top), and WDV-2720/WDV-1370 (bottom) primer pairs (Table 1), respectively. Lane 1, WDV-W positive control (infected wheat plant, top) and negative control (healthy wheat plant, bottom); lane 2, *Aegilops kotschki*; lane 3, *Avena fatua*; lane 4, *Bromus commutatus*; lane 5, *Eremopoa persicsa*; lane 6, *Hordeum murinum*; lane 7, *Lolium persicum*; lane 8, *Sorgum halepense*; lane 9, *Cynodon dactylon*; lane 10, *Hordeum spontaneum*; lane 11, negative control (top) and positive control (bottom); M, 1 Kb GeneRuler™ DNA ladder (Thermo Scientific).

(3-5) were sown in pots containing peat and sand (1 : 1) and grown at 20-25°C in a greenhouse. Seedlings at two- to three-leaf stage were used for the tests.

Leafhoppers were collected in the fall of 2012 in a maize field in Fars province, Iran, using a D.Vac machine and were immediately transferred into the lab. A number of leafhoppers were kept in 70% ethanol for morphological identification. Discoloration process of the insects was carried out by keeping them in 10% KOH solution for 24 h. Genitalia of the male insect placed in a glycerol drop on a microscope slide, dissected under a stereomicroscope and photographed. Published morphological descriptions were used to identify the leafhopper species (Greene, 1971; Nielson, 1968). Some of the leafhoppers were caged on healthy wheat and barley plants to assess possible presence of the virus.

To prepare virus-free insect colonies, a male and a female adult or a pregnant female of the leafhoppers were selected and caged on healthy barley plants and the resulting nymphs were transferred to new healthy plants for three generations. Freedom from WDV was verified by the absence of symptoms on source plants and negative PCR results with specific WDV-W and WDV-B primer pairs (Table 1).

Virus sources. To obtain WDV-W and WDV-B sources for transmission experiments, leafhoppers collected from the cereal fields in Fars province, Iran, were caged on healthy barley plants. PCR assay was performed using WDV-2730/WDV-1430 and WDV-2720/WDV-1370 primer pairs (Table 1) to detect WDV-W- and WDV-B-infected plants, respectively. The PCR-positive plants were then subjected to amplification of the virus full-length genome using Platinum *Taq* DNA polymerase high fidelity (Invitrogen™, USA) and four primer pairs as listed in Table 1. The resulting fragments were purified using gel extraction kit (Qiagen, Germany) and sequenced at GATC Biotech (Konstanz, Germany). Base calling was performed with software Chromas (Technelysium) and the sequences were then assembled using SeqMan Pro software (DNASTAR™ Lasergene, ver. 8) in order to achieve the full-length genome sequence. Sequence data were deposited in the GenBank under the accession Nos. KX212082 and KX212083 for Bajgah (Shiraz, Iran) isolates of WDV-W and WDV-B, respectively. The plants infected by each strain were separately used as the WDV-W and WDV-B sources for further experiments.

For virus acquisition, second and third instar nymphs were caged on infected plants. Nymphs are allowed to feed on young symptomatic leaves for one- or seven-day acquisition access periods. They were then caged on healthy wheat and barley plants for six days to transmit the virus. The number of insects per plant was 1, 2, 4 or 8, each with 10 replicates. At the end, the plants were sprayed with 1% Imidacloprid insecticide (Biesterfeld, Greece). The plants were kept in greenhouse for 3-4 weeks and checked for the development of symptoms. Leaf tissues (200 mg from each plant) were collected from plants inoculated with viruliferous leafhoppers at 7, 14, 21 and 28 days post-incubation (dpi) and subjected to PCR with strain-specific primers.

Statistical analysis. Transmission rate, which was calculated as a percentage, was compared among different treatment groups. The percentages were first transformed to arcsine square root values for analysis of variance (ANOVA) using SAS computer software (ver. 9.00). Duncan's multiple range test was used to determine any significant difference at $P=0.01$ and $P=0.05$ levels among the treatments.

