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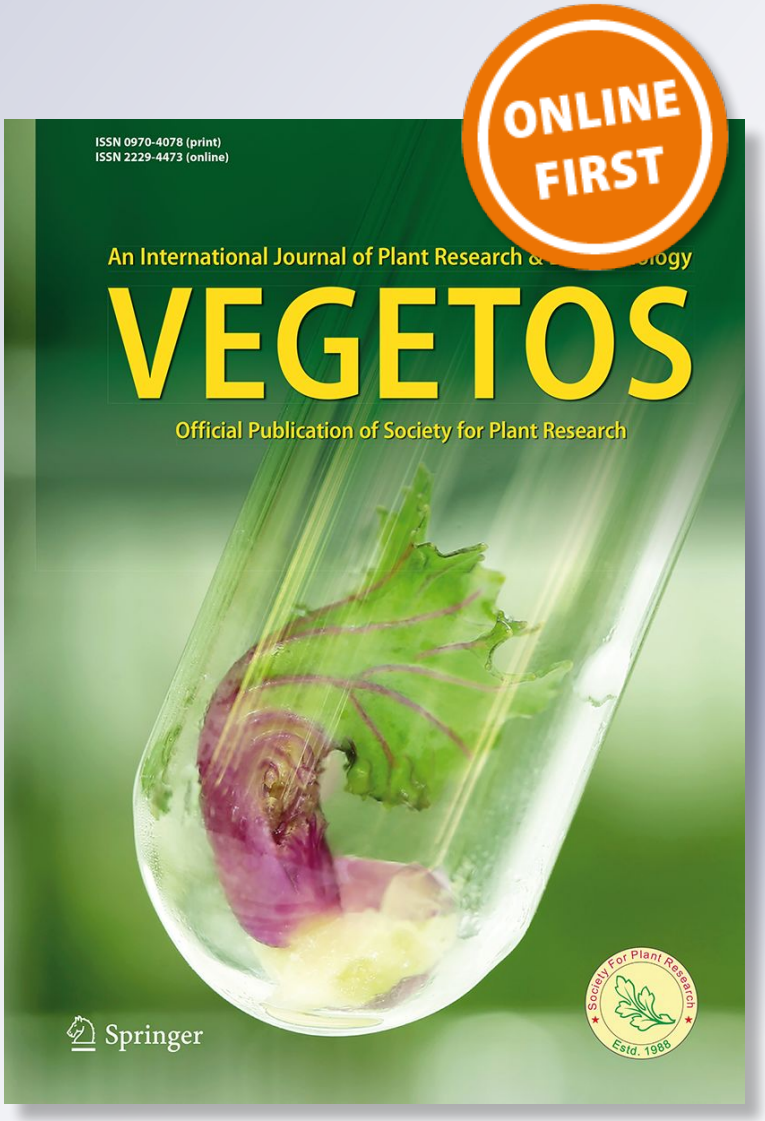

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REVIEW ARTICLES

Wheat grains damaged by *Fusarium graminearum*: alterations in yield, toxicity and protein composition

L. M. Ortega[1] · M. C. Moure[1] · A. L. Astoreca[1] · E. J. Alberione[2] · T. M. Alconada[1]

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Abstract
The aim of this study was to investigate alterations on wheat grains, in relation to yield and quality parameters, such as infection degree, protein composition and toxicity caused by isolates of *Fusarium graminearum* with different aggressiveness. The disease degree on wheat samples was evaluated by the parameters like incidence, severity, damaged kernels percentage, yield, deoxynivalenol content and protein constitution, which were analyzed employing accurate quantitative statistics. High and positive correlations among the disease degree parameters were found, as well as negative correlations between them and yield, meanwhile, the total protein content was correlated with yield but was not correlated with the disease degree. The data analysis showed that the effect of the isolate was more significant than the cultivar in the disease degree for almost all the studied variables. The variability in the content of the deoxynivalenol mycotoxin showed a positive tendency in relation to the infection degree. As a novel approach, the aggressiveness of the different isolates ranged from high to low allowing us to evaluate a wide range of the aftereffects of the disease.

