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Differences in responses to *Wheat dwarf virus* infection in contrasting wheat cultivars Ludwig and Svitava

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Abstract: The two contrasting cultivars, the very susceptible Ludwig and moderately susceptible Svitava, previously evaluated in field trials to determine their resistance levels to *Wheat dwarf virus* (WDV), were analysed by four different test methods to allow for the comparison of the methods and to consider whether the resistance level of cv. Svitava can be an effective component of wheat protection from the virus. The differences in the proportion of the diseased plants were observed after the inoculation by the viruliferous leafhoppers. The differences in the cultivar resistance levels were evaluated by comparing the biomass production after the infection. The amount of viral DNA was determined using quantitative polymerase chain reaction (PCR). Concurrently, the cultivar response to the virus infection after the controlled inoculation by the leafhoppers [*Psammotettix alienus* (Dahlbom, 1850)] under field conditions was further evaluated. The infected plants of cv. Svitava produced five times more dry matter in the vegetative growth stage than cv. Ludwig. The quantitative PCR analysis confirmed the resistance in cv. Svitava. The plant inoculation under a standardised infection pressure showed that the probability of the WDV infection after the inoculation feeding is smaller in the cv. Svitava plants. The comparison of the results from the different test methods shows that the field resistance tests are necessary to obtain a proper assessment of the possibilities for the cultivars' utilisation in the crop protection.

Keywords: *Wheat dwarf virus*; resistance; winter wheat; cv. Ludwig; cv. Svitava

The *Wheat dwarf virus* (WDV), a geminivirus of graminaceous plants, is transmitted by the leafhopper *Psammotettix alienus* (Dahlbom, 1850). The WDV infects wheat (*Triticum aestivum* Linnaeus), barley (*Hordeum vulgare* Linnaeus) and some other economically important cereals, together with more than ten wild species of the *Poaceae* family (Vacke 1971; Lindsten & Vacke 1991; Vacke & Cibulka 1999; Mehner et al. 2003). A WDV infection in wheat plants manifests serious symptoms that include dwarfing, yellowing and premature death at the vegetative phase of the plant development. The viral etiology of the disease was first described in the former Czechoslovakia (Vacke 1961). In the Czech Republic, the virus regularly causes harm in lowland areas, where cereals are intensively grown, and

serious damage to winter wheat has been recorded in several seasons over the last twenty years. Since the beginning of the present millennium, insecticide spraying of fields for crop protection of winter cereals has been introduced in the Czech Republic. Habekuš et al. (2009) found a positive correlation between higher temperatures and the incidence of the *Barley yellow dwarf virus* and expected that insect transmitted viruses will become more important in the future due to global warming.

In field trials conducted between the years 1969–1971, the growth of eight out of a total of 32 winter wheat cultivars was less affected by the WDV infection (Vacke 1971). Later, several trials aimed at identifying wheat cultivars with resistance to the WDV were performed. Vacke and Cibulka

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(2000) found ten moderately susceptible cultivars, while Širlová et al. (2005) found two moderately susceptible winter cultivars, namely, Svitava and Banquet. In these two experimental sets, the plant resistance was evaluated under field conditions with respect to the height and grain yield reduction in the infected plants. The tested cultivars were categorized into very susceptible, susceptible and moderately susceptible groups by Vacke and Cibulka (2000) and Širlová et al. (2005). The plants of the cultivars rated as moderately susceptible had a yield reduction of 82.5–93.1% compared to the non-infected plants. When the differences in the relative virus content in the leaf samples of the infected plants were investigated using DAS-ELISA (double antibody sandwich enzyme-linked immunosorbent assay), significant differences were found between the very susceptible and moderately susceptible cultivars.

The glasshouse experiments performed by Benkovics et al. (2010) have confirmed the resistance of two Hungarian wheat cultivars. Using the quantitative PCR, low amounts of WDV were detected in the infected plants and a lower frequency in the diseased plants was observed following the inoculation for the cultivars Mv Dalma and Mv Vekni. Furthermore, the variation in response to the WDV infection has recently been tested in several species of wild and domesticated relatives of the hexaploid bread wheat and Nygren et al. (2015) have suggested *Aegilops tauschii* as a potential genetic resource for the improvement of the WDV resistance in wheat.

