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Original Research

Using a Protective Treatment to Reduce *Fusarium* Pathogens and Mycotoxins Contaminating Winter Wheat Grain

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Abstract

Fungi of the genus *Fusarium* infect cereal crops during the growing season and cause head blight (FHB). Their secondary metabolites (mycotoxins) contaminate grain. Mycotoxins are not degraded during standard food and feed processing operations or *in vivo* digestion, and are dangerous to human health. In a three-year field experiment we evaluated the effects of biological control agents and a plant biostimulator (as well as fungicides) on the development of Fusarium head blight (FHB), winter wheat grain colonization by *Fusarium* fungi, and the accumulation of ergosterol, deoxynivalenol, nivalenol, zearalenone, beauvericin, enniatins, and moniliformin in winter wheat grain. The biological control agents were bacterial isolates of the genera *Sphingomonas* and fungal isolates of *Aureobasidium pullulans*. All protective treatments inhibited kernel tissue penetration by *Fusarium* pathogens, and contributed to a natural reduction in *Fusarium* spp. populations after six months of grain storage. In comparison with the untreated control, bacterial isolates reduced the abundance of fungal pathogens by 23.5% at harvest and by 100% after a six-month storage period, yeasts by 34.1% and 40.9%, fungicides by 22.1-65.5% and 100%, and the plant biostimulator by 68.1% and 100%, respectively. Fungicides were most effective in reducing wheat grain contamination with deoxynivalenol, whereas bacteria and the plant biostimulator with nivalenol. *A. pullulans* can effectively inhibit the proliferation of a wide spectrum of *Fusarium* toxins (deoxynivalenol, nivalenol, enniatins, and moniliformin).

Keywords: *Fusarium* toxins, *Sphingomonas* sp., *Aureobasidium pullulans*, plant biostimulator, fungicides

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Introduction

In the temperate climate, fungi of the genus *Fusarium* are major producers of toxic compounds in winter wheat grain, including group B trichothecenes, mainly deoxynivalenol (DON) and its acetylated derivatives and nivalenol (NIV); group A trichothecenes (mainly T-2 and HT-2 toxins); fumonisins (FBs); and zearalenone (ZEA) [1]. *Fusarium* pathogens colonize plants already in the field, and their development and species composition as well as the type and concentrations of the synthesized toxins are determined mainly by air humidity and temperature during the growing season [2]. Subject to region, the main *Fusarium* species that colonize wheat spikes and grain are *F. graminearum* [3], *F. culmorum* [4], *F. poae* [5], and *F. avenaceum* [6]. Toxins produced by *Fusarium* fungi are highly stable, and they are not degraded during standard food and feed processing operations or *in vivo* digestion. According to numerous studies, the most resistant toxins produced by *Fusarium* pathogens are DON and NIV [7].

The latest research on the prevention of mycotoxin contamination of grain focuses on identifying the genotypes of wheat that are not sensitive to *Fusarium* pathogens, and efforts are made to develop new biological control methods [8-11]. The use of bacteria in protecting winter wheat against Fusarium head blight and in limiting DON concentrations in the field has been investigated by very few studies [12]. This is the first study that describes the results of a three-year field experiment testing various combinations of bacterial and yeast isolates that effectively limit the contamination of winter wheat grain with *Fusarium* fungi and reduce the concentrations of fusariotoxins in grain. The objective of this study was to evaluate the effectiveness of biological control agents, a plant biostimulator, and fungicides in protecting winter wheat against *Fusarium* spp. and their toxic metabolites.

