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Interactions between *Barley yellow dwarf virus* and *Fusarium* spp. affecting development of Fusarium head blight of wheat

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Abstract

Interactions between *Barley yellow dwarf virus* (BYDV) and *Fusarium* species causing Fusarium head blight (FHB) in winter wheat cvs Agent (susceptible to FHB) and Petrus (moderately resistant to FHB) were studied over three years (2001–2003) in outdoor pot experiments. FHB developed more rapidly in cv. Agent than in cv. Petrus. The spread of FHB was greater in BYDV-infected plants than in BYDV-free plants. Thousand grain weight (TGW) was reduced more in *Fusarium*-infected heads of cv. Agent than in cv. Petrus. A highly significant negative correlation was found between disease index and TGW in cv. Agent ($r = -0.916$), while in cv. Petrus the correlation was less significant ($r = -0.765$). Virus infection reduced TGW in cv. Petrus more than in cv. Agent. In plants with both infections, TGW reductions in cv. Petrus corresponded to those of BYDV infection, and in cv. Agent TGW was more diminished than in BYDV infection. Effects of different treatments determined over three years on ergosterol contents in grain were generally similar to effects on disease indices. Grain weight per ear and ear weight of the different treatments of both cultivars largely corresponded with the TGW results. Deoxynivalenol (DON) content in grain of cv. Agent infected with *Fusarium* spp. was 11–25 times higher compared to the corresponding treatments in cv. Petrus. The DON content in grain of plants of the two cultivars infected with both pathogens was higher than that of plants infected only with *Fusarium* over the three years.

Introduction

Fusarium head blight (FHB) is one of the most important diseases of wheat and other cereals in many cereal-growing areas of the world because of quantitative and qualitative yield losses (Parry et al., 1995; Paulitz, 1999). *Fusarium* species produce mycotoxins in the grain that can harm animals and humans when contaminated grain products are consumed. The two most important mycotoxins in wheat grain are deoxynivalenol (DON) and zearalenone (ZEA). In warmer regions of the world, including parts of the USA, Canada, Australia, China and central Europe, *Fusarium graminearum* is the most important species causing FHB (Parry et al., 1995; Wang, 1997). *Fusarium culmorum* tends to predominate in the cooler maritime regions (Parry et al., 1995). Results from several European countries indicate an increase in the importance of *F. graminearum* as a major pathogen of wheat in temperate climates. For instance, while in the Netherlands *F. culmorum* was the major component of the FHB complex in the 1980s and 1990s (Daamen et al., 1991; De Nijs et al., 1997), there was a shift in the composition of the FHB complex probably since the mid 1990s. Analyses of *Fusarium* isolates in 2000 and 2001 revealed that *F. graminearum* was the most important species causing FHB (Waalwijk et al., 2003). Among the causal factors involved in this shift, an increase in corn production has been suggested.

Trichothecene mycotoxins produced by *Fusarium* species are secondary metabolites and in general they are not essential for normal growth or survival of the fungi. However, they may be involved in pathogenesis (Desjardins and Hohn, 1997). Trichothecenes also exert phytotoxic effects such as chlorosis, necrosis and wilting. Several studies reported correlations between tolerance of DON in cereal cultivars and resistance to FHB (Muthomi et al., 2002; Wanyoike, 2002).

Barley yellow dwarf is the most widely distributed and the economically most important virus disease of wheat. It is caused by a group of luteoviruses called barley yellow dwarf luteoviruses (BYDVs). BYDVs are restricted in host range to Poaceae such as barley, oats, wheat, rye and maize as well as many annual and perennial grasses (Oswald and Houston, 1953; Watson and Mulligan, 1960). Their wide host range within the grass family is associated with numerous aphid species that are vectors of BYDVs. BYDV strains and their principal vectors are RPV (*Rhopalosiphum padi*), RMV (*R. maidis*), MAV (*Sitobion avenae*), SGV (*Schizaphis graminum*) and PAV (*R. padi*). In northern Europe, *R. padi* and *Macrosiphum* (= *Sitobion*) *avenae* are the prevalent aphid vectors and, therefore, BYDV-PAV and -MAV are of special importance in this area (Huth, 2000). BYDVs are transmitted in a persistent manner by aphids. Severity of the disease in a crop depends on several interacting factors, including cultivars, age and physiological conditions of the host plant at the time of infection, number of vectors, BYDV strain and environmental conditions (Irwin and Thresh, 1990). The most characteristic symptom is a loss of green colour of the leaves, which is most evident in older leaves of infected plants. Discolouration begins 7–20 days after inoculation (dai), mostly along the margins or at the tips. The discoloured areas enlarge in the basipetal direction, and finally entire leaves may become discoloured. Infected leaves of wheat usually turn yellow and sometimes red. Leaves of BYDV-infected plants may be more erect and stiffer than those of non-infected plants.

BYDV infections also cause pronounced physiological alterations in barley, oats and wheat plants. While in infected tissues total fresh weight decreased, dry weight in general increased (Jensen, 1968). Starch and soluble carbohydrates, especially reducing sugars accumulated in infected wheat and barley leaves (Watson and Mulligan, 1960). In BYDV-infected wheat leaves, fructose and glucose concentrations increased (Fereres et al., 1990).

The pronounced effects of BYDV infection on the metabolism of cereal hosts suggest that interactions with other cereal pathogens may occur (Monneveux et al., 1992). Studies on such interactions between BYDV infections and infections by other pathogens have received little attention recently, but were studied in the past (Pelletier et al., 1974). Study of effects of BYDV infection in oats and durum wheat on the development of root rot disease caused by *Cochliobolus sativus* showed that plants diseased by both pathogens were more severely affected than plants infected by each pathogen alone (Scott, 1968). Koch and Huth (1997) concluded that susceptibility of wheat plants to *F. culmorum* would be increased in epidemic years of BYDV.