In this paper, we report detailed analyses of the differences in the responses to the WDV infection between two winter wheat cultivars, including the very susceptible cv. Ludwig and the moderately susceptible cv. Svitava.

MATERIAL AND METHODS

Proportion of infected plants under standardised infection pressure

Fifty cv. Ludwig and fifty cv. Svitava plants were cultivated outdoors in a net house. Seeds were sown individually in 0.45 L plastic pots filled with 70% chernozem and 30% garden substrate B. Before sowing, 1.5 g of 15–15–15 NPK fertilizer was individually dosed inside every pot. More than one hundred leafhoppers were kept in one insect isolator for 4 weeks of acquisition feeding on the WDV infected wheat plants cv. Rapsodia to ensure the same properties of the leafhoppers. Upon reach-

ing the growth stage BBCH 11, the seedlings were inoculated with the WDV using the viruliferous leafhoppers. The plants were isolated separately under small insect isolator cages consisting of mesh-covered wire frames and inoculated with only one leafhopper per plant. The inoculation feeding lasted for five days when the leafhoppers and isolators were removed. The plants on which the leafhoppers were found dead were excluded from the evaluation of the standardised infection pressure, but we left them for the symptomatic and enzyme-linked immunosorbent assay (ELISA) diagnosis. The plants were then left to grow during the autumn in a leafhopper free net house until the time of the evaluation. Three months later at the tillering stage, a symptomatic diagnosis was performed. Afterwards, a sample for the DAS-ELISA test was collected from each plant.

The DAS-ELISA testing was performed using the commercial kits WD-TRA 0480 (Sediag, France) with rabbit polyclonal antibodies, alkaline phosphatase coupled antibodies and substrate p-nitrophenyl phosphate. We followed the manufacturer's protocol.

Vegetative biomass production of infected and healthy plants

The plants from the experiment which focused on the proportion of the infected plants under the standardised infection pressure, were removed from the soil after three months of growth in the net house and their roots were carefully washed. Any surface water was removed using a paper towel, and the plants were weighed in a fresh state. The plants were then individually placed in paper bags, oven-dried and then weighed.

Amount of viral DNA in plants

Ninety-four cv. Ludwig and 94 cv. Svitava seeds were sown individually in pots (soil composition and fertilising as described above) outdoors in a net house. Once the majority of the seedlings reached growth stage BBCH 11, the pots with the prematurely and poorly developed plants were eliminated along with the poorly germinated or ungerminated seeds. 82 cv. Ludwig and 90 cv. Svitava plants remained after the selection. Each plant was isolated using a small insect isolator cage and individually inoculated using three viruliferous leafhoppers. The inoculation feeding lasted for 18 h after which the leafhoppers and isolators were removed. Immediately after the inoculation, the plants were transferred to a growth

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chamber and grown at 17.1 °C (for 14 h of light) and at 9.0 °C (for 2 hours of light and 8 h of darkness (1 l–8 d – 1 l)) until the samples were collected for the quantitative PCR analysis. The temperatures given here are an average of the temperatures obtained from the two control temperature probes placed in the growth chamber during the experiment. The first samples were taken on the 5th day after the inoculation feeding ended and then every 5th day thereafter to the 40th day for both cultivars (days 5, 10, 15, 20, 25, 30, 35, 40) and on the 45th day only for the Svitava cultivar. At the same time, the fresh biomass weight of each sampled plant was measured.

Quantitative PCR analysis. The DNA isolation was performed according to the procedure mentioned in Cejnar et al. (2019). The plant tissue was ground into a fine powder in liquid nitrogen. The DNA isolation was performed using the guanidine thiocyanate method (extraction solution: 1 M of guanidine thiocyanate, 0.02 M of Na₂H₂EDTA (ethylenediaminetetraacetic acid disodium salt), 0.1 M of MOPS (3-(N-morpholino)propanesulfonic acid), a pH of 4.6, 0.2% of 2-mercaptoethanol, deionized H₂O). The proteins were removed by extraction with a phenol-chloroform-isoamyl alcohol solution and treated with proteinase K. The DNA was precipitated and washed with 70% (w:v) pre-cooled ethanol. The DNA pellet was dissolved in 50 µL of deionised water.

