International Journal of Pest Management

ISSN: 0967-0874 (Print) 1366-5863 (Online) Journal homepage: http://www.tandfonline.com/loi/ttpm20

The effects of various plant protection methods on the development of Zymoseptoria tritici and Cephalosporium gramineum, grain yield and protein profile

Urszula Wachowska, Iwona Konopka, Adrian Duba, Klaudia Goriewa & Marian Wiwart

To cite this article: Urszula Wachowska, Iwona Konopka, Adrian Duba, Klaudia Goriewa & Marian Wiwart (2018): The effects of various plant protection methods on the development of Zymoseptoria tritici and Cephalosporium gramineum, grain yield and protein profile, International Journal of Pest Management

To link to this article: https://doi.org/10.1080/09670874.2018.1474282

Published online: 28 May 2018.

INTERNATIONAL JOURNAL OF PEST MANAGEMENT, 2018
https://doi.org/10.1080/09670874.2018.1474282

Taylor & Francis
Taylor & Francis Group

The effects of various plant protection methods on the development of *Zymoseptoria tritici* and *Cephalosporium gramineum,* grain yield and protein profile

Urszula Wachowska[a], Iwona Konopka[b], Adrian Duba[a], Klaudia Goriewa[c] and Marian Wiwart[c]

[a]Department of Entomology, Phytopathology and Molecular Diagnostics, University of Warmia and Mazury in Olsztyn, 10-721 Olsztyn, Poland; [b]Chair of Plant Food Chemistry and Processing of Faculty of Food Sciences, University of Warmia and Mazury in Olsztyn, 10-726 Olsztyn, Poland; [c]Department of Plant Breeding and Seed Production, University of Warmia and Mazury in Olsztyn, 10-724 Olsztyn, Poland

ABSTRACT

Septoria leaf blotch progresses rapidly, leading to the development of *Zymoseptoria titici* forms resistant to fungicides. Cephalosporium stripe is caused by *Cephalosporium gramineum*. The aim of this study was to evaluate the effectiveness of selected pesticides in limiting the symptoms of both diseases on winter wheat leaves, and to determine their influence on grain yield and the content and composition of protein fractions in wheat kernels. Propiconazoles were most effective in inhibiting the development of Septoria leaf blotch (symptoms were reduced from 54.7% to 78.6%). Strobilurins were less effective due to the presence of isolates with the G143A mutation. Symptoms of Cephalosporium stripe were rarely observed, and protective treatments did not reduce their severity. The highest content of grain protein (14.81%) was found in plants most intensely protected with the fungicides containing fenpropimorph, pyraclostrobin and epoxiconazole. The principal component analysis revealed that the plant protection method influenced the grain protein profile. The accumulation of HMW glutenins and α/β gliadins was mutually interrelated and higher in high-input treatments; control grain was characterized by close relationships between ω-gliadins, LMW glutenins, albumins and globulins, whereas low-input treatments influenced mostly γ-gliadins.

ARTICLE HISTORY
Received 30 November 2016
Accepted 3 May 2018

KEYWORDS
Fungicides; plant resistance inducer; plant biostimulator; G143A mutation; grain protein fractions

1. Introduction

The leaves of winter wheat are susceptible to colonization by *Zymoseptoria tritici* (Desm.) Quaedvlieg & Crous which causes Septoria leaf blotch (SLB), a serious disease responsible for food production losses around the globe (Quaedvlieg et al. 2011). SLB can decrease wheat yields by 30%–50%, and 70% of fungicide costs are dedicated to combating this disease (Leroux et al. 2007; O'Driscoll et al. 2014; Rodrigo et al. 2015). All winter wheat cultivars are susceptible to infections caused by the above pathogens, and disease can reach epidemic proportions during exceptionally wet periods (Eriksen and Munk 2003; Mirzwa-Mróz et al. 2005). Three types of fungicides are applied to protect winter wheat against SLB: quinone outside inhibitors (QoI), also known as strobilurins (Gisi et al. 2002), sterol demethylation inhibitors (DMIs), also known as azoles (Leroux et al. 2007) and succinate dehydrogenase inhibitor (SDHI) (Dooley et al. 2016). Type QoI fungicides were first used in 1996, but resistant forms of *Z. tritici* were noted already after the first few years of application (Mavroeidi and Shaw 2005; Leroux et al. 2007; Torriani et al. 2008; Curvers et al. 2015). Resistant isolates carried a mutation resulting in the replacement of glycine by alanine at codon 143 (Gisi et al. 2002; Fraaije et al. 2005; Curvers et al. 2015). The influence of plant resistance inducers, biostimulators and growth regulators on the development of *Z. tritici* has been insufficiently investigated, and these products are increasingly often used to control cereal growth.

Cephalosporium stripe (*Cephalosporium gramineum* Nisikado et Ikata) is a vascular wilt disease of cereals (Vazquez et al. 2015). Characteristic symptoms include dry yellow stripes that form along veins in leaf sheaves and leaves heavily colonized by *C. gramineum*. Severe infections lead to wilting and plant death, which lowers yield (Morton et al. 1980; Martyniuk et al. 1995; Mundt 2010). The main sources of the inoculum are infected plant residues, less frequently grain, where the pathogen can overwinter (Murray 2006). Short crop-rotation, cool and wet autumns and root damage caused by soil freezing create supportive conditions for Cephalosporium stripe (Quincke et al. 2014). The disease was first identified in Japan in 1931 (Nisikado et al. 1934), and later in North America (Bruehl 1956) and Europe (Richardson and Rennie 1970; Martyniuk et al. 1995). At present, there are no approved chemical treatments for controlling the disease (Martyniuk et al. 2006; http://www.minrol.gov.pl/eng/Ministry/Online-database-on-plant-protection-products).

CONTACT Marian Wiwart marian.wiwart@uwm.edu.pl

The aim of this study was to evaluate the effectiveness of selected plant protection products in limiting the symptoms of Septoria leaf blotch and Cephalosporium stripe on winter wheat leaves. The influence of the tested products on grain yield, and the content and composition of protein fractions in wheat kernels was also determined.

2. Materials and methods

2.1 Field experiment

A field-plot experiment with a randomized complete block design with four replications was carried out in 2010–2012. Winter wheat cv. Bogatka (Polish National List ... 2016) was sown in plots with an area of 25 m^2 and protected with the evaluated control agents twice (Fung1, Fung4) or three times (Fung2, Fung3, Biot) during the growing season in stages BBCH 31 (stem elongation stage) (Meier 2003), BBCH 55 (heading stage) and BBCH 71 (water ripe stage) (Table 1). Plants were sprayed with a backpack sprayer (Marolex Titan 12, Poland) in the afternoon, on windless and cloudy days, at a temperature of 15–25 °C. The fungicides, growth retardant and biostimulator were applied at dates and doses given in Table 1. Unprotected plots were the control. Winter wheat was fertilized with N/P/K at 120/26/60 kg per ha, respectively. Grain was harvested in the over-ripe stage (BBCH 92) with a plot harvester, and yield was expressed in terms of grams per m^2.