RESULTS

Natural host plants of WDV. A total of 11 species of gramineous wild grass collected from wheat and barley fields were tested by PCR using WDV-W and WDV-B specific primer pairs (Table 1). Among them 8 species including *Avena fatua* (AF), *Aegilops kotschyi* (AS), *Bromus commutatus* (BC), *Cynodon dactylon* (CD), *Eremopoa persica* (EP), *Hordeum murinum* (HM), *Lolium persicum* (LP) and *Sorghum halepense* (SH) showed WDV-W infection (Fig. 1; Table 3). When the same samples were subsequently subjected to PCR assay using WDV-2720/WDV-1370 primer pair to detect WDV-B, only *H. spontaneum* showed infection (Fig. 1).

Sequence analysis. Comparison of nucleotide (nt) sequence of a 1300 bp fragment of Rep-coding region of

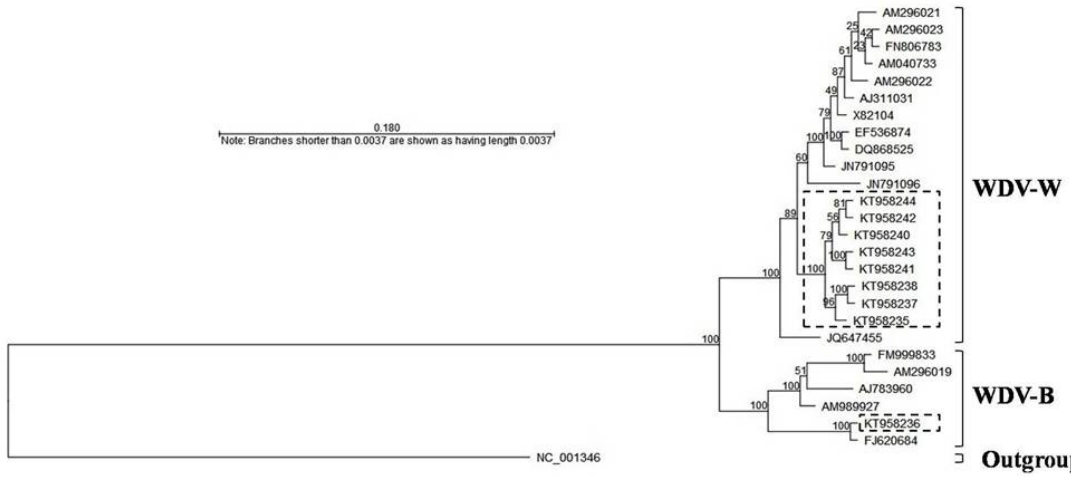

Fig. 2. Phylogenetic tree obtained from the alignment of a genome region encompassing Rep gene sequences from diverse mastreviruses forming the nearest relatives of WDV with reference to their nt sequence similarity as obtained using BLASTn search. One strain of MSV was used as outgroup reference. The phylogenetic tree was constructed using neighbor joining and bootstrap (100 replicates) of CLC Main Workbench 7.6.2. Vertical branches are arbitrary, horizontal branches show approximate mutation distances. The bar represents the expected substitutions per site. The clustering of wheat and barley strains of WDV (WDV-W and WDV-B, respectively) and MSV (outgroup) is indicated. Iranian wheat and barley strains of WDV which have been isolated from wild grasses are boxed (----). See Table 2 for source of isolates.

WDV-W samples isolated from wild grasses in this study with the nt sequence of the same region of other WDV isolates available in the GenBank (Table 2) revealed the highest homology (96-97%) with an Iranian isolate of WDV-W (WDV-Whe[Iran:Sh:Whe], accession No. JN791095). The only barley strain from *H. spontaneum* exhibited more than 92% identity to an Iranian isolate of WDV-B (WDV-IR[Bar], FJ620684). Also, Iranian WDV-W isolates from different weed species characterized in this study shared 97-99% homology while WDV-B sequence from *H. spontaneum* exhibited only 87-88% homology to WDV-W sequences from other wild grasses. Multiple alignments of a region including replication associated protein (Rep) gene sequence were performed and resulted in clustering of 8 weed-derived samples (AF, AK, BC, CD, EP, HM, LP and SH) with other WDV-W isolates available in the GenBank (Fig. 2). WDV-B isolates available in the GenBank also clustered together with the only WDV-B isolated from another weed, i.e. *H. spontaneum*.