Material and Methods

Origin of Winter Wheat Grain

Winter wheat grain (cv. Bogatka) [13] was obtained from a field plot experiment with a randomized block design with four replications conducted in northeastern Poland (53°35′42″N, 19°51′11″E) in three growing seasons: 2008/2009, 2009/2010, and 2010/2011. Bogatka is an early maturing cultivar characterized by high processing suitability (quality class B – bread wheat) and relatively high resistance to Fusarium head blight (FHB) (7.7 points on a nine-point scale). Winter wheat was sown in plots of 20 m^2. Pre-sowing mineral fertilization was applied at 20 kg N, 26 kg P, and 60 kg K (per ha). A nitrogen fertilizer (34% ammonium nitrate, Grupa Azoty S.A., Poland) was additionally applied at 40 kg at the beginning of the growing season and at the first node stage (BBCH 31). Biological control agents (Bac, Ap), a plant biostimulator (Biost), and fungicides (Fung1, Fung2) were applied on the dates indicated in Table 1. The commercial control agents (Biost, Fung1, Fung2) were applied twice at the first node stage (BBCH 31) and in the middle of heading (BBCH 55). The Bumper 250 SC fungicide was applied at 0.5 dm^3 per ha, and Fandango 200 EC, Alert 375 SC, and Soprano 125 SC fungicides were each applied at 1 dm^3 per ha. Asahi SL plant biostimulator was applied at 0.6 dm^3 per ha. All protective treatments were carried out with the use of a backpack sprayer (Marolex, Poland). Unprotected plants were the control. The antagonistic activity of isolates obtained from winter wheat grain against fungi of the genus *Fusarium* sp. had been previously confirmed under *in vitro* conditions. Biological control involved a combination of bacterial isolates (*Sphingomonas* sp., GenBank accession number JX444564 [14]) or yeast isolates (*Aureobasidium pullulans,* KX444670 [14]) in the form of cell suspensions with concentrations of 10^8 (Bac) and 10^6 (Ap) CFU in 1 cm^3 of sterile water. The suspensions were sprayed on plants with a backpack sprayer (Marolex, Poland) in the afternoon on windless and cloudy days of 15-25ºC. Spike, stem, and leaf surfaces were evenly coated with the microbial suspension. Each plot was sprayed with 250 cm^3 of the suspension diluted in two liters of water. Grain harvested at the overripe stage (BBCH 92), with a relative moisture content of 14.5%, was stored in paper bags at 11ºC. Mycological analyses were conducted at harvest and after six months of storage. Grain for chemical analyses was stored at -20ºC.

Severity of FHB Symptoms and Biometrical Measurements

The health status of spikes was evaluated at the dough stage (BBCH 75) of winter wheat grain. The severity of FHB was estimated based on the percentage of spike surface area with disease symptoms and the incidence of spikes with disease symptoms, according to the Bulletin of the European Plant Protection Organization [15]. One hundred spikes (25 from each plot) were randomly sampled for analysis.

Isolation and Identification of Fusarium Fungi

Grain was subjected to microbiological analyses at harvest and after six months of storage in dark, in paper bags, at low relative air humidity, and at 11ºC. Epiphytes were washed from 10 g grain samples by shaking randomly selected kernels in 250 cm^3 flasks containing 90 cm^3 of sterile water (Elpin+ 378 S shaker table, Poland). Endophytes were obtained from grain that was surface-disinfected in 1% sodium hypochlorite solution. Grain was dried and ground in a mill (Prespol, Poland) to particles with a diameter of 1-3 mm. Ten-g samples of kernel homogenates were shaken in 250 cm^3 flasks containing 90 cm^3 of sterile water (Elpin+ 378 S shaker table, Poland). The resulting fungal suspensions (colony-forming unit, CFU) were diluted with sterile water at 1:1000, and 0.1 cm^3 specimens were transferred to Petri

Table 1. Details of biological and fungicidal treatments on winter wheat.

Treatment		BBCH 31 (First node at least 1 cm above tillering node)	BBCH 55 (Middle of heading)
Bac	Biological	*Sphingomonas* sp., *Bacillus* sp., *Pseudomonas* sp.	*Sphingomonas* sp., *Bacillus* sp., *Pseudomonas* sp.
Ap	Biological	Aureobasidium pullulans	Aureobasidium pullulans
Fung1	Fungicide	Bumper 250EC[1]	Fandango 200EC[2]
Fung2	Fungicide	Alert 375SC[3]	Soprano 125SC[4]
Biost	Plant biostimulator	Asahi SL[5]	Asahi SL[5]