During the experiment, winters were mild, and temperatures did not drop below −9 °C in the coldest years of 2010 and 2012, which supported plant overwintering. In 2011, spring was warmer than in the remaining years, but summer months were somewhat cooler than in 2010 with highly abundant rainfall in July (202.8 mm) (Table 2). In 2010 and 2011, April was characterized by very low precipitation levels (18.2 mm and 22.5 mm, respectively), which adversely affected plant growth and development. Heavy rainfall was noted in June 2010 (131.9 mm).

Table 2. Rainfall and average temperatures in growing seasons of 2009 - 2012 in the study area.

Year	MAR	APR	MAY	JUN	JUL	AUG
	Temperature [°C]					
2009	1.3	9.4	12.4	14.9	20.4	17.6
2010	2.1	8.1	12.0	16.4	21.1	19.3
2011	1.6	9.1	13.1	17.1	17.9	17.6
2012	3.0	7.8	13.4	15.0	19.0	17.7
	Rainfall [mm]					
2009	59.0	4.8	52.9	137.0	48.3	19.3
2010	36.7	18.2	131.9	84.8	80.4	95.3
2011	16.3	22.5	51.1	81.7	202.8	82.1
2012	24.7	73.1	51.7	103.2	121.0	45.1

2.2 Severity of plant infections

The percentage area of leaves covered with spots and fruit bodies of *Z. tritici* (pycnidia and pseudothecia) was determined separately for penultimate leaves (PL) and flag leaves (FL) of 100 plants in each treatment (25 plants per plot). Penultimate leaves (PL) were evaluated in the watery ripe stage (BBCH 71), and flag leaves (FL) – in the early milk stage (BBCH 73). On the second evaluation date, the number of plants displaying symptoms of Cephalosporium stripe was counted in each plot. Plant health was evaluated based on the scale proposed by the European and Mediterranean Plant Protection Organization – EPPO PP 1/26 (https://bip.minrol.gov.pl/content/download/28570/156734/version/1/file/fung%20-%20choroby%20li%C5%9Bci%20zb%C3%B3%C5%BC.pdf). The results were expressed as the mean percentage leaf area displaying symptoms of *Z. tritici* infection and the average prevalence of plants showing symptoms of *C. graminеum* infection.

2.3 Isolation and identification of pathogens

Infected leaves were surface disinfected in 1% sodium hypochlorite (Abchem, Poland) and ground in a mortar for pathogen isolation. Spore suspensions were placed on sterile Petri plates with a diameter of 9 cm

Table 1. Pesticide treatments in winter wheat under experimental conditions.

Treatment	BBCH 31	BBCH 55	BBCH 71
Fung1 (low-input 1)	Alert 375 SC [1] (flusilazole – 125 g l^{-1}, carbendazim – 250 g l^{-1}) rate: 1l ha^{-1}	no treatment	Tarcza Łan 250 EW [2] (tebuconazole – 250 g l^{-1}) rate: 1 l ha^{-1}
Fung2 (high-input 1)	Corbel 750EC [3] (fenpropimorph – 750 g l^{-1}) rate: 1 l ha^{-1}	Opera Max 147,5SE [4] (pyraclostrobin – 85 g l^{-1}, epoxiconazole 62.5 g l^{-1}) rate: 2 l ha^{-1}	Tarcza Łan 250 EW (tebuconazole – 250 g l^{-1}) rate: 1 lha^{-1}
Fung3 (with growth regulator)	Moddus 250EC [5] (trinexapac-ethyl – 250 g l^{-1}) rate: 0.4 l ha^{-1}	Amistar 250 SC [6] (azoxystrobin – 250 g l^{-1}) rate: 1 l ha^{-1}	Tarcza Łan 250EW (tebuconazole – 250 g l^{-1}) rate: 1 l ha^{-1}
Biot (biotechnological)	Asahi SL [7] (*o*-nitrophenol – 2%, *p*-nitrophenol – 3%, 5-nitroguaiacol – 2%) rate: 0.6 l ha^{-1}	Biochicol 020 PC [8] (chitosan 20 g l^{-1}) rate: 2.5 l ha^{-1}	Biochicol 020 PC (chitosan 20 g l^{-1}) rate: 2.5 l ha^{-1}
Fung4 (low-input 2)	no treatment	Bumper 250 EC [9] (propiconazole – 25.1%) rate: 0.5 l ha^{-1}	Topsin M 500 SC [10] (thiophanate-methyl – 500 g l^{-1}) rate: 1.4 l ha^{-1}

[1] Du Pont International Operations Sarl, Switzerland, [2] Organika-Sarzyna SA, Poland, [3,4] BASF SE, Germany, [5] Syngenta, Switzerland, [6] Syngenta, United Kingdom, [7] Asahi Chem. MFG Co. Ltd., Japan, [8] Gumitex Poli-Farm Sp. z o.o., Poland, [9] Makhteshim Chemical Works Ltd, Israel, [10] Nippon Soda Co. Ltd., Japan.

and immersed in Martin agar (Martin 1950). Colonies characteristic of pathogens were isolated in pure culture and identified under a light microscope (Nikon 200 E, Japan). Seven isolates of *C. gramineum* (Cg2, Cg3, Cg4, Cg5, Cg6, Cg7, Cg8) and nine isolates of *Z. tritici* (Zt1, Zt2, Zt3, Zt4, Zt5, Zt6, Zt7, Zt8, Zt9) were isolated and analyzed. Isolate filaments were stored at −80 °C. DNA was isolated with the use of the DNA Genomic Mini AX kit (A&A Biotechnology, Poland). Fragments containing ITS 1, 5.8S and ITS 2 rDNA regions were amplified with species-specific primers ITS5 (F) GTATCGGACGGAGATCCAGC and ITS4 (R) TTGCTCAGTGCATTG TCGG (White et al. 1990) with the use of the FailSafe PCR system (Epicentre, Poland). The reaction was performed on 20 ng DNA in the Mastercycler Ep Gradient thermocycler (Eppendorf, USA). The reaction had the following thermal profile: 95 °C for 3 min, followed by 34 cycles of: 95 °C for 1 min, 58 °C for 1 min, 74 °C for 3 min and 74 °C for 10 min. Electrophoresis of amplicons with the size of 750 bp was carried out in 1.2% agarose gel (Prona, Poland) in TBE buffer (Sigma, Poland). Amplicons were sequenced at the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw. Sequence similarity was determined in the BLAST program of the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Detection of mutations conditioning resistance to azoxystrobin