Preparation of the DNA standard: Plasmid pGEM-T easy (Type, Promega, USA) with a cloned viral DNA (primer pair UnivWDVfw, UnivWDVrv, Gadiou et al. 2012) was cultivated in the DH5alpha strain of *Escherichia coli* and the DNA was isolated by a GenElute Plasmid Miniprep Kit (Sigma-Aldrich, USA). The purity of the isolated plasmid was confirmed by electrophoresis and its concentration was estimated spectrophotometrically (Nanodrop 2000, ThermoFisher Scientific, USA). The isolated plasmid was stored in aliquots at – 80 °C.

Real-time PCR: The absolute quantification of the DNA viral copies was performed by using a TaqMan kit from Applied Biosystems, the primer pair UnivWDVfw, UnivWDVrv (Gadiou et al. 2012) and a 6-FAM-TCATCAACTACTCGTTCGCCTC-CG-TAMRA probe. All the samples were run on a LightCycler® 480 Multiwell Plate 384 (Roche, Switzerland). The qPCR cycle was performed as follows: 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s in a 12 µL final volume containing 6 µL of the master mix, 3.0 µL of the DNA solution (20 ng DNA/µL), 0.6 µL of the primer solution

(20 mM of each primer, 20 mM of the TaqMan probe), 2.4 µL of deionised H₂O. The qPCR was followed by a melting analysis of the qPCR product to control the specificity of the reaction.

The amount of WDV in each plant sample was calculated with a standard curve generated from the serially diluted plasmid DNA (ten-fold serial dilution, plasmid number 10⁶–10¹²). All the samples and calibrators were measured in triplicate.

Cultivar evaluation under field conditions

The Ludwig and Svitava cultivars were sown at the end of September in small 1 m² sized neighbouring plots. The plant spacing was 10 × 6 cm. The field trials were repeated for three years (growing seasons 2008/2009, 2009/2010, 2010/2011) and consisted of two variants: (1) a control variant – without infection, (2) a variant with the infection. After sowing, the plots were covered with polypropylene isolators. During the second leaf stage (BBCH 12), the plants from the variant with the infection were inoculated with the WDV using viruliferous leafhoppers, as described in Vacke and Cibulka (2000). 14 days later, the leafhoppers were killed using a Vaztak 10 SC insecticide (ai alpha-cypermethrin). During the growing season, the plots were kept free of insect vectors by insecticide spraying. The trials were assessed after winter in the following growth season. The following traits were observed: the proportion of the plants with symptoms, the proportion of the heading plants, the aboveground biomass weights, the stalk lengths and the grain yields.

The *Psammotettix alienus* leafhopper individuals and the WDV wheat strain were obtained from the virus collection maintained at the Crop Research Institute, Prague.

We calculated the confidence intervals with a 95% confidence level for the dry matter weight of the young plants and the stalk length of the mature plants and with a 90% confidence level for the concentration of the viral DNA. The graphs were prepared in Microsoft Excel (version 2010).

RESULTS

Proportion of infected plants under standardized infection pressure

On five cv. Ludwig and four cv. Svitava plants, the leafhoppers died during the inoculation feeding and none of these plants were infected according to the ELISA test. Among the successfully inoculated

plants, the proportion of the plants that tested positive by ELISA reached 57.8% (26 infected out of 45) and 19.6% (9 out of 46) for cv. Ludwig and Svitava, respectively. In cv. Ludwig, the symptoms of the WDV infection were intensive and the diagnosis according to the visible symptoms and by the ELISA technique gave the same results. The symptomatic diagnosis of the Svitava cultivar proved unreliable and difficult as the specific symptoms of the WDV infection were not visible. Thus, according to the ELISA results (9 infected out of 50 Svitava plants), the symptomatic diagnosis did not reveal two infected plants of cv. Svitava, while five plants that tested negative for the WDV in the ELISA test were incorrectly identified as infected by the symptomatic diagnosis.