Both diseases, BYDU and FHB, of wheat plants differ significantly in their nature of parasitism and spreading in host plants. BYDV is a biotrophic pathogen, which is restricted to the phloem in which it spreads systemically in the entire host plant and causes pronounced morphological and physiological changes in diseased wheat plants. FHB, on the other hand, is predominantly a disease of wheat spikes. Studies on the infection process of spikelets by *Fusarium* spp. suggest that, after a short biotrophic relationship, *Fusarium* spp. change to necrotrophic parasitism during spread in the spike tissue (Kang and Buchenauer, 2000).

The objectives of this work were to study effects of BYDV infection on FHB development, caused by *F. culmorum* and *F. graminearum* in cvs Agent and Petrus, which differ in susceptibility to FHB, in outdoor pot experiments during three seasons. In addition yield parameters as well as ergosterol- and DON-contents in grain were determined.

Materials and methods

Plant material

Two winter wheat (*Triticum aestivum*) cultivars, Agent and Petrus, were used. They differ in their susceptibility to FHB; according to the 'Beschreibende Sortenliste' (Anon., 1997), cv.

Agent is classified as 7 and cv. Petrus as 2, within a rating scale from 1 (highly resistant) to 9 (highly susceptible). The seeds were sown on 19 and 20 December in 2000, 2001 and 2002 in 10 l pots filled with loamy organic soil and plants cultivated under semi-field conditions (outdoor pot experiments). After growth stage (GS) 25 (Zadoks et al., 1974) plants were fertilized weekly with 100 ml of 1% (v/v) solution of the fertilizer Wuxal (N-P-K: 8–8–6; Aglukon, Germany). At GS 31 (first node detectable) all tillers were eliminated, allowing only the main shoots to grow, and the plants were thinned out to 15 plants per pot. The experimental design was as follows: A, control; B, infection with BYDV by virus-bearing *Rhopalosiphum padi*; C, infestation with virus-free *R. padi*; D, infection with *Fusarium* spp.; E, infection with BYDV and *Fusarium* spp.; F, infestation with virus-free *R. padi* and infection with *Fusarium* spp. Each treatment consisted of at least 6 pots (90 plants) and the pots of each treatment were arranged in blocks.

BYDV vector and virus inoculation

Oat seedlings (*Avena sativa*) of cv. Jumbo were infected with a BYDV-PAV strain which was kindly provided by Dr W. Huth (BBA, Braunschweig, Germany). The aphid species *Rhopalosiphum padi* was used as the vector for transmission of the virus from infected to healthy oat seedlings. The BYDV-PAV-bearing aphids and virus-free aphids were fed separately in screened cages in different cabins under greenhouse conditions (13 h daylight, at 23 °C; 11 h dark, at 20 °C). At GS 51 (beginning of heading; 16 and 14 days before inoculation of wheat spikes with *F. culmorum* in 2001 and with *F. graminearum* in 2003, respectively) and at GS 39 (flag leaf stage; 22 days before inoculation of wheat spikes with *F. graminearum* in 2002), approximately 10 viruliferous aphids were placed in a small triangular paper bag, which was attached to the abaxial surface of the flag leaf. Simultaneously, the same number of virus-free aphids was transmitted to the control wheat plants. After a feeding period of 7 days, the aphids were killed by spraying the plants with the insecticide Tamaron® (0.15%, a.i., methamidophos; Bayer AG). Seven days after transmission by virus vectors, the infection of the wheat plants by BYDV was determined with ELISA (enzyme-linked immunosorbent assay; Clark and Adams, 1977) using a small section of the tip of the flag leaf of each inoculated plant.

Inoculum production of Fusarium culmorum *and* F. graminearum*, inoculation of wheat heads and disease evaluation*

The fungal species *F. culmorum* (isolate Fc 46) and *F. graminearum* (isolate Fg 18.7) were grown on oat grain according to the procedure described by Wanyoike (2002). One litre bottles were each filled with 400 g presoaked oat grain (in tap water, 12 h), plugged with cotton wool and autoclaved three times (each 121 °C, 1.2 bar, 1 h). For inoculation of oat grain, conidia of *F. culmorum* or *F. graminearum* were produced on SNA-liquid medium (special low nutrient agar, Nirenberg, 1976) by stirring at 21 °C and, after an incubation period of 4 days conidia were allowed to settle, the supernatant was decanted and spores were suspended in sterilized tap water. The autoclaved oat grain was inoculated with 5 ml conidial suspension of *F. culmorum* or *F. graminearum* (each 1×10^5 conidia ml^{-1}). The flasks were incubated for 3 weeks at 21 °C and a 16/8 h day/night cycle. To obtain uniform colonization of the grain with the fungal species, bottles were shaken every other day. Conidial production of the fungal isolates was stimulated after spreading flat layers of the oat grain in plastic trays (40×60 cm) and exposing them to NUV-light (near ultraviolet light, Osram tubes, L36 W/73) for 3 weeks. During this incubation period, the oat grain layers were moistened and mixed every other day. Afterwards the oat grain with *Fusarium* growth was stored in plastic bags at 4 °C until used.

In order to prepare conidial suspensions for inoculation of the wheat plants, conidia of *F. culmorum* or *F. graminearum* were washed from the oat grain with tap water, and the suspensions were filtered through two layers of muslin. The resulting conidial concentration was counted using a hemacytometer and adjusted with tap water to 1×10^5 conidia ml^{-1}. At mid-flowering (GS 65), 10 μl of conidial suspension of *F. culmorum* in 2001 and *F. graminearum* in 2002 and 2003 were pipetted into the cavity between the lemma and palea of a floret in the middle of a spike. The heads were covered with a plastic bag tied to the stalk to maintain high relative humidity for 24 h.

Fusarium head blight (FHB) was evaluated 7, 14, 21 and 28 days after inoculation (dai). Disease severity of each spikelet of the spike was determined using the following scheme:

0 = all three florets per spikelet healthy, 1 = one of three florets per spikelet showing discolouration, 2 = two of three florets per spikelet showing discolouration, 3 = all three florets per spikelet showing discolouration.

$$\text{Disease index} = \frac{\sum_{i=1}^{n} X}{nY \times 3} \times 100$$

X = sum of florets showing disease symptoms/spike
Y = average number of spikelets/spike (average number of spikelets per spike of cv. Agent = 21; average number of spikelets per spike of cv. Petrus = 24)
n = total number of spikes evaluated/pot.