Keywords Wheat · *Fusarium graminearum* isolates · Gluten proteins · Deoxynivalenol · Statistic principal component analysis

Introduction

Wheat (*Triticum aestivum* L.) is one of the three most widespread crops in the world, along with maize and rice, and the most extensively consumed by people, with a wide variety of food and industrial applications (FAO 2015). The crops are exposed to severe fungal diseases, which produce significant crop losses with great economic impact. *Fusarium* Head Blight (FHB) disease has caused severe epidemics worldwide, altering yield and quality parameters of grains, with the additional risk of contamination with mycotoxins. *Fusarium graminearum* is the main etiological agent in regions with warm and humid climate (Castañares et al. 2014). The aggressiveness of the pathogen on crops is mainly related to its ability to produce enzymes and mycotoxins (Pariaud et al. 2009). The damage caused by the disease is evaluated mainly by measurements of yield—weight loss in the grains per spike and the weight of a thousand grains—and variables resulting of visual damage—incidence and severity (Alberione et al. 2016). Damaged grains present changes in their chemical composition, altering their physical and chemical properties and consequently the quality of the resulting flour (Torbica et al. 2007; Horvat et al. 2014). The protein constitution of gluten—gliadins and glutenins—is directly related to the quality of flours. Moreover, glutenins are classified into high and low molecular weight proteins, called HMW-GS and LMW-GS respectively (Eggert et al. 2010). Deoxynivalenol (DON), the main mycotoxin related to the disease, which produces adverse effects on animal and human health, is considered as an indicator of the infection (Alvarez et al. 2010; Astoreca et al. 2017).

Our objective was to determine the effects of the infection on the wheat according to parameters indicative of the grains quality such as the protein content, presence of DON mycotoxin and yield. The obtained data was analyzed using suitable statistical tools, which allowed us to establish correlations and groupings among the variables.

✉ T. M. Alconada
alconada@biotec.quimica.unlp.edu.ar

[1] Centro de Investigación y Desarrollo en Fermentaciones Industriales, UNLP-CONICET, Facultad de Ciencias Exactas, La Plata, Argentina

[2] Patología Vegetal del Cultivo de Trigo, Instituto Nacional de Tecnología Agropecuaria, Marcos Juárez, Córdoba, Argentina

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Materials and methods

Biological materials

During the 2013–2014 growing season wheat samples were obtained from ten wheat cultivars independently infected with three *F. graminearum sensu stricto* isolates of different aggressiveness in the experimental station belonging to the National Agricultural Technology Institute (INTA) located in Marcos Juárez (32°42′ South, 62°06′ West) province of Córdoba, Argentina. From the employed wheat cultivars, eight belonged to the wheat breeding program of INTA: SRM Nogal, Sy 200 and BIOINTA 1005 (SN, S2 and BI1 susceptible cultivars); Buck Meteoro and AGP Fast (BM and AF moderately susceptible cultivars); Sy 100, BIOINTA 2005 and Klein Tigre (S1, BI2 and KT moderately resistant cultivars) and two were resistant international varieties used as controls: Sumai 3 and Soba Komugi (control cultivars). The *F. graminearum s.s.* isolates were previously characterized by their enzymatic activity (protease and polygalacturonase) and toxicogenic potential (deoxynivalenol mycotoxin) (Ortega et al. 2016).

The assays were carried out according to Alberione et al. (2016), consisting of 102 plants, distributed in 4 blocks of 25 plants each and 2 uninfected controls. Each block was designed with all the possible combinations between the three isolates and the eight cultivars, in addition to an internal control (a total of 25 plants per block). For inoculation, each hill plot was hand sprayed with a suspension adjusted to 3×10^5 conidia/mL. All the spikes of each hill plot were manually harvested and stored in envelopes of wood paper for later observation and evaluation. The threshing was done manually to avoid the use of automatic equipment, which generally uses air that carries the lighter diseased grains.

Disease evaluation

Infection parameters

From the harvested spikes the disease evaluation was estimated employing the parameters incidence, severity and damaged grains. Twenty spikes were randomly selected from each treatment for the evaluation of the incidence of disease (percentage of diseased spikes every 20). Then, six spikes were taken and the percentage of severity calculated as percentage of infected spikelets per spike and the damaged grains percentage (%GD) was measured as the percentage of damaged grains per spike among the total of diseased spikes selected. Finally, the yield was measured counting and weighing the grains of each wheat sample and extrapolating it to the thousand grain weight (TGW).