The rate of WDV infection in the two sampling dates was individually calculated for each plant species (Table 3). Among the nine virus positive species, *H. murinum*, *S. halepense*, *B. commutatus* and *A. fatua* showed over 80% incidence in October survey while in May survey *H. spontaneum* had the highest incidence rate of over 70%. In contrast, *C. dactylon* and *L. persicum* had the lowest incidence in both sampling dates. The virus incidence in all species was found to be higher in October than in May.

Vector identification. The adults of *Psammotetix* genus are morphologically characterized by ground color creamy of their head, pronotum and scutellum (Greene, 1971). The wings are transparent and the veins of hind wing are cleared (Fig. 3A). The leafhopper samples collected from a maize farm in Shiraz and those which were able to transmit WDV to barley seedlings were subjected to identification process based on the male aedeagus (Greene, 1971). All samples showed a state of depression in the expanded

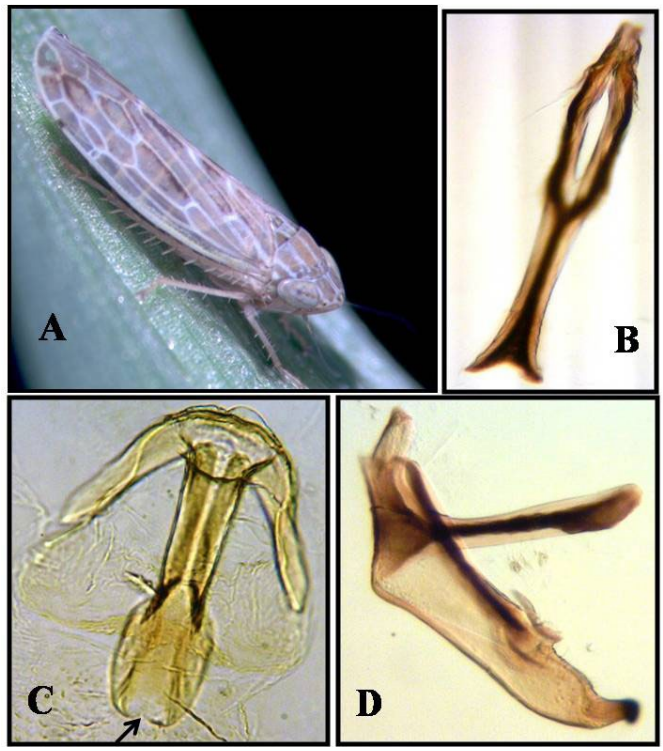

Fig. 3. Adult morphology (A) and microscopic photographs (10X) of the male genitalia of *Psammotettix alienus* collected from a maize field in Fars province, Iran; (B) connective; (C) dorsal view of aedeagus; (D) style. Black arrow in (C) shows depression in the aedeagus.