Determination of thousand grain weight (TGW)

After harvesting, the wheat spikes were threshed per hand. All the grain was counted and TGW was calculated.

Measurement of ergosterol content in grain

Ergosterol contents were determined using the method described by Schwadorf and Müller (1989). From the harvested and ground wheat grain, 5 g were taken and homogenized with 120 ml methanol, 50 ml ethanol and 5 g potassium hydroxide in a 250 ml round-bottomed flask. The mixture was saponified at 80 °C for 30 min. After cooling to room temperature, the saponified mixtures were filtered, the samples were transferred into 500 ml separating funnels, and 100 ml water and 80 ml petrolether were added. After shaking and partitioning of the layers, the petrolether fraction was collected and dried by passing through anhydrous sodium sulphate. The aqueous phase was again partitioned in 50 ml petrolether and the petrolether fraction was collected and dried in anhydrous sodium sulphate. The combined petrolether fraction was reduced to dryness by evaporation. The residue was dissolved in acetonitrile/methanol mixture (7:3 v:v) and centrifuged. The supernatant was used for analysis. Ergosterol was measured by high performance liquid chromatography (HPLC). Extraction of grain samples with known amounts indicated that extraction efficiencies were within the range of 87–92%.

Ergosterol contents were determined with a computer-controlled HPLC system, equipped with an L-6200 gradient pump (Merk-Hitachi, Japan), an autosampler (AS-4000A, Merck-Hitachi, Japan) and a photodiode array detector (Waters 991, Germany), which was set at 282 nm to identify ergosterol. The HPLC-column consisted of a LUNA RP-18 (Phenomenex, Germany), 250 mm × 4 mm i.d., particle size 5 μm, and 30 μl of each sample extract were analysed.

All solvents were of analytical grade and degassed prior to use. For separation, a flow rate of 0.75 ml min^{-1} was used, resulting in back pressure of 8.8 MPa. The following eluents were used: A, 10 mM phosphate buffer (pH 2.4), in 10% acetonitrile/methanol (7:3, v:v); B, water/10% acetonitrile (Mallinkrodt-Baker; Griesheim, Germany). Isobaric conditions for separation of ergosterol were maintained for 20 min. The column was regularly flushed with both pure water and acetonitrile to remove impurities and prolong column life. Quantification was performed by the external standard mode. The ergosterol contents in the ground samples were identified by comparing the retention times and the absorption spectrum of the standard. HPLC analysis of ergosterol was performed at a temperature of 35 °C maintained by a Merck T-6300 column thermostat.

Analysis of trichothecene mycotoxins in grain

Trichothecenes in wheat grain were determined using gas chromatography/mass spectrometry (GC/MS) for verification analysis. The method described by Walker and Meier (1998) was used. Ground grain (5 g) was homogenized with 35 ml acetonitrile:water (84:16, v:v) using an ultraturrax (IKA T25, Janke & Kunkel; Staufen, Germany) for 3 min. The mixture was adjusted to 50 ml using acetonitrile:water (84:16, v:v) and filtered, and 6 ml of extract were passed through a Mycosep column (No. 227 Coring Systems; Gernsheim, Germany). One ml of the purified extract was transferred to a graduated test tube for cleaning. Saturated sodium chloride (0.5 ml) and 3 ml ethyl acetate solution were added and mixed for 1 min

using a Vibrax shaker (IKA-Vibrax-VXR, Janke & Kunkel; Staufen, Germany) at 900 rpm. After settling for 2 min, the mixture formed two layers. The top organic layer was removed and collected. The lower aqueous phase was extracted again using 2 ml ethyl acetate. Then the aqueous phase was discarded. The combined organic fraction was dried by passing it through anhydrous sodium sulphate and was evaporated to dryness under a gentle stream of nitrogen at 50 °C using a temperature controlled evaporation system (Barkey, Germany). The residue of the sample extract was redissolved in 1 ml of 4-(dimethylamino)-pyridine (DMAP)/toluene mixture (DMAP, 2 mg ml^{-1} in toluene) and 50 μl heptafluorobutyric anhydride and derivatized in a 60 °C water bath for 20 min. After cooling to room temperature, 1 ml aqueous sodium hydrogen carbonate solution (0.03 g ml^{-1} in water) was added, the mixture shaken for 10 s on a Vibrax shaker and layers allowed to separate for 2 min. The lower phase was removed and the top organic layer washed with 1 ml water. The top (toluene) layer was transferred to GC vials and analysed by GC/MS. For identification and quantification purposes a mixed standard containing nivalenol (NIV), deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-A-DON) and 15-acetyl-deoxynivalenol (15-A-DON) (Sigma-Aldrich) was prepared. DON concentration was adjusted to 20 $\mu g\ l^{-1}$ with isooctane while the others were adjusted to 30 $\mu g\ l^{-1}$. Quantification was done by external standard mode. The limit for quantification of all of the trichothecene derivatives was 10 $\mu g\ kg^{-1}$ flour.

Gas chromatography (GC)/electron impact (EI)-mass spectra were recorded by a Varian® 3800 Gas Chromatograph, 8200 CX autosampler, coupled to an ion trap mass spectrometer operating in EI mode (Varian®, Saturn 2000). The trichothecene-containing sample (1 μl) in isooctane was injected at a continuous flow of 1.4 ml min^{-1} on a Varian-Chrompack SIL−24 CP MS column (30 m × 0.25 mm i.d.). Column temperature ranged from 80 to 290 °C (temperature ramp 10 °C min^{-1}), while the injector and detector (ion trap) temperature were 250 and 225 °C, respectively. Full-scan EI-mass spectra (m/z: 100–600) were recorded for peak identification. The retention times (min) of the trichothecenes were as follows: NIV, 10.84; DON, 12.52; 3-A-DON, 15.87; 15-A-DON, 15.47.