[1]propiconazole 25.1% (Makhteshim Chemical Works Ltd, Israel)
[2]fluoxastrobin 100 g l^{-1}, prothioconazole 100 g l^{-1} (Bayer SAS, France)
[3]fusilasol 125 g l^{-1}, carbendazim 250 g l^{-1} (Du Pont International Operations Sarl, Switzerland)
[4]epoxiconazole 125 g l^{-1} (Makhteshim Chemical Works Ltd, Israel)
[5]*o*-nitrophenol 2%, *p*-nitrophenol 3%, 5-nitroguaiacol 2% (Asahi Chem. MFG Co. Ltd., Japan)

dishes with a diameter of 9 cm. Selective Martin's medium [16] cooled to 42°C was poured into the plates. The number of colonies in plates was counted, and culture fragments were transferred to Petri dishes filled with potato dextrose agar (PDA, Merck) to obtain pure cultures. Colonies were identified to the species level based on their sporulation characteristics [17].

Determination of Ergosterol and Mycotoxin Concentration in Grain

Standards of mycotoxins (DON, NIV, ZEA, BEA, ENNs (A, A1, B and B1), FBs (FB1-FB3), MON), and ERG and organic solvents (HPLC grade) were purchased with a standard grade certificate from Sigma-Aldrich (Steinheim, Germany). All chemicals used for extraction and purification of ERG and mycotoxins were purchased from POCh (Gliwice, Poland). Water for the HPLC mobile phase was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

All mycotoxins and ERG were analyzed in the healthy looking kernels (HLK) fraction as well as in the fraction of *Fusarium*-damaged kernels (FDK). Wheat kernels were manually divided into fractions based on their appearance evaluated visually under a stereoscopic microscope; FDK were poorly developed, wrinkled and/or discolored. Fungal metabolites were extracted and purified according to the detailed procedures described by Goliński et al. [18], Stępień and Waśkiewicz [19], and Waśkiewicz et al. [20]. The eluate was evaporated to dryness at 40ºC under a stream of nitrogen. Dry residue was stored at -20ºC until HPLC analyses.

The chromatographic system consisted of a Waters 2695 high-performance liquid chromatograph (Waters, Milford, USA) with detectors: a Waters 2996 photodiode array detector with a Nova Pak C-18 column (300 3.9 mm) for DON, NIV (max = 224 nm) and MON (max = 229 nm) analysis, a Waters 2996 photodiode array detector with a Nova Pak C-18 column (150 3.9 mm) for ERG (max = 282 nm), BEA (max = 205nm) and ENN (max = 205 nm) analysis, a Waters 2475 multi-fluorescence detector (λex = 274 nm, λem = 440 nm) and a Waters 2996 photodiode array detector with a Nova Pak C-18 column (150 3.9 mm) for ZEA analysis, and a Waters 2475 multi-fluorescence detector (λex = 335 nm, λem = 440 nm) with an XBridge column (3.0100 mm) for FB (FB1-FB3) analysis.

Quantfying mycotoxins was performed by measuring the peak areas at retention times according to the relevant calibration curve. The limits of detection (LODs) were: 10 μg kg^{-1} for ERG, 1 μg kg^{-1} for ZEA, 3 μg kg^{-1} for DON, 2 μg kg^{-1} for NIV, 3 μg kg^{-1} for BEA, 2 μg kg^{-1} for ENNs, 1 μg kg^{-1} for MON, and 1 μg kg^{-1} for FBs (FB1-FB3).

Statistical Analysis

The abundance of *Fusarium* fungi was determined based on the number of dilutions of fungal suspensions and the weight of grain samples. The number of *Fusarium* colonies isolated from each treatment was calculated from the following formula: colony-forming units (CFU) = $\Sigma C / (n_1 + 0{,}1n_2)\, d$, where ΣC is the sum of colonies counted on all the dishes retained, n_1 is the number of dishes retained in the first dilution (-3), n_2 is the number of dishes retained in the second dilution (-4), and d is the dilution factor corresponding to the first dilution. The results of grain mycological analyses were log transformed (CFU+1). The significance of differences between mean values was estimated by a factorial analysis of variance, and the mean values were compared by the multiple Student-Newman-Keuls (SNK) test at $p<0.01$ using the Statistica 12 program [21]. The concentrations of secondary *Fusarium* metabolites were subjected to principal component analysis (PCA).