Analytical measures

Deoxynivalenol mycotoxin analysis

For analytical measures, grain samples were milled with an electric grinder obtaining the corresponding flours. Five grams of each flour sample was placed in 50 mL centrifuge tube with 20 mL of distilled water, incubated with agitation for 30 min and centrifuged at 3000*g* for 10 min afterwards. Then, the samples were diluted taking 6 mL of the supernatant—in a new 50 mL centrifuge tube—and adding 15 mL of distilled water. For DON extraction the ISOLUTE MYCO 60 mg/3 mL column (Biotage, D'Amico Sistemas S.A., Argentine) were conditioned with 2 mL of acetonitrile and equilibrated with 2 mL of distilled water. Each pre-treated sample (3 mL) was loaded into the column at a maximum flow rate of 1 mL/min, then the column was washed with 3 mL of distilled water and the DON eluted with 10% (v/v) of acetonitrile in water (3 mL). Chromatographic analysis was performed in an ACQUITY UPLCTM system (Waters, Milford, MA, USA) equipped with a binary solvent delivery system, degasser, autosampler and column heater. Chromatographic separation was performed using an Acquity UPLC BEH C18 column (100 mm × 2.1 mm), with 1.7 m particle size, from Waters. MS/MS detection was performed using an Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK), equipped with an electrospray ionization interface (ESI) operating in positive ion mode. For instrumental control, data acquisition and processing, MassLynx and QuanLynx software version 4.1 (Waters) were used (Valle-Algarra et al. 2011). The calibration curve was performed by injecting dilutions of 5, 10, 20, 50 and 100 ng/mL of a standard solution of 1 mg/mL (Sigma Aldrich Co., St. Louis, MO, USA, purity > 99%). A correlation coefficient (r) of 0.969 was obtained for the range of the concentrations used. The limits of detection and quantification were 0.3 and 1.0 ng/mL respectively.

Protein analysis

Determination of total proteins

The total protein content in flours was estimated with the Kjeldahl method according to the standardized international protocol (ISO 2013).

UV-spectrophotometry analysis of gluten proteins

The extraction of the gluten's proteins from flours for the UV spectrophotometry was carried out according to the diagram of (Hernández Espinosa et al. 2013). The extractions were performed in duplicate as follows: 10 µg of wheat

flour was weighed into 2 mL plastic microtube. The soluble monomeric fraction (SMF), rich in gliadins, was extracted with 1.8 mL of NaI solution 2.3% w/v and 3.75% v/v 2-propanol. The mixture was centrifuged at 16,500×*g* for 5 min at 25 °C and the supernatant was collected. To obtain the soluble polymeric fraction (SPF), 1.8 mL of 2% w/v of sodium dodecyl sulfate (SDS), 0.75% w/v Tris and 40% v/v 2-propanol were added to the previous precipitate and centrifuged at 16,500×*g* for 5 min at 25 °C, separating the supernatant. Finally, the insoluble polymer fraction (IPF) was obtained from the precipitate by the addition of 1.8 mL of 2% w/v dithiothreitol (DTT), 50% v/v 2-propanol and incubated for 75 min at 55 °C. The readings were performed at 280 nm using a T60 UV–visible spectrophotometer (PG instruments). The results were expressed, for each cultivar, as the percentage of each fraction in relation to the total.

Statistical analysis

All data were analyzed using the STATISTIC7 program. Analysis of variance (ANOVA), Fisher's test or LSD (low significant differences), Pearson's correlation test, and principal component analysis (PCA) were performed to observe the relationship among the variables studied (Horvat et al. 2014).

Results and discussion

Infection effect on spikes and grains

As a result of the artificial infection of different wheat cultivars with *F. graminearum* isolates of diverse aggressiveness, a varied degree of infection was observed (Fig. 1).