Statistical analysis

Tukey's studentized range test was used to compare means and test for normality using SAS. Data were then evaluated by analysis of variance and correlation analysis. The data of disease index were angularly transformed before analysis.

Results

Relative humidity and temperature

In 2001, 2002 and 2003, the time periods from inoculation with *Fusarium* spp. (GS 65, mid-flowering) until the last evaluation of Fusarium head blight (FHB) of both cultivars were 28, 28 and 21 days, respectively. The average temperatures during these time periods in 2001, 2002 and 2003 were 17.2, 19.2 and 21.8 °C (Figure 1). The number of days with temperatures above 20 °C was 6, 13 and 18 days, respectively. The average relative humidities during these experiment periods were 71.4, 69.7 and 68.0%, respectively.

The grain filling periods from GS 71 (grain watery ripe) to GS 87 (hard dough) in the years of the experiments were 28, 28 and 21 days, respectively, with average temperatures during these periods of 18.3, 19.2 and 21.3 °C, respectively. The average relative humidities in these time periods were 65.8, 71.9 and 67.9%, respectively, and the average hours of sunshine per day during these periods were 9.3, 8.8 and 8.3 h, respectively.

Fusarium head blight development

The two winter wheat cultivars Agent and Petrus differed clearly in the development of FHB after single-floret inoculations with *Fusarium*. In 2001, at the first evaluation time (7 dai), the disease index of the susceptible cv. Agent inoculated with *F. culmorum* still exhibited a low value (Figure 2a). With increasing incubation time head blight disease spread continuously in the spikes and reached at the last evaluation time, 28 dai, a disease index of 32%. A similar course of head blight was found in plants that had been previously infested with *R. padi* free of BYDV. In wheat plants infected with BYDV, FHB values were slightly, but significantly, higher at the last disease assessment compared to the values of the other two treatments F (only

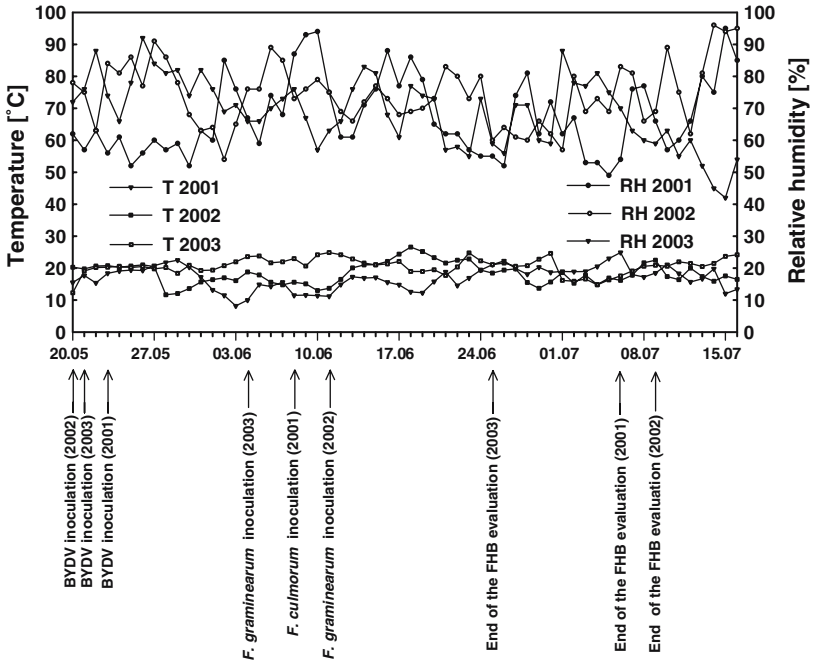

Figure 1. Average temperature [T] and relative humidity [RH] during the time period from BYDV inoculation until growth stage GS 87 (hard dough; grain content solid) of the three experiment years 2001–2003.

Fusarium) and AF (virus-free aphids plus *Fusarium*). In cv. Petrus development of FHB was markedly suppressed (Figure 2b); disease index in all three treatments was lower than 7%; however, the FHB disease of treatment VF was significantly higher than those of AF and F treatments.

In 2002, 28 dai a higher disease index of 68% in plants of cv. Agent infected with BYDV was determined, while in plants exclusively inoculated with *F. graminearum* and in plants pre-infested with virus-free *R. padi*, head blight developed much more slowly and values of the disease index reached 25 and 23%, respectively, which differed significantly from treatment VF (Figure 2c). There was less spreading of FHB in cv. Petrus in the treatments F (infected with *F. graminearum*) and AF (pre-infested with virus-free *R. padi* and infected with *F. graminearum*) (Figure 2d) compared to cv. Agent. In BYDV-infected plants disease expression of *F. graminearum* in spikes increased after the third disease assessment and the disease index reached 27% 28 dai; it was significantly higher than those of treatments F and AF.

In 2003, head blight severity rapidly progressed in spikes of cv. Agent and 3 weeks after inoculation a disease index of 59% was obtained exclusively in *F. graminearum*-inoculated plants (Figure 2e). Plants pre-infested with *R. padi* showed a similar course of FHB severity in the heads as plants exclusively inoculated with *F. graminearum*. On the other hand, wheat plants infected with BYDV responded as more susceptible to FHB infection. At all evaluation times there was more disease development than in the years before, resulting in a significantly higher disease index (81%) compared to treatments F and AF 3 weeks after inoculation. In cv. Petrus, FHB spread was slow in treatments F and AF, but both disease values differed significantly. On the other hand, in BYDV-infected plants FHB increased (Figure 2f). Three weeks after inoculation a disease index of 47% was recorded in the VF treatment (inoculation with virus and *Fusarium*) which differed significantly from treatments F and AF. The spikes of treatments C (Control), V (only virus infected) and A (infested with aphids) but not inoculated with *Fusarium* species showed no FHB