Vegetative biomass production of infected and healthy plants

Three months after inoculation, the reduction of the dry matter production due to the infection was 83.9% for cv. Ludwig and 20.4% for cv. Svitava in comparison with the healthy plants (Table 1). The severe growth retardation and reduction of the tillering in the infected cv. Ludwig plants were observed, the developed disease was characterized by dwarfing, growth termination, yellowing and dying of the older leaves and the whole plants in cv. Ludwig. In cv. Svitava, the disease symptoms were only slight. In most of the infected plants, the reduced plant growth and light yellowing in the older leaves were observed.

Table 1. Comparison of the dry matter production (expressed in gram per plant) in the healthy and WDV-infected plants three months after inoculation by the leafhopper *Psammodettix alienus*

Group	Cv. Ludwig		Cv. Svitava	
	healthy (n = 24)	diseased (n = 26)	healthy (n = 41)	diseased (n = 9)
$\bar{\mu} \pm \Delta$	5.78 ± 0.54	0.93 ± 0.24	5.93 ± 0.34	4.72 ± 0.96

$\bar{\mu} \pm \Delta$ shows the mean and 95% confidence interval

Amount of viral DNA in plants

In the Ludwig cultivar, the virus DNA concentrations rose rapidly over time two to three weeks after infection. The curve showing the virus concentration in relation to time followed an exponential course (Figure 1A). During this period, the virus replicated in the target cells and spread through the plant, leading to a systemic infection, while the virus concentration in the plant tissue rapidly increased.

Afterwards, the propagation of the virus slowed down, which coincided with the severe disruption of the plant growth and the emergence of the typical wheat dwarf symptoms. In the cv. Svitava, virus concentration (over the time investigated) was lower (Figure 1). The maximum mean viral DNA concentration in the infected cv. Svitava plants under this virus multiplication experiment was more than ten times lower than for cv. Ludwig. Concurrently, we observed that the amount of fresh biomass increased faster for cv. Svitava (Figure 2).

Cultivar evaluation under field conditions

The symptoms of the virus infection in cv. Ludwig appeared in early spring after the plant growth restoration. The first obvious symptoms appeared over time from the end of the first third of March to the beginning of April. Distinct differences between the healthy and diseased plants occurred from the second third of April. The infected plants rarely passed the growth stage BBCH 30. The diseased plants died prematurely in May and failed to produce any kernels (Table 2).

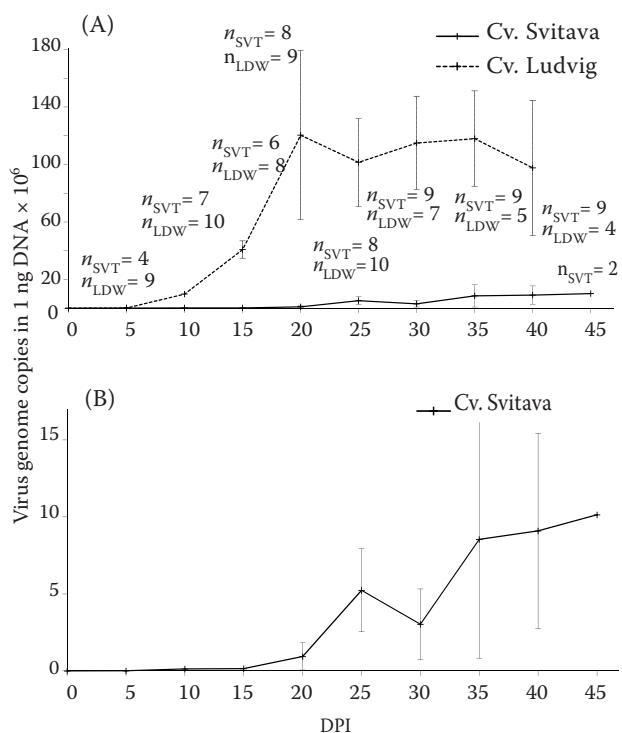


Figure 1. (A, B) The accumulation of the *Wheat dwarf virus* in the infected plants of the cultivars Svitava and (A) Ludwig observed by qPCR within 45 days post-inoculation (DPI). The bars represent 90% confidence intervals SVT – Svitava cultivar; LDW – Ludwig cultivar