The ANOVA test showed a significant effect of the isolate's genotype on all the variables studied ($p < 0.05$). However, the influence of the cultivar's genotype was observed only for the variables %GD and TGW ($p < 0.05$). Pearson's test showed significant correlations between the measured variables, both positive—between incidence, severity, %GD—and negative—between those parameters and yield (Table 1A). Lastly, LSD test was employed to determinate

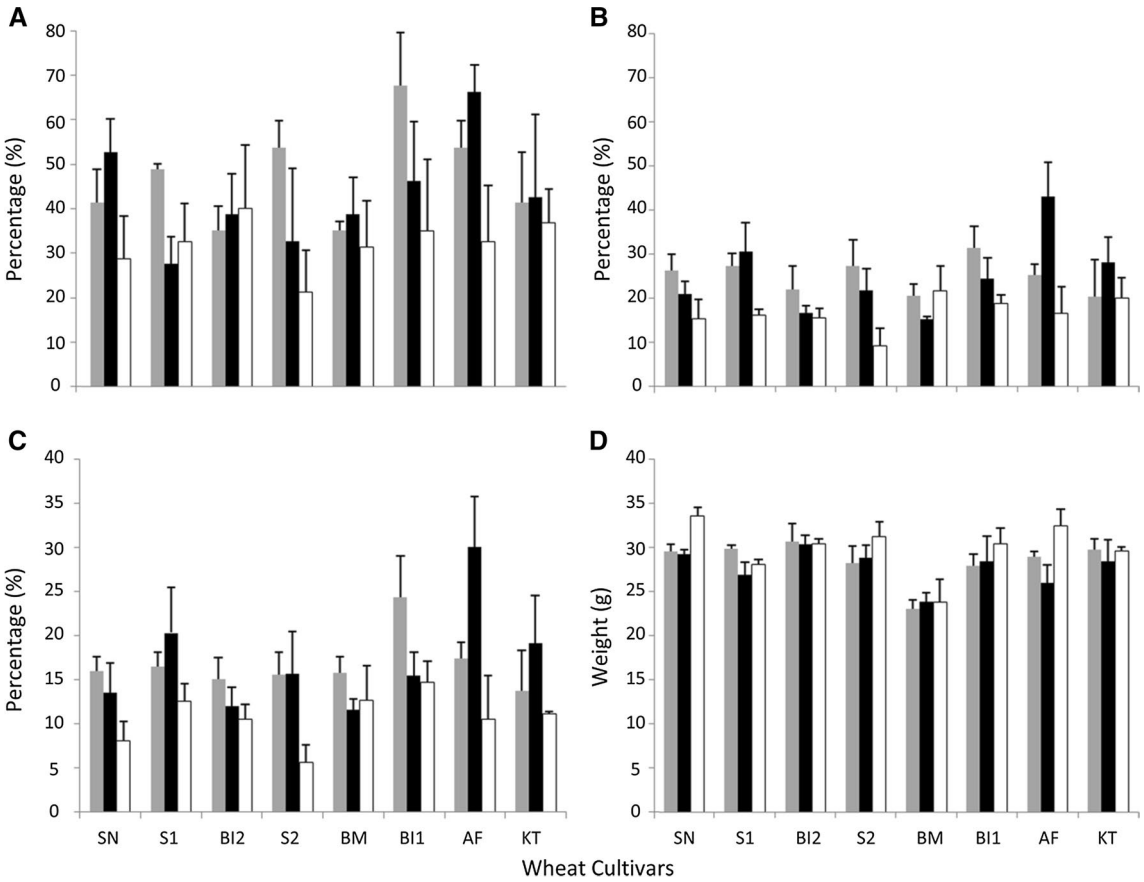

Fig. 1 Evaluation of the infection degree on wheat grains inoculated with different *Fusarium graminearum* isolates. **a** Incidence; **b** severity; **c** %GD and **d** TGW. Isolate 1 (white bars); Isolate 2 (grey bars); Isolate 3 (black bars). The values represent the average of four independents samples ± SE

Table 1 Pearson's correlation between infection variables and protein values (A). Assignment of homogeneous groups by Fisher test (LSD) for *Fusarium graminearum* isolates (B)

Variable	Total protein	Incidence	Severity	%GD	TGW
A					
Total protein	–	0.00	−0.14	−0.10	−0.42*
Incidence	0.00	–	0.51*	0.47*	−0.25*
Severity	−0.14	0.51*	–	0.88*	−0.43*
%GD	−0.10	0.47*	0.88*	–	−0.54*
TGW	−0.42*	−0.25*	−0.43*	−0.54*	–
Isolate	$\bar{X}$	$\bar{X}$	$\bar{X}$	$\bar{X}$	$\bar{X}$
B					
1	11.96[a]	32.07[a]	16.78[a]	10.93[a]	29.71[b]
3	11.95[a]	43.12[b]	24.83[b]	16.81[b]	28.37[a,b]
2	11.88[a]	47.03[b]	24.83[b]	17.23[b]	27.65[a]

%GD percentage of damaged grains, *TGW* thousand grain weight

*Significant differences ($p < 0.05$), $\bar{X}$ mean values. Different letters mean significant differences among the treatments

the influence of each isolate on the variance of the parameters (Table 1B).