Isolates of *Z. tritici* were cultured on PDA (Merck, Poland) with and without the addition of 0.1 μg azoxystrobin in 1cm^3 PDA. Suspensions of spores at a concentration of 10^4 ml^{-1} of water were cultured in the amount of 10 μl. Spontaneous point mutations in the cytochrome b gene, responsible for strobilurin resistance, were detected by the mismatch amplification mutation assay developed by Siah et al. (2010). Primers were designed with a mismatch on the penultimate nucleotide of the 3' end of the forward primer, where the ultimate nucleotide was at the point mutation position of codon 143 of the cytochrome b gene. Sensitive isolates were identified with the use of StroSNP2fwd (5′-CTTATGGTCAAATGTCTTTATGATG-3′) and StrobSNP1rws (5′-GGTGACTCAACGTGATAGC-3′) primers, and the resulting amplicon had the size of 639 bp. For resistant isolates, a fragment with the length of 302 bp was amplified with primers StrobSNPrcF7 (5′-CAATAAGTTAGTTATAACTGTTGCGG-3′) and StrobSNPrcR1 (5′-CTATGCATTATAACCCTAGCGT-3′). The reaction was carried out in samples of 50 μL with 50 ng DNA of selected isolates. The reaction had the following thermal profile: 94 °C for 10 min, followed by 40 cycles of: 94 °C for 1 min, 55 °C for 30 s, 60 °C for 30 s for primers StroSNP2fwd and StrobSNP1rws or 72 °C for 1 min for primers StrobSNPrcF7 and StrobSNPrcR1, and 72 °C for 10 min. The products were visualized by electrophoresis in 1.5% agarose gel (Prona, Poland) in TBE buffer (Sigma, Poland).

2.5 Biology and control of Cephalosporium gramineum by chemical methods

The pathogenicity of *C. gramineum* isolates was analyzed in Koch's test. *C. gramineum* spores were rinsed from 2-week PDA cultures, and 100 ml of the suspension at a concentration of 10^3 ml^{-1} was combined with soil and placed in pots with a diameter of 12 cm. Twenty kernels of winter wheat cv. Bogatka and spring variety Sumai 3 (reproduced in the Department of Plant Breeding and Seed Production of the University of Warmia and Mazury in Olsztyn) were sown per pot at a depth of 1 cm. Seedlings were grown in a controlled environment (19/23 °C, 8 h dark / 16 h light) and observed for 4 weeks. After 4 weeks, seedlings were measured and the severity of infection was graded on a 4-point scale (0 – healthy seedling, 1 – mild symptoms consisting of yellow stripes along leaf sheath veins, 2 – moderate symptoms, 3 – strong symptoms, 4 – wilting seedlings). Uninoculated seedlings were the control.

Isolates of the analyzed pathogens were also proliferated on Petri plates containing selected commercial fungicides (fenpropimorph at a concentration of 2.50 μl ml^{-1}, pyraclostrobin + epoxiconazole at 5.00 μl ml^{-1}), the biostimulator at a concentration of 1.20 μl ml^{-1} (*p*-nitrophenol, *o*-nitrophenol, 5-nitroguaiacol), the growth regulator at a concentration of 2.50 μl ml^{-1} (trinexapac-ethyl) or the plant resistance inducer (chitosan) at a concentration of 6.25 μl ml^{-1}. These concentrations corresponded to the doses of the preparations applied in the field. Agar discs overgrown by 7-day-old filaments of *C. graminearum* were placed on 9 cm Petri plates containing PDA (Merck, Poland) with the addition of the tested substances. Colonies were scanned after 4 days of incubation (scanner Epson Perfection, USA). Petri plates containing PDA without the tested substances were the control. The area colonized by the pathogen was determined after 4 days in the ImageJ program (Rasband 2016). The experiment was performed in three replications.

2.6 Determination of protein content and composition

Grain was dried to approx. 14% moisture, manually cleaned from broken kernels and ground in a type A10 IKA Labortechnic mill to obtain particles smaller than 300 μm for protein analysis. The content and composition of proteins were determined according to the method proposed by Konopka et al. (2007). Albumins, globulins, ω, α/β, γ gliadins and high-molecular-weight (HMW) and low-molecular-weight (LMW) glutenins

were extracted based on the procedure developed by Wieser and Kieffer (2001). The protein content of each extract was determined in the Bio-Rad Bradford Protein Assay using bovine serum albumin (Bio-Rad) for the quantification of albumins and globulins, and the gliadin standard (Sigma-Aldrich) was applied for the quantification of gluten proteins. Extracts were separated in the Agilent Technology 1050 series chromatography system (Palo Alto, CA, USA) using a RP-18 Vydac 218TP54 column with 5 μm bead size and 300 Å pore size, 250 × 4.6 mm; a Zorbax 300SB-C18 pre-column, 4.6 × 12.5 mm, with column temperature of 45 °C, mobile phase flow rate of 1 ml min^{-1}, and injection volume of 20 μl. A two-component gradient was used. Mobile phase component A: 75% in 0 min, 65% in 5 min, 50% in 10 min, 25% in 17 min, 15% in 18 min, and 75% in 19 min. The first component (A) was water with 0.1% TFA and the second (B) was ACN with 0.1% TFA. The absorbance spectra of eluted proteins were determined by a diode-array detector (HP 1050) at 210 nm. The integration procedure was performed using HPLC 3D ChemStation software. Peaks were classified as ω, α/β, γ gliadins and HMW and LMW glutenins according to the procedure proposed by Konopka et al. (2007). The retention times of peaks and the second derivative of their UV spectra, which represents differences in the tryptophan to tyrosine ratio between the main subunits, were used. The protein content of each fraction was expressed in g 100 g^{-1}.

2.7 Statistical analysis

The results were processed statistically using STATISTICA 12 software (Statsoft Inc. 2014). The significance of differences between means was estimated by analysis of variance, and mean values of average infection severity and yield were estimated by the Student–Newman–Keuls (SNK) test at $p < 0.01$ The effectiveness of the tested fungicides was calculated with the use of the formula (1):

$$^{*}\text{Effectiveness} = 100\% - \left(\frac{\text{T}}{\text{C}} \times 100\%\right) \qquad (1)$$

where

T - severity of symptoms on protected plants
C - severity of symptoms on unprotected plants.

The sequences of the ITS region in experimental and reference isolates were compared in the MEGA 6 program (http://en.bio-soft.net/tree/MEGA.html) to determine their phylogenetic affinity. The phylogenetic tree was developed with the use of the neighbor-joining method. Data were verified in the Jukes-Cantor model. The reliability of the phylogenetic tree was estimated by bootstrapping with 2000 replications. The results obtained for protein fractions were subjected to a principal component analysis (PCA).

3. Results

During the experiment, the predominant diseases affecting winter wheat plants were SLB and Cephalosporium stripe, whereas other diseases, such as powdery mildew and leaf rust, were sporadically observed. High precipitation levels in May 2010 contributed to the development of SLB, and symptoms of disease were observed on 33.3% of leaf area in PL and 14.3% of leaf area on FL on average (Tables 2–4). Propiconazole (Fung 4) used in BBCH 55 was most effective in protecting PL (symptoms were reduced by 54.7% on average in comparison with control) and FL (78.6%). The average effectiveness of the high-input treatment with intensive fungicide use (Fung2) was estimated at 38.3% for PL and 45.2% for FL. The remaining fungicides did not deliver satisfactory results, and chitosan failed in limiting the spread of SLB.