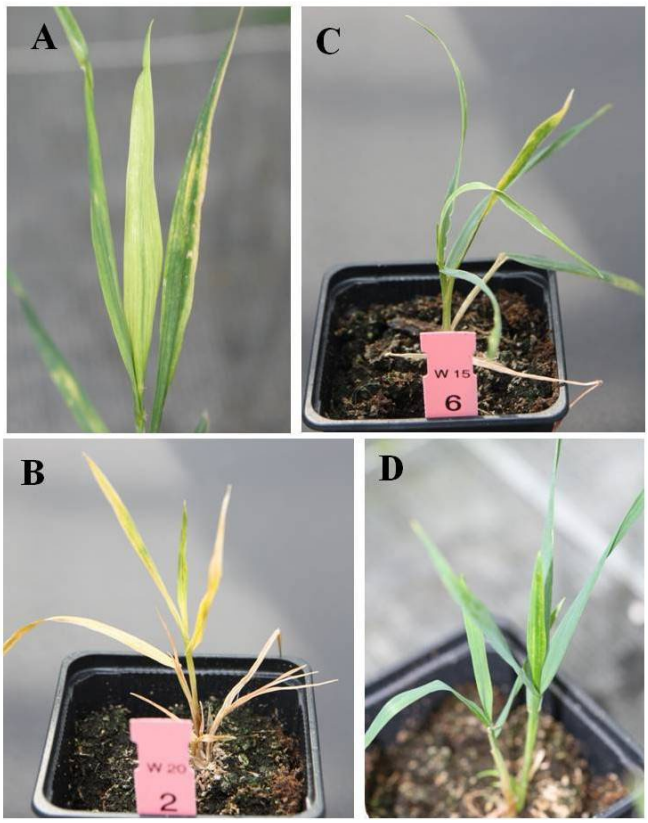

Fig. 4. Symptoms of vector-transmitted WDV-W in wheat plants including leaf yellowing (A-D), leaf necrosis (B), leaf curling (C) and dwarfing (B-D).

apex of the aedeagus (Fig. 3C), which is a distinguishing character of *P. alienus*. Hence, the collected leafhoppers were identified as *P. alienus*. Other features of male genitalia including style, the small paired appendages on the male subgenital plate (Fig. 3D), and connective, longitudinal cord of nerve fibers connecting successive genitalia (Fig. 3B) confirmed the insect identification.

Transmission efficiency of WDV-W and WDV-B by *P. alienus*. The ability of *P. alienus* in transmission of WDV-W and WDV-B to both wheat and barley seedlings was tested by placing 1, 2, 4 or 8 insects on each seedling. The insects previously fed on either a WDV-W-wheat infected plant or a WDV-B-barley infected plant as the WDV-W and WDV-B sources, respectively. Typical symptoms including yellowing, necrosis and reduced growth were clearly observed 30 dpi (Fig. 4). To detect the virus and to distinguish the virus strain, two sets of primers were used (Table 1). Fig. 5 shows the percentage of WDV-W- and WDV-B-infected plants with different treatments included in the experiment. In both wheat and barley, transmission of both WDV-W and WDV-B was achieved even with one adult of *P. alienus* per plant. The leafhoppers with 7-day acquisition access period could transmit WDV to wheat and barley plants more efficiently than those with 1-day acquisition access period (Fig. 5A). Furthermore, using different number of leafhopper per plant led to significantly different ($P<0.01$) infection rates by WDV-W and WDV-B in the two hosts (Fig. 5B). Regarding the number of insect per plant on wheat, the results of Duncan's multiple range tests showed that there were significant differences in transmission rate among all treatments except for the data comparing 4 and 8 insects per plant in which no significant difference was observed, whereas in barley plants there was no significantly difference between the treatments with 1, 2, 4 and 8 insects per plant (Fig. 5B). However, increasing the number of insect per plant up to 4 and/or 8 led to a significant difference between the infection rates. The infection rate of wheat and barley plants caused by WDV significantly increased ($P<0.01$) with time after inoculation (7, 14, 21 and 28 dpi) during the experiment (Fig. 5C). Comparison of infection rates caused by WDV-W and WDV-B in the two plant hosts indicated a significant difference between the infection rates achieved by the two strains. WDV-W was more efficiently vector transmitted to either wheat or barley than WDV-B (Fig. 5D). There was no significant difference among the infection rates of each strain in wheat and barley plants, when their infection rate was assessed individually.

DISCUSSION

Wild grasses are potential hosts and may act as reservoirs for cereal-infecting viruses. In a study carried out by Bisnieks *et al.* (2006), it was shown that the incidence of perennial grasses infected with *Barley yellow dwarf virus*-PAV (BYDV-PAV), *Barley yellow dwarf virus*-MAV (BYDV-MAV) and *Cereal yellow dwarf virus*-RPV (CYDV-RPV) was as high as 19% in randomly collected samples which can explain the relatively high incidence of B/CYDVs in grasses in Lativa. ELISA technique detected WDV infection in a relatively large number of symptomless wild grasses (Mehner, 2005). However, in another survey in Sweden, it was surprisingly found only a low number of samples (8/1098) from three grass species and triticale were found to be WDV positive by ELISA and PCR (Ramsell *et al.*, 2008). This low incidence of infected wild grasses was found in the vicinity of winter wheat fields with reported 50% incidence of WDV (Lindblad and Sigvald, 2004). In our study, 91 out of 149 grass samples (61.07%) collected from cereal farms in October 2013 were infected by WDV while the average infection rate of the same hosts was 32.47% (38 infected out of 117 collected samples) in the following spring (May 2014). This relatively high rate of infection among the gramineous weeds is in agreement with earlier report of high WDV incidence (86.3%) in Fars province, Iran (Lotfipour *et al.*, 2013b). Nearly all virus positive grass species (8/9) exhibited WDV-W infection while only one species (*H. spontaneum*) was found to be infected by WDV-B which is consistent with relatively higher incidence of WDV-W than that of WDV-B in Iran (Lotfipour *et al.*, 2013b). Similarly, only