As it was observed in this work, high variability of the disease parameters on infected wheat plants was found in several studies worldwide, being the most reported ones, severity and TGW (Horvat et al. 2014; Alvarez et al. 2010; Capouchová et al. 2012). The significant values found in the Pearson's tests indicate that the evaluated parameters are closely related with each other. Similarly, Malbrán et al. (2012) observed a negative tendency among severity and TGW, meanwhile, Alvarez et al. (Horvat et al. 2014) reported a low correlation between these variables. As it is shown in the results, the isolate 1 (low aggressive) produced the lowest degree of disease and the highest yield on wheat.

Infection effect in flour

Total protein analysis

The average value of total protein content in ours samples estimated by Kjeldahl method, was in accordance with technical reports for the growing season 2013 in Argentina (Guarda et al. 2004). Significant differences ($p < 0.05$) through ANOVA were observed, attributable to the genotype of the cultivar, and no significant differences were found when considering the isolate's genotype in the infection which was similar to the reported by other authors (Horvat et al. 2014) (Table 1B). Table 1A shows Pearson's correlations between total protein content and the variables analyzed in the infected wheat plants. It should be noted that the ratio among the total protein content was inversely proportional to TGW (−0.42), in accordance with previous studies (Guarda et al. 2004). This behavior represents one of the main challenges of wheat breeding programs, which have as one of their principal objectives the detection of cultivars to optimize their yield and their protein concentration in the grains.

Changes in gluten composition

As it is shown in Table 2, the IPF fraction—rich in HMW-GS—was the most abundant fraction for all the treatments. The ANOVA test showed significant differences on the FPS fraction among treatments, influenced by the isolate employed; whereas, for the remaining fractions, only the cultivar used produced significant differences. From LSD test differences on the FPS fraction between isolate 1 and 2 were found. The values for the glutenin fraction observed in this research were similar to the results reported by DuPont et al. (DuPont et al. 2005), whereas Hernández Espinosa et al. (2013) found lower average values. In concordance with the results of the LSD test, the correlation between the protein content of the FPS fraction and the severity showed negative values, indicating an inversely proportional relation between these variables.

Deoxynivalenol mycotoxin

Deoxynivalenol mycotoxin was present in 32% of the samples analyzed, within a wide range of values, approximately half of which exceeded the limit of 2 µg established by the current grain trade legislation (Table 3) (FAO 2003).

Even if the mycotoxin content was dispersed, the data analysis showed a positive tendency between DON content and severity (data not shown), although this relationship is controversial according to the bibliography (Alvarez et al. 2010; Mesterházy 2002).

Table 2 Composition of gluten protein fractions in infected flours

Wheat flours	SMF		SPF		IPF	
	%	s	%	s	%	s
SN	28.05	0.55	8.71	1.00	63.24	0.82
KT	28.44	1.45	8.90	0.55	62.66	1.46
BI2	30.06	1.71	8.52	0.65	61.42	1.70
S2	28.58	1.17	8.38	0.42	63.05	1.56
AF	39.02	1.30	9.76	1.05	51.22	0.41
BM	36.17	1.17	10.43	1.19	53.41	2.24
BI1	36.16	0.17	10.48	1.61	53.35	1.60
S1	35.57	3.14	9.67	0.67	54.76	3.10

SMF soluble monomeric fraction, *SPF* soluble polymeric fraction, *IPF* insoluble polymeric fraction, *s* standard deviation

Table 3 Content of deoxynivalenol in infected flours

Wheat flour	*F. graminearum* isolate	Concentration range (µg/g)	Median (µg/g)[a]
SN	2	nd–13.18	5.48
	3	nd–8.26	0.47
	1	–	–
S1	2	–	–
	3	nd–1.66	0.00
	1	nd–18.17	0.00
BI2	2	nd–5.33	2.22
	3	nd–5.84	0.00
	1	–	–
S2	2	nd-3.06	1.05
	3	–	–
	1	nd–0.71	0.00
BM	2	nd–0.02	0.00
	3	nd–6.71	0.00
	1	nd–4.22	0.00
BI1	2	nd–6.91	3.01
	3	nd–23.24	9.02
	1	nd–1.23	0.00
AF	2	–	–
	3	nd–18.56	3.80
	1	nd–1.25	0.00
KT	2	nd–48.55	0.74
	3	nd–0.29	0.00
	1	nd–16.05	0.00

[a]To calculate the median value, the undetected values (nd) were considered as 0

Principal components analysis

To evaluate the relationship among the parameters measured, the PCA statistical analysis was carried out, obtaining two main components that explained 81.29% of the total variance.