Molecular analyses confirmed the identification of the studied isolates to species *Z. tritici* and *C. gramineum*. The phylogenetic analyses revealed that the evaluated species formed separate clades (Figure 1). In the group of nine analyzed isolates of *Z. tritici*, seven isolates were capable of growth on media containing azoxystrobin. The presence of the G143A mutation in the *CYTb* gene was confirmed in their genotypes (Figure 2).

In 2012, symptoms of Cephalosporium stripe were observed in 1.5% plants on average (Figure 3). The spread of the disease was not effectively controlled by any of the treatments applied during the growing season. Only after the application of Biot treatments, the incidence of Cephalosporium stripe was insignificantly lower. High variation of percentage of infected plants was the reason for the lack of statistical significance of the mean values differentiation. Koch's test revealed that the rate of infection caused by *C. gramineum* was

Table 3. F-values obtained in two-way ANOVA evaluating the severity of Septoria leaf blotch (SLB), % plants infected by *C. gramineum* and grain yield.

Source of variation	DF	Severity of SLB in heading stage (PL)	Severity of SLB in milk stage (FL)	Percentage of plants infected by *C. gramineum*	Grain yield
Year (Y)	2	76.82**	6.02*	24.21**	180.11**
Treatment (T)	5	7.91**	1.04	0.71	0.54
YxT	10	4.79**	0.65	0.47	1.59

** significant at $p < 0.01$; * significant at $p < 0.05$
PL - penultimate leaves, FL - flag leaves

Table 4. Average severity (% infected leaf area) of Septoria leaf blotch (± standard error) on flag leaves (FL) and penultimate leaves (PL) in the heading stage and the milk stage across treatments and years.

	Heading stage (PL)				Milk stage (FL)			
Year Treatment	2009–10	2010–11	2011–12	Mean	2009–10	2010–11	2011–12	Mean
Control	24.5^{bc} (±5.0)	8.4^{a} (±0.9)	11.5^{a} (±2.7)	12.8^{AB} (±2.6)	16.0^{a} (±0.3)	1.3^{a} (±0.3)	1.3 (±0.2)	4.2 (±2.1)
Fung1	33.0^{b} (±11.0)	5.6^{ab} (±2.7)	11.8^{a} (±2.4)	13.6^{AB} (±4.8)	15.0^{a} (±1.6)	0.2^{b} (±0.1)	0.9 (±0.2)	3.5 (±2.4)
Fung2	48.0^{a} (±1.2)	3.6^{b} (±1.6)	2.1^{c} (±1.1)	7.9^{BC} (±2.2)	20.0^{a} (±5.5)	0.02^{b} (±0.01)	0.04 (±0.02)	2.3 (±2.2)
Fung3	35.0^{b} (±13.0)	8.3^{a} (±1.8)	7.8^{ab} (±1.7)	11.1^{BC} (±3.0)	18.0^{a} (±2.2)	0.4^{ab} (±0.2)	1.4 (±0.5)	2.8 (±1.9)
Biot	55.5^{a} (±1.5)	6.6^{ab} (±1.8)	12.5^{a} (±4.5)	18.7^{A} (±6.2)	18.0^{a} (±2.1)	1.2^{ab} (±0.7)	19.1 (±9.0)	11.7 (±7.4)
Fung4	14.0^{c} (±3.2)	5.1^{ab} (±3.1)	4.6^{bc} (±0.1)	5.8^{C} (±1.2)	7.0^{b} (±1.2)	0.03^{b} (±0.01)	0.2 (±0.1)	0.9 (±0.7)
Year	33.3^{X} (±5.3)	5.7^{Y} (±0.5)	8.4^{Y} (±1.0)		14.3^{X} (±1.9)	0.4^{Y} (±0.1)	2.9^{Y} (±2.3)	

Values denoted by the same letter (A - C for treatment; X, Y for year; a - c for treatment for each year separately - the significances of differences were tested using separate one-way analyses of variance) do not differ significantly at $p < 0.01$.

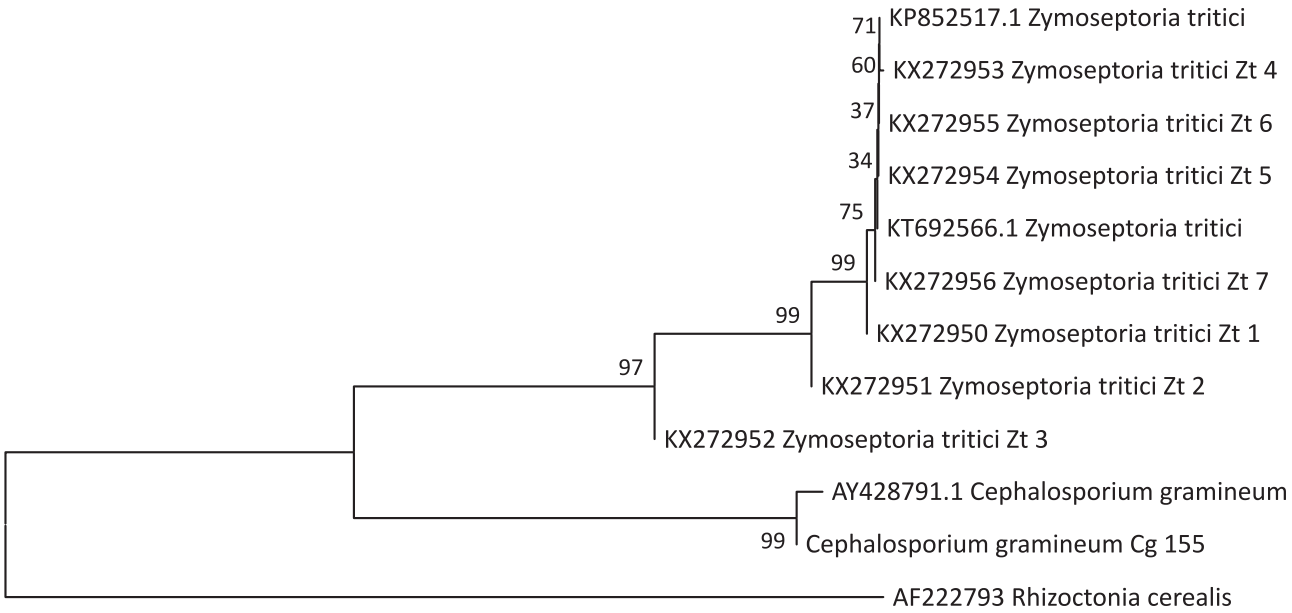

Figure 1. A phylogenetic tree of experimental and reference isolates developed by the neighbor-joining method to determine phylogenetic affinity between the identified fungal species. ITS4 and ITS5 primers were used to identify pathogens. The phylogenetic tree was processed statistically by bootstrapping with 2000 replications. Bootstrap support > 70%.