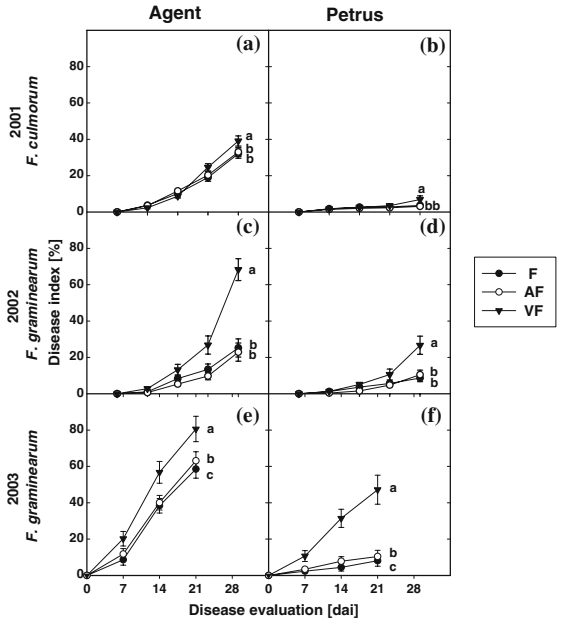

Figure 2. Disease index of head blight of the winter wheat cvs Agent and Petrus after single-floret inoculation at growth stage GS 65 with a conidial suspension of *Fusarium culmorum* in 2001 and with a conidial suspension of *F. graminearum* in 2002 and 2003. Treatments: F = *Fusarium* spp.-infected spikes, AF = *Rhopalosiphum padi*-infested plants plus *Fusarium* spp.-infected spikes, VF = BYDV-infected plants plus *Fusarium* spp.-infected spikes; dai = days after inoculation with *Fusarium* spp. Means designated with the same small letters are not significantly different according to Tukey's test, $P = 0.05$. Error bars are one standard error.

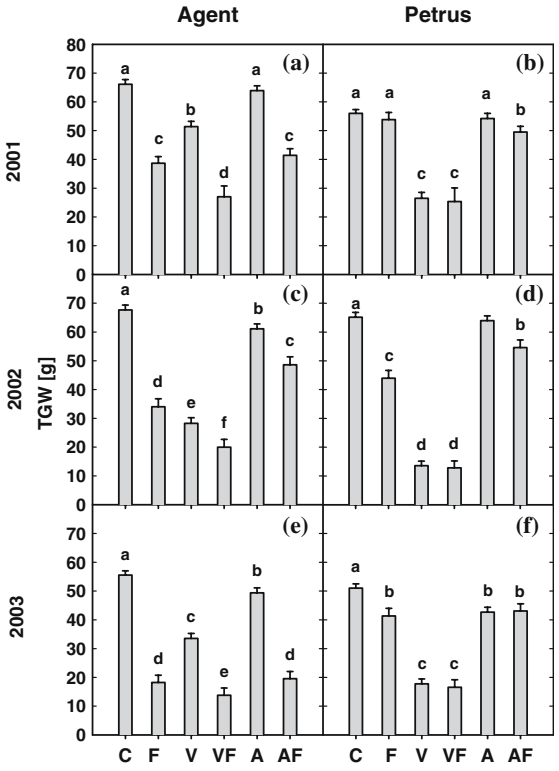

Figure 3. Thousand grain weights [TGWs] of the winter wheat cvs Agent and Petrus from different experimental treatments: C = control plants, F = *Fusarium culmorum*-infected spikes in the year 2001, *F. graminearum*-infected spikes in the years 2002 and 2003, V = BYDV-infected plants, VF = BYDV-infected plants plus *Fusarium* spp.-infected spikes, A = *Rhopalosiphum padi*-infested plants, AF = *R. padi*-infested plants plus *Fusarium* spp.-infected spikes. Means designated with the same small letters are not significantly different according to Tukey's test, $P = 0.05$. Error bars are one standard error.

symptoms during the three years of the experiment.

Thousand grain weight (TGW)

During the three years of the experimental period, thousand grain weights (TGWs) of both cultivars were evaluated (Figure 3). The TGWs of control treatments (C) of cv. Agent were: 66 g (2001), 68 g (2002) and 56 g (2003). For cv. Petrus the corresponding values were 56 g (2001), 65 g (2002) and 51 g (2003). In 2001, inoculation of cv. Agent with *F. culmorum* (treatment F) resulted in a significant reduction of TGW by 42% compared to the uninoculated control treatment (treatment C) (Figure 3a). Infection with BYDV (treatment V) significantly diminished TGW by 22%. When BYDV-infected plants were inoculated with *F. culmorum* (treatment VF), TGW was most markedly diminished by 59% and differed significantly from all other treatments. In plants pre-infested with aphids (treatment A) no effect on TGW was observed; however, when plants of this treatment were inoculated with *F. culmorum* (treatment AF) TGW was reduced (37%) to a similar extent as in treatment F. FHB severities were negatively associated with TGWs (Table 1). Infection of spikes of cv. Petrus with *F. culmorum* (treatment F) did not affect TGW compared to the control treatment. On the other hand, virus infection (treatment V) and double infection (treatment VF) significantly decreased TGWs by 53 and 55%, respectively, although values of the head blight index were very low (Figure 3b).

Table 1. Correlations between disease index and contents of ergosterol and total DON in grain, thousand grain weight (TGW), grain weight per ear and ear weight of cvs Agent and Petrus in the years 2001–2003

	TGW		Grain weight		Ear weight		Ergosterol		DON	
	Agent	Petrus	Agent	Petrus	Agent	Petrus	Agent	Petrus	Agent	Petrus
Disease index	-0.916^{***}	-0.762^{*}	-0.819^{**}	-0.797^{*}	-0.836^{**}	-0.804^{**}	0.766^{*}	0.733^{*}	0.695^{*}	ns
TGW			0.916^{***}	0.988^{***}	0.925^{***}	0.986^{***}	-0.830^{**}	ns	-0.794^{**}	-0.565^{*}
Grain weight					0.998^{***}	0.999^{***}				
Ergosterol									0.900^{***}	ns

ns = Not significant; ***, **, * Significant at 0.001, 0.01 and 0.05 probability levels, respectively.