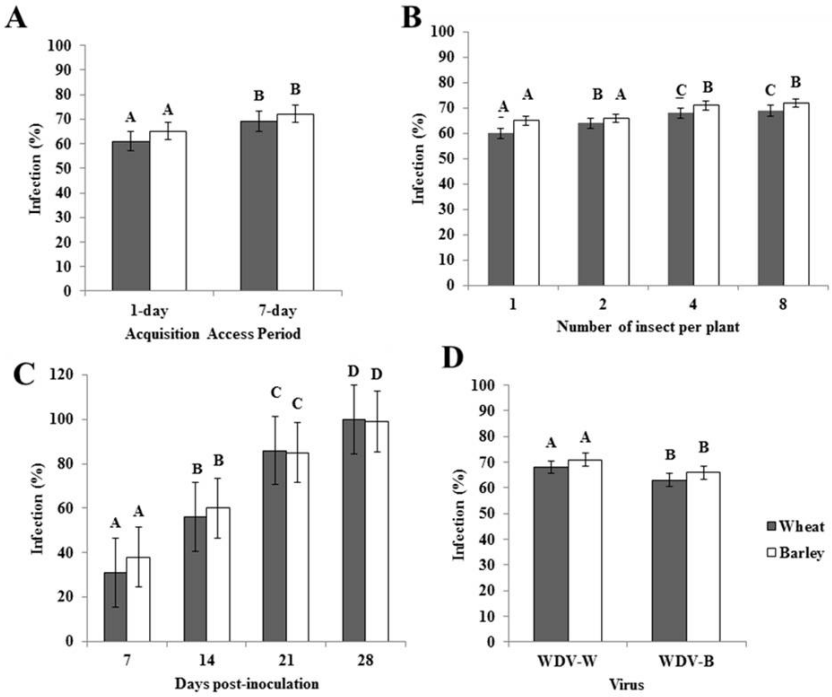

Fig. 5. The effects of different treatments including acquisition access period (A), number of leafhoppers (*Psammotettix alienus*) (B), time intervals (C) and virus strain (D) on the infection rate (%) of WDV-W and WDV-B in wheat and barley plants. The data presented in (A), (B) and (D) are the mean value of the data of four time intervals (7, 14, 21 and 28 days post-inoculation).

WDV-W has been detected among wheat samples (Lotfipour *et al*., 2013b). It seems that WDV-B cannot naturally infect wheat while barley samples can be infected by both WDV-W and WDV-B. This may also show the better adaptation of WDV-B towards its natural host. The high infection rate of WDV-B in *H. spontaneum* might be explained by the fact that this weed is closely related to the cultivated species, *H. vulgare*, than the other wild grasses. Similarly, it has been already determined that the WDV accumulated more in the domesticated wheat group than in wild plant group, suggesting more virus amplification and/or movement within the domesticated plants (Nygren *et al*., 2015). These annual weed species are common in wheat and barley fields in Iran and therefore seem to have significant effect on the epidemiology of WDV infection. Among the wild grasses in which WDV incidence was detected, *S. halepense* and *C. dactylon* are the most probable reservoirs, as these are common perennial grasses in Iran and may thus carry WDV infection between growth seasons.