higher in the seedlings of wheat cv. Sumai 3 compared with cv. Bogatka (Table 5). On average, 50% of cv. Sumai 3 seedlings were infected, but the severity of disease was mild. Seedlings inoculated with isolates Cg2 (cv. Bogatka) and C6 (cv. Sumai 3) were respectively 14.8 and 12.6% shorter than non-inoculated ones. Under in vitro conditions, fenpropiomorph and pyraclostrobin+epoxiconazole completely inhibited the

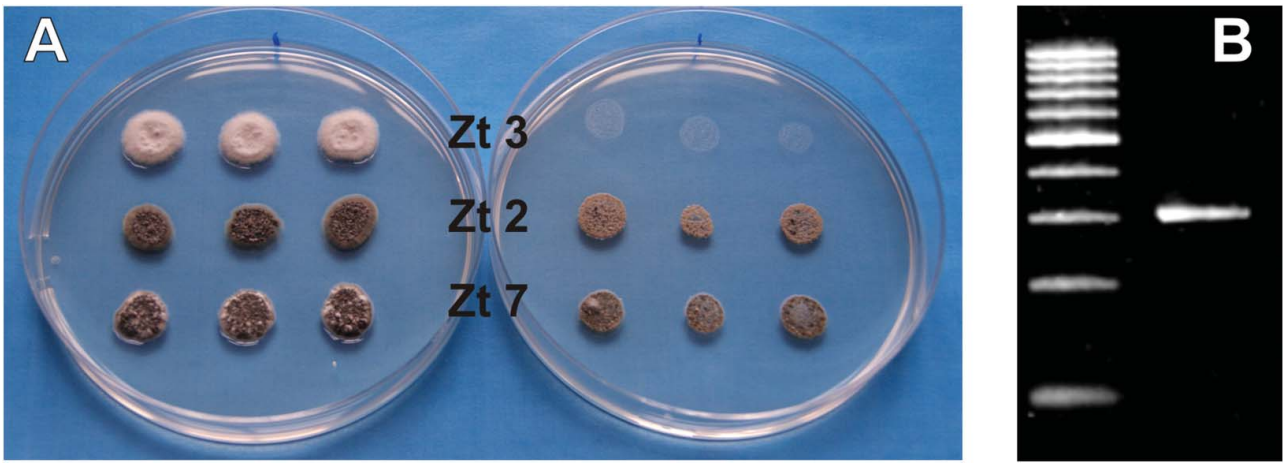

Figure 2. A) *Z. tritici* isolates Zt3 (KX272952), Zt2 (KX272951), Zt7 (KX272956) cultured without (left) and with (right) 0.1 μl cm^{-3} azoxystrobin; B) 302 bp-long amplicon of *Z. tritici* isolate Zt2 (KX272951) resistant to strobilurin fungicides.

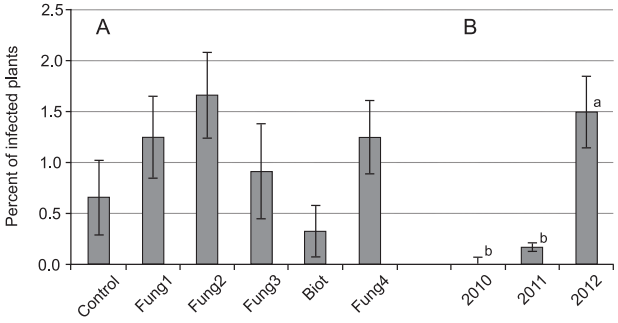

Figure 3. Average percentage of plants infected with *C. gramineum* across treatments (A) and years (B). Bars marked by the same letter differ not significantly at $p < 0.05$.

growth of *C. gramineum* colonies and the effectiveness of chitosan was determined at 84.9% in comparison with control (Table 6).

In 2011, wheat yield increased in response to all protective treatments, whereas in 2010, similar results were achieved only in treatments with pyraclostrobin and epoxiconazole (Fung2) and propiconazole (Fung4) (Table 7). In 2010 and 2011, yields increased by 5.1% and 9.5% in high-input treatments (Fung2) and by 3.4% and 7.0% in response to propiconazole (Fung4), respectively, in comparison with control. The protein content of wheat grain varied across treatments from 13.80 to 14.81 g in 100 g of dry matter (Table 8).

Table 5. Characterization of the studied *C. gramineum* isolates in Koch's test.

	Percentage inhibition of seedling growth relative to control		Average severity of seedling infection on a 4-point scale (%) *	
Isolate	cv. Sumai 3	cv. Bogatka	cv. Sumai 3	cv. Bogatka
Cg2	0 ^{c}	14.8 ^{a}	1.1 ^{x} (86)	1.5 ^{x} (93)
Cg3	0 ^{c}	0 ^{c}	0 ^{z}	0 ^{z}
Cg4	0 ^{c}	4.3 ^{b}	1.3 ^{x} (62)	0 ^{z}
Cg5	4.8 ^{b}	7.2^{b}	1.7 ^{x} (82)	0.8 ^{y} (17)
Cg6	12.6 ^{a}	5.1^{b}	1.2 ^{x} (79)	1.5 ^{x} (83)
Cg7	6.3 ^{b}	0 ^{c}	1,5 ^{x} (93)	0.5 ^{y} (35)
Cg8	0 ^{c}	0.6 ^{c}	0 ^{z}	0.5 ^{y} (48)
Mean	3.4 ^{A}	4.6 ^{B}	0.9 ^{Y} (50)	0.7 ^{X} (35)

* - percentage of seedlings with symptoms of infection caused by *C. graminearum*. Values denoted by the same letter (a - c for inhibition of seedling growth; x - z for average severity of disease; A, B, X, Y for mean values of the above parameters) do not differ significantly at $p < 0.01$.

Table 6. *In vitro* effectiveness of the analyzed pesticides in limiting the growth of *C. gramineum* colonies on PDA.

Treatment	Dose [μl ml^{-3}]	Colony area [cm^2]	Effectiveness * [%]
Control		5.3 ^{c}	
o-nitrophenol + *p*-nitrophenol + 5-nitroguaiacol	1.50	5.1 ^{c}	3.8
fenpropimorph	2.50	0.0 ^{a}	100
pyraclostrobin + epoxiconazole	5.00	0.0 ^{a}	100
trinexapac-ethyl	1.00	4.8 ^{ab}	9.5
chitosan	6.25	0.8 ^{a}	84.9

*Effectiveness $= 100\% - \left(\frac{\text{colony area on PDA with treatment}}{\text{colony area in control}} \times 100\%\right)$

a-c – values denoted by the same letter do not differ significantly at $p < 0.01$

Table 7. Yield of winter wheat grain ± standard error (g per m^2) under experimental conditions.