In 2002, TGWs in cv. Agent were significantly reduced in the *F. graminearum*- (treatment F), BYDV- (treatment V) and BYDV plus *F. graminearum*- (treatment VF) infected plants by 50, 58 and 71%, respectively, compared to the uninfected control plants. While TGW reduction differed significantly between treatments F (50%) and AF (18%) (Figure 3c), FHB severity of both treatments was similar (Figure 2c). In 2002 in cv. Petrus *F. graminearum* infection significantly reduced TGW by 33% (Figure 3d). TGWs were most drastically decreased in treatment V (79%) as well as in treatment VF (80%) and these values differed significantly from all other treatments. In plants of treatment AF TGWs were reduced to a significantly lesser extent (16%) than in those exclusively fungus-infected (F; 33%).

In 2003, TGW of *F. graminearum*-treated plants (F) in cv. Agent (Figure 3e) was drastically reduced by 67% compared to the control. TGWs were diminished to similar extents in the treatments VF and AF by 75 and 65%, respectively. In BYDV-infected plants TGW was reduced by 40%. The values of all treatments differed significantly from each other. In cv. Petrus TGWs were most significantly reduced in virus- (treatment V; 65%) as well as in virus plus fungus- (treatment VF; 68%) infected plants. In the other treatments, TGWs were significantly decreased between 16% (treatments A and AF) and 19% (treatment F) compared to the control (Figure 3f).

Very high significant negative correlations were found between disease index and TGW in cv. Agent, while in cv. Petrus the correlation was only significant at $P = 0.05$ (Table 1).

Ear weight and grain weight per ear

Results of ear weights and grain weights per ear of the different treatments of both cultivars determined over the three years largely resembled the results on TGWs (Figure 3). Exceptions were the values of the treatments V (BYDV infection) and A (*R. padi*-pre-infested) of cv. Agent in the year 2001. Reductions in ear weights and in grain weights per ear were more pronounced than those of TGWs.

Very high positive correlations between TGW and both grain weight per ear and ear weight as well as between grain weight and ear weight in both cultivars were obtained (Table 1). There were highly significant negative correlations between FHB disease index and grain- and ear weights in both cultivars.

Ergosterol content in grain

Ergosterol contents in grain of the three *Fusarium*-treatments (F, VF and AF) of both cultivars determined over three years (Figure 4) largely reflected disease indices. In grain of the susceptible cv. Agent markedly higher contents of ergosterol were found in the different treatments compared to the values determined in the grain of cv. Petrus. In grain of cv. Agent infected with *F. culmorum* in 2001 and infected with *F. graminearum* in 2002 and 2003, 7.1, 5.3 and 11 μg ergosterol g^{-1} grain were obtained, respectively. On the other hand, in cv. Petrus, ergosterol contents in grain of the different treatments were below detection level in 2001 and in grain of *F. graminearum*-treatment (F) in 2002 and 2003, 1.9 and 2.2 μg g^{-1} grain were obtained, respectively.

Grain from plants of both cultivars infected with BYDV plus *Fusarium* spp. (treatment VF) contained significantly more ergosterol compared to grain of the treatment with only *Fusarium* spp. In cv. Agent, ergosterol contents in plants of treatment VF were 1.9, 2.4 and 1.8 times higher than in grain of treatment F in 2001, 2002 and

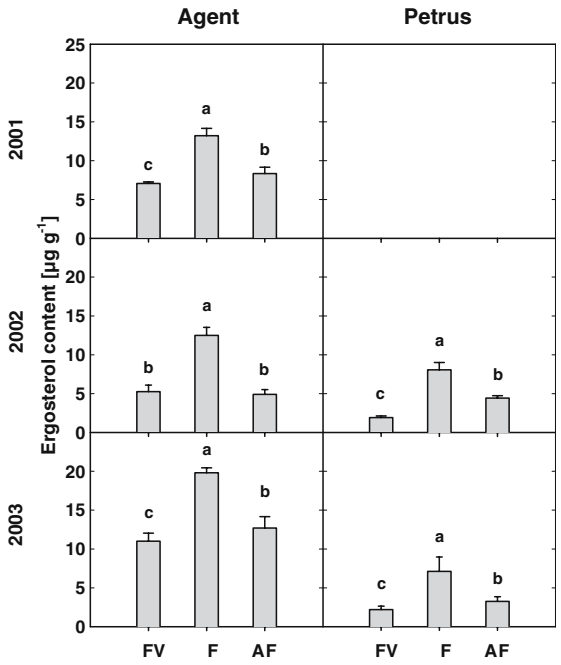

Figure 4. Ergosterol content [μg g^{-1}] in grain of the winter wheat cvs Agent and Petrus in different experimental treatments: F = *Fusarium culmorum* infection in the year 2001, *F. graminearum* infection in the years 2002 and 2003, AF = *Rhopalosiphum padi* infestation plus *Fusarium* spp. infection, VF = BYDV infection plus *Fusarium* spp. infection. Error bars are half standard error. Means designated with the same small letters are not significantly different according to Tukey's test, $P = 0.05$. Error bars are one standard error.

2003, respectively. Ergosterol contents in grain of treatment VF of cv. Petrus were 4.2 and 3.2 times higher than in grain of treatment F in 2002 and 2003, respectively.

In general, grain from plants pre-infested with *R. padi* (treatment AF) contained significantly higher ergosterol values compared with grain of the treatment only with *Fusarium* spp. There were significant positive correlations between disease index and ergosterol contents in the grain of both cultivars. Negative correlations between TGWs and ergosterol contents were determined in cv. Agent while in cv. Petrus no significant relationship was found (Table 1).