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Table 2. Analysis of the healthy and the wheat dwarf-diseased harvested plants. The field tests are for seasons 2009, 2010 and 2011

Group	Cv. Ludwig		Cv. Svitava	
	healthy (1)	diseased (2)	healthy (1)	diseased (2)
Stalk length ($\bar{\mu} \pm \Delta$) (cm)	81.8 \pm 2.9	0.2 (near 0)	64.9 \pm 2.8	17.6 \pm 6.1
Heading (%)	100	0	100	41.7
Ears per plant (number)	3.3	0	3.4	0.6
Aboveground biomass (g/plant)	15.4	0.05	16.1	1.7
Grain yield (g/plant)	7.10	0	8.81	0.25

$\bar{\mu} \pm \Delta$ – the mean and 95% confidence interval of the stalk length for the years 2009, 2010 and 2011; 1 – plants taken from the control variant; 2 – plants taken from the variant with the infection; the diseased plants were identified by the symptomatic diagnosis

The first symptoms in cv. Svitava were observed later – from the second third in April. Unambiguous differences between the diseased and healthy plants occurred from May, always coming after the growth stage BBCH 32. A minority of the diseased plants matured earlier than the healthy plants. However, the majority of the plants matured at the same time as the healthy ones. In contrast to the other experiments conducted using the growth chamber and the net house, we observed evident wheat dwarf symptoms in the older cv. Svitava plants under the field conditions, which enabled us to reliably distinguish the diseased from the healthy plants by a symptomatic evaluation (Table 2). The symptomatic diagnosis performed during the growth season during the years 2009–2011 showed the wheat dwarf infection at 50.0%, 64.3% and 40.0% for cv. Ludwig and 4.2%, 15.4% and 31.9% for cv. Svitava, respectively.

DISCUSSION

Our investigation confirmed the differences in response to the WDV infection between the cvs. Ludwig and Svitava as previously reported by Širlová et al. (2005). The reduced virus concentration in the cv. Svitava plants confirmed the occurrence of the resistance to WDV in this cultivar. Therefore, the differences observed in the biomass production between the infected plants of cvs. Ludwig and Svitava are associated with the resistance.

The results presented in this work seem to suggest the potential for the use of these individual test methods in resistance screening tests. The plant pot testing of young plants after infection to evaluate the biomass production can deliver fast results, without the need for expensive laboratory equipment.

Under infectious conditions, the relatively higher biomass production and grain yield of a particular genotype compared to the susceptible and sensitive genotypes can be caused by the resistance or tolerance. Resistance is defined as the ability of the host to hinder a pathogen or a disease-causing agent (Robinson 1969). Thus, resistant cultivars reduce the multiplication or spreading of the virus in the plant (Cooper & Jones 1983). The concept of plant tolerance is based on the ability of the host to limit the damage without the necessity to repress the pathogen growth and reproduction. According to Schafer (1971), plant tolerance was defined as "that capacity of a cultivar resulting in less yield or quality loss relative to disease severity or pathogen development when compared with other cultivars or crops." Tolerance reduces the effect of the infection on the yield without necessarily minimising the pathogen multiplication (Little et al. 2010). Resistance and tolerance are, therefore, two fundamentally different strategies to deal with pathogens: resistance reduces the risk of infection and/or the replication rate

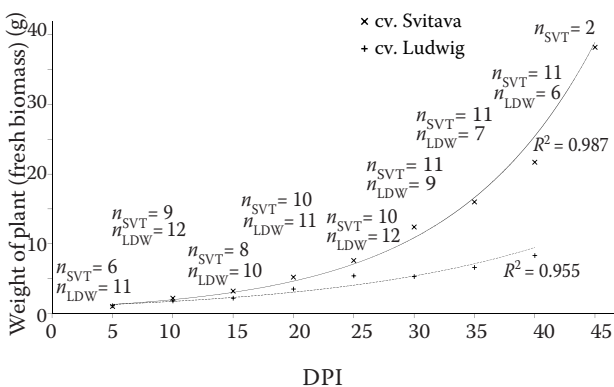


Figure 2. The fresh biomass weights of the young infected plants of the cultivars Svitava and Ludwig within 45 days post-inoculation (DPI) with the WDV.