Year Treatment	2010	2011	2012	Mean for treatments
Control	639.7 ^{de} (±37.6)	917.3 ^{a} (±21.5)	750.7 ^{bc} (±20.4)	769.2 (±37.1)
Fung1	599.0 ^{e} (±41.2)	974.3 ^{a} (±53.7)	714.8 ^{bcd} (±25.2)	762.7 (±49.9)
Fung2	672.3 ^{cde} (±37.3)	1005.0 ^{a} (±17.8)	726.0 ^{bcd} (±16.2)	801.1 (±48.4)
Fung3	635.8 ^{de} (±40.6)	987.3 ^{a} (±18.6)	643.7 ^{de} (±38.2)	755.6 (±52.5)
Biot	623.3 ^{de} (±25.8)	958.6 ^{a} (±33.4)	777.5 ^{b} (±25.4)	786.5 (±44.5)
Fung4	661.3 ^{cde} (±18.1)	981.7 ^{a} (±20.6)	673.0 ^{cde} (±34.4)	772.0 (±47.6)
Mean for years	638.5 ^{X} (±12.1)	970.7 ^{Y} (±10.5)	714.3 ^{Z} (±11.0)	

Values marked by the same letter (a - e for treatment and year; X, Y for year) do not differ significantly at $p < 0.05$.

Table 8. The content of the major protein fractions in wheat grain (g of protein per 100 g of dry matter ± SD) under experimental conditions.

		Gliadins				Glutenins			
Treatment	Albumins + globulins	ω	α/β	γ	Total gliadins	HMW	LMW	Total glutenins	Total protein
Control	2.34 ^{a} (±0.01)	1.10 (±0.25)	4.25 (±0.16)	2.31 (±0.07)	7.66 (±0.48)	1.43 ^{b} (±0.01)	3.28 ^{a} (±0.08)	4.71 ^{a} (±0.08)	14.70 ^{ab} (±0.58)
Fung1	2.18 ^{b} (±0.01)	0.84 (±0.04)	4.36 (±0.24)	2.40 (±0.09)	7.59 (±0.37)	1.41 ^{b} (±0.01)	3.21 ^{ab} (±0.01)	4.62 ^{ab} (±0.02)	14.39 ^{ab} (±0.39)
Fung2	2.14 ^{bc} (±0.02)	0.97 (±0.01)	4.60 (±0.03)	2.31 (±0.03)	7.88 (±0.07)	1.54 ^{a} (±0.00)	3.26 ^{ab} (±0.01)	4.80 ^{a} (±0.01)	14.81 ^{a} (±0.10)
Fung3	1.96 ^{d} (±0.06)	0.77 (±0.04)	4.16 (±0.26)	2.35 (±0.07)	7.28 (±0.37)	1.41 ^{b} (±0.01)	3.23 ^{a} (±0.03)	4.64 ^{ab} (±0.04)	13.89 ^{b} (±0.47)
Biot	2.05 ^{cd} (±0.04)	0.79 (±0.05)	4.11 (±0.25)	2.32 (±0.11)	7.22 (±0.42)	1.43 ^{b} (±0.01)	3.10 ^{bc} (±0.01)	4.53 ^{b} (±0.02)	13.80 ^{b} (±0.48)
Fung4	2.05 ^{cd} (±0.01)	0.84 (±0.04)	4.40 (±0.19)	2.42 (±0.02)	7.66 (±0.26)	1.41 ^{b} (±0.02)	2.97 ^{c} (±0.03)	4.38 ^{c} (±0.05)	14.09 ^{b} (±0.31)

Mean values in a column marked by the same letter do not differ significantly at $p < 0.01$.

The albumin and globulin fraction accounted for 14.1%–15.9%, the gliadin fraction – for 52.1%–54.4%, and the glutenin fraction – for 31.1%–33.4% of total proteins. Only a negative tendency (not statistically significant) was observed between the severity of SLB symptoms on flag leaves and the content of γ gliadins (r = - 0.680) and α/β gliadins (r = - 0.429), whereas a positive correlation was noted between the severity of symptoms on flag leaves and the content of HMW (r = 0.625) and LMW (r = 0.423) glutenins. All protection treatments contributed to a significant decrease in the content of albumins and globulins, relative to control grain (Table 8). In general, the grain of plants subjected to the high-input treatment (Fung2) had the highest content of gluten proteins. The results of the PCA revealed noticeable differences in grain protein profiles across treatments (Figure 4). The combined effect of PC1 and PC2 explained 74.23% of observed variation. A biplot was used to present points corresponding to each treatment (PC1-PC2 in a Cartesian coordinate system) and points corresponding to the analyzed protein fractions (variables) in a single diagram. In the biplot, variable points are distributed inside a circular area with a radius of 1, which corresponds to the maximum (+1) and minimum (−1) value of the correlation coefficient between the variable and PC. The closer a given variable point is located to the circle, the stronger its influence on discrimination. A point's location indicates whether the correlation coefficient has a positive or negative value (+ or -). The results revealed a very low content of the α/β gliadin fraction in the grain harvested from treatments Biot and Fung3 (Table 8). It should be stressed that protein profiles were most similar in these two treatments. The content of each fraction in fungicide-protected treatments (Fung 1, Fung 2 and Fung 4) was completely different from that of unprotected grain (control). Such strong discrimination resulted mainly from a relatively high content of γ gliadins in Fung1 and Fung4 (3.9 and 4.8% higher in relation to the control, respectively) and significantly higher content of HMW glutenins and a very high content of α/β gliadins in Fung2 in relation to the control (7.7 and 8.2% higher, respectively)(Figure 4, Table 8). The protein profile of control grain was different from that of grain in protected treatments, particularly in comparison with Fung 2 and Fung 4 (Figure 4).

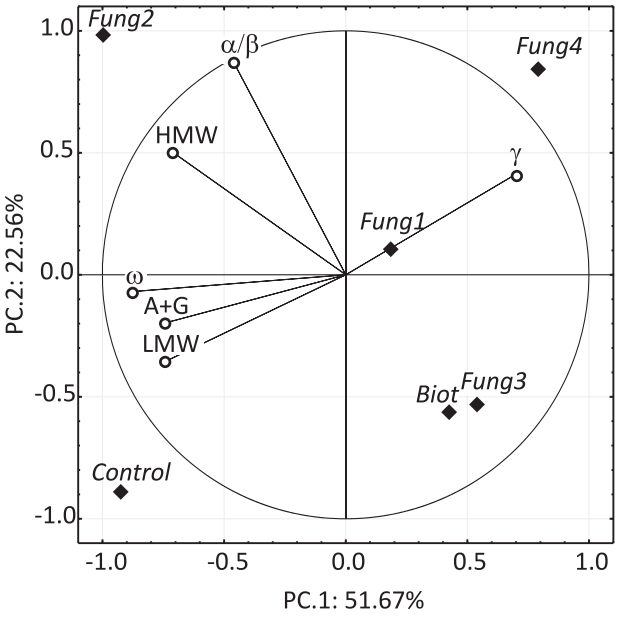

Figure 4. A biplot presenting the results of PCA, including all protein fractions examined in wheat grain (marked with small circles) and in six experimental treatments (marked with black diamonds). For a detailed description of the treatments, refer to Table 1. HMW – high-molecular-weight glutenins; LMW – low-molecular-weight glutenins; A+G – albumins + globulins ω - ω gliadins α/β - α/β gliadins.