As shown in Fig. 2 the PCA analysis grouped the disease's parameters, leaving out the TGW and total protein value. Meanwhile, the mycotoxin content was positioned outside the cluster, but close to it. Therefore, this analysis allowed us to find grouping of the variables that correlated positively in the prior analysis mentioned above, leaving out those which correlated negatively with these variables (as TGW) or which showed no correlation (as total protein value). Furthermore, the near position of the mycotoxin content to this group could indicate a relationship between these variables. Similar results were found by Horvat et al. (2014) between DON and TGW and by Siuda et al. (2010) between protein and TGW, which may indicate that there is a tendency among the behavior of these variables. However, the results obtained by Horvart et al. (2014) by analyzing protein fractions showed that those were positioned close to TGW and opposite to DON, which may indicate that while the total protein content is not affected, the composition of each protein fraction in the infected flour may display a different behavior.

Conclusions

The data obtained was analyzed using suitable statistical tools, which allowed us to establish correlations and groupings among the variables. By ANOVA and LSD, the differential effect of *F. graminearum* isolates on wheat grains evidenced as alterations in yield, toxicity and protein composition. The data analysis showed interaction among the measured parameters—observed by significant Pearson's correlations and by grouping of the variables by PCA—which indicates that the evaluated parameters are closely related with each other. The near position of the mycotoxin content to this grouping could indicate a relationship between them. The IPF gluten fraction—rich in HMW-GS—proved to be the most abundant for all the treatments. These results tend to improve the comprehension of the damage

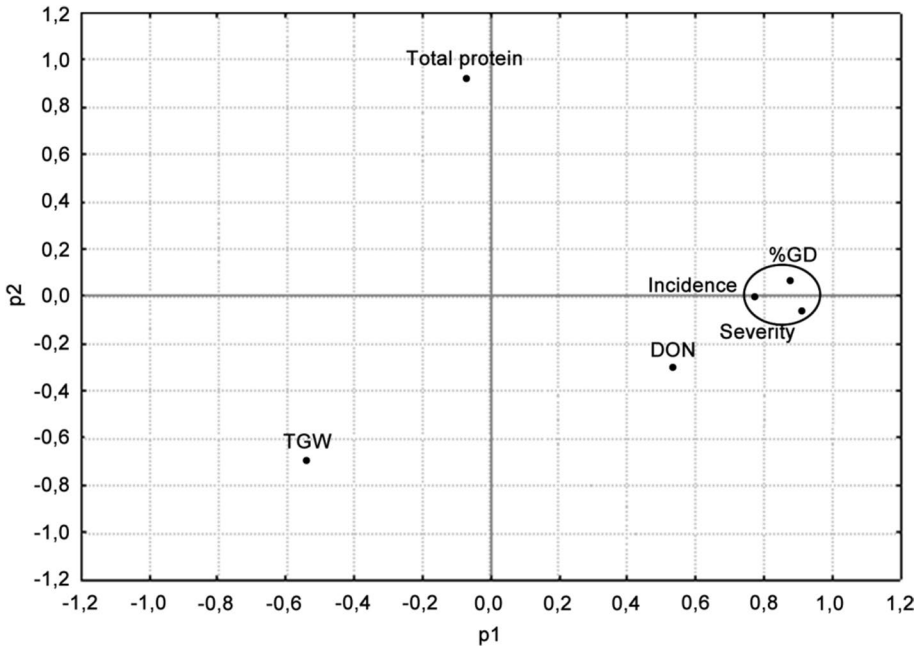

Fig. 2 PCA Score-plot of the disease evaluation parameters. *%DK* percentage of damaged kernel, *TGW* thousand grain weight; deoxynivalenol content and total protein content

caused by *F. graminearum* on grains and their changes in quality.

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