SVT – Svitava cultivar; LDW – Ludwig cultivar; the lines show the exponential regression for the fresh biomass weight of the infected plants

of the pathogen in the host, whereas tolerance does not (Råberg 2014). Resistance and tolerance are generally not mutually exclusive and can coexist in the same plant (Pagán & García-Arenal 2018).

Taking the tests for the biomass production of young plants in combination with the test under the appropriate standardised infection pressure and with the test aimed at the amount of viral DNA may help to distinguish the cultivars with the virus resistance from the tolerant ones. In our study, the reduction of the dry matter production due to the infection of the young plants was lower in cv. Svitava compared to cv. Ludwig. The standardised infection pressure testing revealed that the probability of the WDV infection after the inoculation feeding is smaller in the cv. Svitava plants. A comparison of the differences in the number of virus genome copies determined for both cultivars did not confirm tolerance as the cause of the reduced formation of the disease symptoms in cv. Svitava, but, on the contrary, the comparison affirmed its resistance to WDV. The use of the qPCR analysis showed that the nature of the cv. Svitava resistance could be caused by the reduced virus multiplication. However, confirmation of this hypothesis requires further investigation.

The most appropriate time for the plant sampling to detect the differences in the virus accumulation between the cultivars by means of the qPCR technique is 3–4 weeks post-infection, since the greatest differences in the virus content under the growing conditions described above will be observed. The rather high differences in the virus content measured in the plants of the same group can be a disadvantage in reliably distinguishing small differences in the cultivar resistance levels (the bars in Figure 1).

The duration of the virus multiplication experiment did not allow us to assess the curve shape describing the virus content in cv. Svitava (contrary to cv. Ludwig). A continuous increase in the number of viral genome copies is evident in the plants of cv. Svitava 45 days after the infection (Figure 1B); however, the highest concentration that would be reached cannot be inferred. A 45-day experiment was too short to obtain the graph curve that better characterises the accumulation of the WDV over time in the infected plants of cv. Svitava.

Evaluating the resistance under the field conditions allows the cultivars to be assessed for their proper place in the systems of crop protection and is, therefore, an irreplaceable method for forecasting cultivar yields. Omission of field resistance tests during the

resistance evaluation leads to the results obtained from tests on young plants under good growth conditions occurring in greenhouses and climate chambers, which, in turn, may lead to higher expectations of the resistance effect on the grain yield. Under field conditions, plants are typically exposed, to some extent, to various stresses, e.g., abiotic stresses during overwintering or drought periods, and these adverse effects manifest more substantially in the infected plants. The results of the field experiments presented in this work show that the resistance level observed in cv. Svitava is insufficient to preserve an adequate grain yield in plants infected during autumn.

On the other hand, the resistance level of cv. Svitava may be useful in cases where infections take place during spring, when the virus infection continues its spread to other healthy plants in the wheat crops infected from autumn. The resistance of cv. Svitava may positively impact the crop protection in the spring before the plants become resistant due to the occurrence of the mature resistance in older plants (Lindblad & Sigvald 2004).

Furthermore, our results demonstrated that the probability of the WDV infection after the inoculation feeding is smaller in the cv. Svitava plants than the cv. Ludwig plants. The differences can be explained by the need of a higher dosage of viral particles required for infection in the case of cv. Svitava; however, the different vector feeding behaviour on the cultivars can also be accountable. The most important advantage of the cv. Svitava resistance under the field conditions is the lower proportion of the infected plants. This will, in turn, result in better grain yields in the Svitava cultivar in the case of the infection pressure, which causes the yield failure in the Ludwig cultivar due to the severe decrease in the number of grain-productive plants in the crop.

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