4. Discussion

SLB is one of the most dangerous leaf diseases of wheat. In this study, its severity was considerably higher than in Portugal (Rodrigo et al. 2015) and Denmark (Eriksen and Munk 2003). The spread of SLB was probably exacerbated by high rainfall in May 2010 which led to severe infection and premature wilting of penultimate leaves and flag leaves. In a study by Rodrigo et al. (2015), the evaluated wheat varieties were also more severely infected in a year with high rainfall than in a year characterized by low precipitation. *Z. tritici* is a hemibiotroph that colonizes plants through stomata only. It initially grows between leaf mesophyll cells without producing haustoria or other infection structures.After another 12–20 days, *Z. tritici* enters an aggressive necrotrophic phase of development. Our results clearly demonstrate that the progression of SLB is most effectively curtailed when protective treatments are applied in the heading stage. Propiconazole, a triazole fungicide, was particularly effective, and similar results were reported by Rodrigo et al. (2015). When combined with epoxiconazole and tebuconazole, pyraclostrobin reduced the severity of SLB and contributed to a 10.13% increase in grain yield. The content of the major protein groups was characteristic of wheat grain and similar to that described by Konopka et al. (2007), Kiefer (2006) and Shewry (2009). In the high-input treatment, an increase was observed in the content of gluten proteins and the HMW glutenin fraction. The above changes are desirable in grain processing because they improve the viscoelastic properties of dough. The high content of total protein, in particular gluten proteins, is the key characteristic feature of wheat grain which determines its processing parameters, such as flour strength and overall baking value (Anjum et al. 2007; Konopka et al. 2007).

The use of only azoxystrobin on the second treatment date (BBCH 55) was not highly effective. The resulting changes in the content and structure of

protein fractions were generally unfavorable due to a significant drop in the content of total protein, albumins and globulins. The results of our study indicate that the effectiveness of azoxystrobin was limited by the formation of *Z. tritici* genotypes resistant to strobilurin fungicides. A mutation was observed in the mitochondrial gene of cytochrome b, where alanine was substituted for glycine at position 143 (G143A) (Gisi et al. 2002). Strobilurin fungicides have been used in Poland since 1998, and the first reports concerning the resistance of Polish isolates of *Z. tritici* to strobilurin were published in 2010, but they were poorly documented (FRAC 2015). Pieczul (2016) reported the presence of *Z. tritici* isolates with decreased sensitivity to strobilurin fungicides associated with the G143A mutation in the cytochrome b gene. *Z. tritici* genotypes resistant to strobilurin fungicides have been isolated in the United Kingdom, France, Denmark and Ireland since 2002 (Fraaije et al. 2005; Torriani et al. 2008), in Germany since 2004 (Torriani et al. 2008), and in the Czech Republic since 2007 (Drabešová et al. 2013). In our study, most *Z. tritici* isolates from plants protected with strobilurin fungicides had the G143A mutation. In Poland, the Amistar 250 SC fungicide has been recently approved for small-area farms, which increased cropland acreage protected with this product.

Chitosan was completely ineffective in eliminating SLB. Chitosan treatments were probably introduced too late to induce resistance because *Z. tritici* infects hosts already in the fall, and it has a very long incubation period. The treatment with biotechnological preparations was the least desirable option because it also led to a significant decrease in the total protein content of grain. Interestingly, the protein profile of grain from biotechnological treatments was highly similar to that noted in the grain of plants protected with azoxystrobin and tebuconazole and subjected to the growth retardant (Fung3).

The presence of Cephalosporium stripe was noted sporadically, but plants showing symptoms of disease were reported more frequently in wheat monocultures, which corroborates the findings of Quincke et al. (2012). *C. gramineum* was highly sensitive to fungicides and the plant biostimulator *in vitro*, and similar observations were made by Martyniuk et al. (2006). The pathogen infects plants through damaged roots in early stages of development and has systemic advance, therefore foliar application of fungicides may be ineffective in combating Cephalosporium stripe (Quincke et al. 2014).

5 Conclusions

Triazole fungicides applied in growth stage BBCH 55 are most effective in reducing the symptoms of SLB, and intensive use of morpholines, triazoles and strobilurins leads to the highest increase in yield and in the content of gluten proteins in grain. Azoxystrobin is moderately effective in limiting the spread of SLB due to the presence of *Z. tritici* isolates with a mutation in the *CYTb* gene which encodes resistance to strobilurin fungicides. *Cephalosporium gramineum* is highly susceptible to fungicides *in vitro*, but their efficacy is limited under field conditions.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

Anjum FM, Khan MR, Din A, Saeed M, Pasha I, Arshad MU. 2007. Wheat gluten: High molecular weight glutenin subunits-structure, genetics, and relation to dough elasticity. J Food Sci. 72:R56–R63.

Bruehl GW. 1956. Cephalosporium stripe disease of wheat in Washington. Phytopathology. 46:178–180.

Curvers K, Pycke B, Kyndt T, Vanrompay D, Haesaert G, Gheysen G. 2015. A high resolution melt (HRM) assay to characterize *CYP51* haplotypes of the wheat pathogen *Mycosphaerella graminicola*. Crop Prot. 71:12–18.

Dooley H, Shaw MW, Mehenni-Ciz J, Spink J, Kildea S. 2016. Detection of *Zymoseptoria tritici* SDHI-insensitive field isolates carrying the SdhC-H152R and SdhD-R47W substitutions. Pest Manag Sci. 72:2203–2207.

Drabešová J, Ryšánek P, Brunner P, McDonald BA, Croll D. 2013. Population genetic structure of *Mycosphaerella graminicola* and Quinone Outside Inhibitor (QoI) resistance in the Czech Republic. Eur J Plant Pathol. 135:211–224.

Eriksen L, Munk L. 2003. The occurrence of *Mycosphaerella graminicola* and its anamorph Septoria tritici in winter wheat during the growing season. Eur J Plant Pathol. 109:253–259.

Fraaije BA, Cools HJ, Fountaine J, Lovell DJ, Motteram J, West JS, Lucas JA. 2005. Role of Ascospores in Further Spread of QoI-Resistant Cytochrome b Alleles (G143A) in Field Populations of *Mycosphaerella graminicola*. Phytopathology. 95:933–941.