Deoxynivalenol (DON) contents in grain

DON contents of the grain of the susceptible cv. Agent of the three *Fusarium*-infected treatments single *Fusarium*-infected (F), BYDV-pre-infected (VF) and aphid-pre-infested plants (AF) were 11–25 times more than DON contents in grain of the treatments of the less susceptible cv. Petrus in the three years (Figure 5). DON contents of the treatment VF were significantly higher than those of the treatments F and AF for both cultivars in all three years.

In 2001, DON contents in grain of cv. Agent in treatments F, VF and AF were 10.3, 44.8 and 25.2 μg g^{-1}, respectively. Grain of cv. Petrus contained markedly lower amounts of DON: 0.6 μg g^{-1} (treatment F), 3.1 μg g^{-1} (treatment VF) and 1.3 μg g^{-1} (treatment AF), respectively.

In 2002, DON contents in grain were generally lower than those determined in 2001 for both cultivars. In cv. Agent, DON contents in grain of the treatments F, VF and AF were 8.9, 10.7 and 3.0 μg g^{-1}, and in the corresponding treatments of

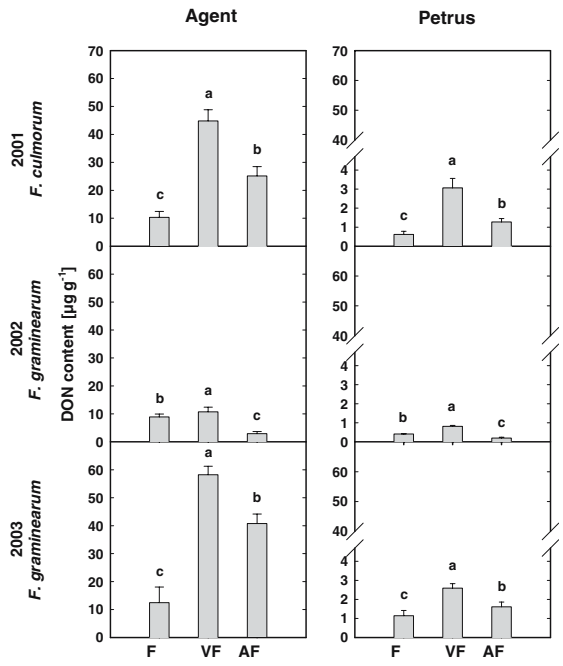

Figure 5. Deoxynivalenol (DON) content [μg g^{-1} ground grain] of the winter wheat cultivars Agent and Petrus in different experimental treatments: F = *Fusarium culmorum* infection in 2001, *F. graminearum* infection in 2002 and 2003, AF = *Rhopalosiphum padi* infestation plus *Fusarium* spp. infection, and VF = BYDV infection plus *Fusarium* spp. infection. Means designated with the same small letters are not significantly different according to Tukey's test, $P = 0.05$. Error bars are one standard error.

cv. Petrus the contents were 0.4, 0.8 and 0.2 $\mu g\ g^{-1}$, respectively.

DON levels determined in grain of the different treatments in 2003 corresponded with those analysed in 2001. DON contents in the grain of treatments F, VF and AF in cv. Agent were 12.5, 58.3 and 40.8 $\mu g\ g^{-1}$ and those in cv. Petrus were 1.1, 2.6 and 1.6 $\mu g\ g^{-1}$, respectively.

Discussion

During the three experimental years (2001–2003), development of FHB differed markedly reflecting the different degrees of resistance of the wheat cvs Agent (susceptible) and Petrus (moderately resistant). Different environmental conditions, especially average temperatures, affected FHB development. The average temperatures from inoculation of the wheat cultivars with *Fusarium* spp. until the last evaluation time of FHB were respectively in 2001, 2002 and 2003, 17.2, 19.2 and 21.8 °C respectively. The relatively low average temperature (17.2 °C) in 2001 was more favourable to development of *F. culmorum* (Parry et al., 1995; Lacey et al., 1999) and 28 dai a low disease index (32%) developed.

In 2002, FHB, caused by *F. graminearum*, reached a relative low disease index of 25% in cv. Agent 28 dai. This relatively low disease value may result from the higher temperature requirement for infection and disease development of *F. graminearum* compared to *F. culmorum* (Parry et al., 1995; Lacey et al., 1999). The higher average temperature (21.8 °C) in 2003 most likely stimulated disease development of FHB caused by *F. graminearum*.

Factors involved in the restricted FHB development of the moderately resistant cv. Petrus compared to the susceptible cv. Agent are still not fully elucidated. Ultrastructural studies revealed that, following single-floret inoculation, spread of *F. culmorum* and *F. graminearum* during early stages of spikelet infection and colonization of the rachilla and rachis was markedly retarded in resistant cvs Arina and Frontana compared to the susceptible cv. Agent (Kang and Buchenauer, 2000; Wanyoike et al., 2002). Plant structural defense reactions, such as formation of thick layered appositions and large papillae, were essentially more pronounced in the infected host tissues of the moderately resistant cvs Arina and Petrus than in cv. Agent (Kang and Buchenauer, 2000; Liu, 2004). While lignin content in cell walls of the infected tissues of the susceptible cv. Agent only slightly increased, lignin accumulated intensely in host cell walls of infected wheat spikes of the moderately resistant cv. Arina (Kang and Buchenauer, 2000). Immunocytochemical localization of β−1,3-glucanase and chitinase demonstrated distinct accumulation of both defense-related enzymes in *F. culmorum*-infected wheat spikes of the moderately resistant cvs Arina and Petrus, whereas in the susceptible cv. Agent both enzymes hardly increased (Kang and Buchenauer, 2002; Liu, 2004).

In *Fusarium*-infected plants of cv. Agent, grain yields (TGW, ear and grain weight per ear) decreased more in the three experimental years than in cv. Petrus. This effect might be due to the reduced spread of *Fusarium* species from floret to floret and by diminished colonization by mycelium in the rachis of cv. Petrus compared to cv. Agent. While translocation of photosynthates into the middle and upper part of the spike was markedly impaired in cv. Agent, high proportions of assimilates were translocated into the middle and upper spike sections of cv. Petrus (Liu, 2004).