Pairwise nucleotide comparison of the sequences from wild grasses showed more than 90% identity to those previously reported from Iran (Fig. 2). However, there was a remarkable difference (13-15%) between WDV-W and WDV-B isolates in the region encompassing Rep-coding sequence which resulted in forming different clusters in phylogenetic analysis as reported by Schubert *et al.* (2007). Furthermore, WDV-W and WDV-B isolates reported in this study formed two separate clades in the phylogenetic tree when the nt sequence of their genome regions encompassing the Rep gene were compared (Fig. 2). Host-dependent clustering of wheat- and barley-derived samples in two separate clades has been also archived on the basis of complete genome sequence comparison of either European or global isolates of WDV (Schubert *et al*., 2013; Wu *et al*., 2015). Further experiments need to be conducted to compare the full-length genome sequences of gramineous weeds isolates of WDV which would be useful for more detailed phylogenetic analysis.

Comparison of WDV infection rates between two sampling times showed that the gramineous weeds are more frequently infected in October than in May. This suggests that due to the absence of main hosts (e.i. wheat and barley) in summer and early fall, the leafhopper vector switches to surrounding plant and consequently feeds on wild grasses leading to high WDV-infection rate among them (Table 3). Our result is supported by the finding that WDV transmission to wild grasses by leafhoppers occurs usually (Vacke, 1961, 1964; Lindsten and Vacke, 1991). However, in the spring, the main hosts are available to suit the presence of the leafhoppers. The difference between WDV-infection rates among the gramineous grasses can also be explained by the population dynamics of the leafhopper vector during the year. It was previously indicated that the occurrence of *P. alienus* leafhoppers as well as their activities as the vector of WDV detected from May to either November or December depending on the geographical region and the year of sampling (Manurung *et al*., 2005). The peak population density of *P. alienus* occurred in winter barley (stubble) field in mid-September (Manurung *et*

al., 2005). Further information on the population dynamics of *P. alienus* in different sampling sites and times are required to build a more reliable relationship between the WDV-infection rates and the population size of the leafhopper vector.

Based on our results it can be generally concluded that the leafhoppers transmit WDV-W and WDV-B to wheat and barley hosts with relatively high transmission efficiency as 100% infection rate was obtained with only one single viruliferous *P. alienus* adult per plant. This might occur due to the rapid transmission of WDV and long retention of the virions in the leafhopper vector (Wang *et al*., 2014). The statistical analysis of transmission data indicated that seven-day acquisition access treatment significantly increased the transmission efficiency of WDV in both wheat and barley hosts (Fig. 5A). This has also been reported for other persistent viruses (Lucio-Zavaleta *et al*., 2001; Ammar *et al*., 1995). The positive correlation between the transmission efficiency and acquisition access period of leafhopper vector obtained in this study was consistent with the results of Yazdkhasti (2012) study in Sweden. These results highlighted the importance of acquisition phase in the process of WDV transmission which triggers an elaborate insect-virus interaction necessitating the retention of virus in the specific locations of vector body (Chen *et al*., 2011). Furthermore, as the number of insect per plant increased, the infection rates caused by WDV were significantly increased in the two plant hosts (Fig. 5B) suggesting that high rate of WDV infection is positively correlated to the population size of leafhopper vector. This has been previously shown in a survey by Manurung *et al*. (2005) in which they found that peak population density of the leafhopper *P. alienus* was associated with high infection rate of winter barley caused by WDV. Comparison of infection rates caused by WDV-W and WDV-B in wheat and barley plants showed that WDV-W was vector transmitted with higher transmission efficiency than WDV-B, indicating WDV-W higher level of virulence.

It seems that the leafhoppers originated from Fars province (Iran) are relatively more efficient vectors of both WDV-W and WDV-B, as the Beijing (China), Quedlinburg (Germany) and Prague (Czech Republic) populations have exhibited lower transmission efficiencies of 75, 58 and 76%, respectively (Wedde *et al*., 2012). This variation among the transmission efficiency of WDV by *P. alienus* might be due to experimental conditions. Alternatively, various parameters such as environmental factors (temperature and humidity), host susceptibility and the level of virus virulence may affect the rate of transmission (Reynaud and Peterschmitt, 1992). Additionally, the development stage of *P. alienus* individuals was found to influence diversely on the transmission efficiency of WDV (Mehner *et al*., 2003). These results highlight the key role of *P. alienus* individuals in the epidemiology of WDV for the virus transmission in Iran, which invokes more research to achieve better understanding about this pathosystem.

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