FRAC. 2015. FRAC list of plant pathogenic organisms resistant to disease control agents. Revised January 2013. http://www.frac.info/docs/default-source/publications/list-of-resistant-plant-pathogens/list-of-resistant-plant-pathogenic-organisms—february-2013.pdf?sfvrsn=4 (accessed 28 Nov. 2016).

Gisi U, Sierotzki H, Cook A, McCaffery A. 2002. Mechanisms influencing the evolution of resistance to Qol inhibitor fungicides. Pest Manag Sci. 58:859–867.

http://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed 28 Nov. 2016).

http://en.bio-soft.net/tree/MEGA.html (accessed 28 Nov. 2016).

http://www.minrol.gov.pl/eng/Ministry/Online-database-on-plant-protection-products (accessed 28 Nov. 2016).

https://bip.minrol.gov.pl/content/download/28570/156734/version/1/file/fung%20-%20choroby%20li%C5%9Bci%20zb%C3%B3%C5%BC.pdf (accessed 28 Nov. 2016).

Kiefer R. 2006. The role of gluten elasticity in the baking quality of wheat. In: Popper L, Schäfer W, Freund W,

editors. Future of flour – a compendium of flour improvement. Clenze, Germany: Verlag Agrimedia; p. 169–178.
Konopka I, Fornal Ł, Dziuba M, Czaplicki S, Nałęcz D. 2007. Composition of proteins in wheat grain obtained by sieve classification. J Sci Food Agric. 87:2198–2206.
Leroux P, Albertini C, Gautier A, Gredt M, Walker AS. 2007. Mutations in the *CYP51* gene correlated with changes in sensitivity to sterol 14 alpha-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. Pest Manag Sci. 63:688–698.
Martin JP. 1950. Use of acid, rose Bengal and streptomycin in the plate method for estimating soil fungi. Soil Sci. 38:215–220.
Martyniuk S, Stachyra A, Wroblewska B. 1995. Disease levels in winter wheat, rye and triticale grown on soil artificially inoculated with *Cephalosporium gramineum*. Eur J Plant Pathol. 101:701–704.
Martyniuk S, Stochmal A, Macias FA, Marin D, Oleszek W. 2006. Effects of some benzoxazinoids on in vitro growth of *Cephalosporium gramineum* and other fungi pathogenic to cereals and on Cephalosporium stripe of winter wheat. J Agric Food Chem. 54:1036–1039.
Mavroeidi VI, Shaw MW. 2005. Sensitivity distributions and cross-resistance patterns of *Mycosphaerella graminicola* to fluquiconazole, prochloraz and azoxystrobin over a period 9 years. Crop Prot. 24:259–299.
Meier U. 2003. Phenological growth stages. In: Schwarz MD, editor. Phenology: an integrative science. Dordrecht/Boston/London: Kluver Academic Publishers; p. 270–238.
Mirzwa-Mróz E, Tvaružek L, Zamorski C, Nowicki B. 2005. Research on the development of *Mycosphaerella graminicola* (Fuckel) Schroeter teleomorph on wheat leaves. Acta Agrobot. 58:59–65.
Morton JB, Mathre DE, Johnston RH. 1980. Relation between foliar symptoms and systemic advance of *Cephalosporium gramineum* during winter wheat development. Phytopathology. 70:802–807.
Mundt CC. 2010. Cephalosporium stripe. Vol 3. In: Bockus WW, Bowden RL, Hunger RM, Morrill WL, Murray TD, Smiley RW, editors. Compendium of wheat diseases and pests. St. Paul: APS; p. 24–27.
Murray TD. 2006. Seed transmission of *Cephalosporium gramineum* in winter wheat. Plant Dis. 90:803–806.
Nisikado Y, Matsumoto H, Yamauti K. 1934. Studies on a new Cephalosporium which causes the stripe disease of wheat. Berichte des Ohara Instituts für Landwirtschaftliche Biologie Okayama Universität. 6:275–306.
O'Driscoll A, Kildea S, Doohan F, Spink J, Mullins E. 2014. The wheat-Septoria conflict: a new front opening up? Trends Plant Sci. 19:602–610.
Pieczul K. 2016. Identification of strobilurin resistant strains of *Mycosphaerella graminicola* by PCR-RFLP. Prog Plant Prot. 56:42–46.
Polish National List of Agricultural Plant Varieties. 2016. Słupia Wielka: Research Center for Cultivat Testing, COBORU, Poland.
Quaedvlieg W, Kema GHJ, Groenewald JZ, Verkley GJM, Seifbarghi S, Razavi M, Crous PW. 2011. *Zymoseptoria* gen. nov.: a new genus to accommodate *Septoria*-like species occurring on graminicolous hosts. Persoonia. 26:57–69.
Quincke MC, Murray TD, Peterson CJ, Sackett KE, Mundt CC. 2014. Biology and control of Cephalosporium stripe of wheat. Plant Pathol. 63:1207–1217.
Quincke MC, Peterson CJ, Mundt CC. 2012. Relationship between incidence of Cephalosporium stripe and yield loss in winter wheat. Int J Agron, Article ID 635219. 1–9. https://doi.org/10.1155/2012/635219.
Rasband WS, 2016. ImageJ. Bethesda, Maryland, USA: U.S. National Institute of Health. http://imagej.nih.gov/ij/, .
Richardson MJ, Rennie WJ. 1970. An estimate of the loss of yield caused by *Cephalosporium gramineum* in wheat. Plant Pathol. 19:138–140.
Rodrigo S, Cuello-Hormigo B, Gomes C, Santamaria O, Costa R, Poblaciones MJ. 2015. Influence of fungicide treatments on disease severity caused by *Zymoseptoria tritici*, and on grain yield and quality parameters of bread-making wheat under Mediterranean conditions. Eur J Plant Pathol. 141:99–109.
Shewry PR. 2009. Wheat. J Exp Bot. 60:1537–1553.
Siah A, Deweer C, Morand E, Reignault Ph, Halama P. 2010. Azoxystrobin resistance of French *Mycosphaerella graminicola* strains assessed by four *in vitro* bioassay and by screening of G143A substitution. Crop Prot. 29:737–743.
StatSoft Inc. 2014. *STATISTICA (data analysis software system), version 12*. www.statsoft.com.
Torriani SFF, Brunner PC, McDonald BA, Sierotzki H. 2008. Qol resistance emerged independently at least 4 times in European populations of *Mycosphaerella graminicola*. Pest Manag Sci. 65:155–162.
Vazquez MD, Zemetra R, Peterson CJ, Mundt CC. 2015. Identification of Cephalosporium stripe resistance quantitative trait loci in two recombinant inbred line populations of winter wheat. Theor Appl Genet. 128:329–341.
White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. New York: Academic Press, Inc.; p. 315–322.
Wieser H, Kieffer R. 2001. Correlations of the amount of gluten protein types to the technological properties of wheat flours determined on a micro-scale. J Cereal Sci. 34:19–27.