In 2001 wheat plants infected with BYDV showed only slightly increased FHB compared to exclusively *F. culmorum*-infected plants of both cultivars. On the other hand, BYDV infection markedly increased FHB development in 2002 and 2003 compared to plants infected only by *F. graminearum*. The small promoting effect of BYDV infection on FHB development in 2001 may be due to the relatively low average temperature (17 °C) from BYDV inoculation until the last evaluation time of FHB, which might have retarded virus disease expression. Duffus (1963) reported that the average latent period of BYDV was halved for each 10 °C rise in the 5–25 °C range. On the other hand, BYDV-infected plants developed markedly more extensive FHB than plants without BYDV in 2002. This effect was more clearly expressed in cv. Agent than in cv. Petrus. The predisposing effect of BYDV infection to FHB may have been caused by inoculation of wheat plants at an earlier growth stage and by a higher average temperature during the experiment period compared to 2001. The time period between BYDV and *Fusarium* inoculation in 2002 was 22 days and thus 6 and 8 days longer than in 2001

and 2003, respectively. The earlier plants are infected by BYDV, the more severe does BYDV affect wheat plants (Mathre, 1997).

The induced susceptibility of BYDV-infected wheat plants to FHB might be caused by profound morphological and physiological alterations. Microscopical studies revealed phloem degeneration in leaves of BYDV-infected host plants very early after virus infection (Esau, 1957). Impairment of phloem functions may result in reduced translocation, leading to accumulation of photosynthates in leaves. Furthermore, chlorophyll contents are reduced which results in decreased photosynthetic rates (Jensen and van Sambeek, 1972). Our results revealed that BYDV infection markedly diminished total pigment contents and photosynthetic fixation in flag leaves and export of photosynthates from flag leaves to plant heads of both cultivars Agent and Petrus during the experimental period (Liu, 2004). Ultrastructural studies showed that BYDV infections caused degenerative changes in the fine structure of chloroplasts in the infected cells of flag leaves of both cultivars Agent and Petrus (Liu, 2004). In addition, chloroplasts of virus-infected tissue contained large starch grains; the accumulation of starch grains may be regarded as consequence of inhibition of export of carbohydrates produced in leaves.

Reduced chlorophyll contents, photosynthesis and translocation of photosynthates due to BYDV infection might result in more diminished grain yields (TGW, ear and grain weight per ear). It was observed during the three years of the experiment that the upper part of the ears of BYDV-infected plants was thinner and contained very small and shrivelled or no grain. In general, BYDV infection affected TGW in cv. Agent more than in cv. Petrus. The more severely reduced TGWs in BYDV-infected plants in both cultivars in 2002 compared to 2001 and 2003 might be the result of the earlier virus infection relative to *Fusarium* inoculation. Grain yield losses caused by BYDV are greater in plants that become infected earlier in their growth stage (Pike, 1990).

When virus-infected plants of cv. Agent were inoculated with *Fusarium* spp., TGWs were more effectively reduced than in virus-free plants. However, in cv. Petrus, TGWs of double infected plants were reduced to comparable degrees as in plants infected exclusively with virus. The results suggest that BYDV infections affect grain yield of wheat cultivars to different extents and wheat cultivars may react differently with interactions between BYD-disease and FHB. Koch and Huth (1997) reported that double infections of winter wheat cultivars Orestis and Kraka with BYDV and *F. culmorum* had significantly greater effects on the yield of wheat plants than single infections.

Ergosterol is an essential component of membranes of fungal species, involved in maintaining structure and function, and it may be regarded as a constituent for determining fungal growth in host tissues (Müller and Schwadorf, 1988). Results obtained in this study revealed that ergosterol contents in the grain of the different treatments of both cultivars Agent and Petrus largely resembled the disease indices determined at the last evaluation. Grain of the susceptible cv. Agent exhibited markedly higher ergosterol content compared to grain of the corresponding treatments of cv. Petrus over the three years. There were significant positive correlations between the FHB-disease index and ergosterol content in both cultivars over the three years of the experiment. Other studies (Bechtel et al., 1985; Miller and Young, 1985) also confirmed associations between FHB severities and ergosterol content.

In our pot experiments of 2001, 2002 and 2003, DON content in grain of *F. culmorum* or *F. graminearum*-infected plants (F, AF and VF) were 11–25 times higher in the susceptible cv. Agent than in the resistant cv. Petrus. Siranidou (2000) also reported marked differences in DON content in grain of cvs Agent (2029 μg kg^{-1}) and Petrus (241 μg kg^{-1}) inoculated with *F. culmorum* strain 46.

In grain of BYDV-infected plants, DON content was markedly higher than in grain of exclusively *Fusarium*-infected heads of both cultivars in 2001 and 2003. The differences in DON content between treatments F and VF were especially pronounced in cv. Agent (treatment F 10.3 μg g^{-1} grain, treatment VF 44.8 μg g^{-1} grain) in 2001. These results are surprising, since FHB severities in both treatments differed only slightly. The data suggest that a rather low degree of BYDV infection had significantly stimulated toxin production in the grain of cv. Agent.

Detection of β−1,3-glucanases and chitinases by immunogold labelling revealed no increase in activity in *F. graminearum*-infected lemma tissues of plants of cv. Agent not diseased or diseased by

BYDV. On the other hand, in cv. Petrus infection with *F. graminearum* induced 3 dai a markedly enhanced activity of both enzymes; the increase of both enzyme activities was less pronounced in virus diseased plants (Liu, 2004). The present studies indicate that BYDV infection significantly diminished resistance responses to FHB by interfering in the induction of both structural and chemical defense reactions. It may be assumed that in virus-infected plants further defense reactions to FHB are impaired.

In conclusion, BYDV infection predisposes wheat plants to an increased susceptibility to FHB and enhances DON content in grain compared to exclusively *Fusarium*-infected plants. These effects may be of practical significance.

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