Results

Weather Conditions

Total precipitation and mean temperatures during the period of the study are presented in Fig. 1. Total

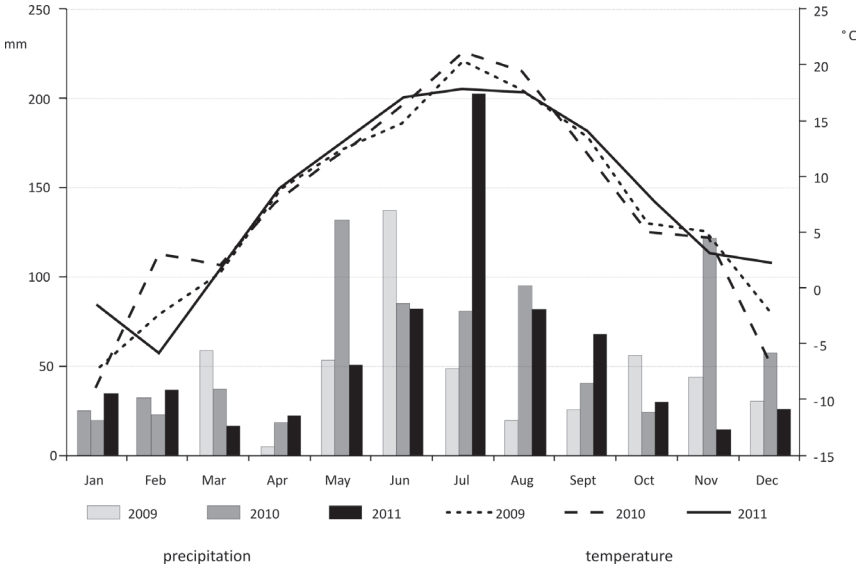

Fig. 1. Total precipitation and average temperatures in the growing seasons of 2009, 2010, and 2011.

annual precipitation exceeded 600 mm, and precipitation patterns were not evenly distributed across experimental years. Precipitation levels were generally low in March and April, in particular in 2011. In June 2009, during wheat flowering, precipitation levels were determined at 136.9 mm. Abundant rainfall was reported in May 2010 (132 mm) and in July 2011 (203 mm). In 2009, lower mean temperatures were recorded in May and June in comparison with 2010 and 2011.

Severity of FHB Symptoms and Percentage of FDK

In 2009 winter wheat kernels were significantly more severely and more frequently infected with *Fusarium* pathogens than in the remaining years of the study (Table 2). The application of fungicides containing carbedazim, flusilazole, and epoxiconazole (Fung2) led to the greatest (but statistically non-significant) decrease (20.3%) in the average incidence of spikes with FHB symptoms in comparison with unprotected plants. Only fungicide treatments significantly decreased FDK percentages in spikes in comparison with control.

Colonization of Winter Wheat Grain by Fusarium Pathogens

The mean percentage of epiphytic *Fusarium* fungi in the populations of filamentous fungi colonizing winter wheat grain (average of the three research years) stored for six months was lower than immediately after harvest (Table 3). This natural trend was generally reinforced by all control agents, in particular in years characterized by lower FHB severity. At harvest and after six months of grain storage, the average counts of epiphytic *Fusarium* fungi isolated from wheat grain treated with the bacterial suspension were reduced by 26.8% and 56.8%, respectively, in comparison with control.

The communities of endophytic *Fusarium* fungi obtained from grain at harvest and six months after harvest were reduced in size 1.47-fold and 4.18-fold,

Table 2. Mean (min-max) values of Fusarium head blight (FHB) symptoms and percentage of Fusarium-damaged kernels (FDK) in conditions of experiment in 2009-11.

Treatment†	FHB symptoms		FDK
	%	FR	(%)
Control	1.42	23.05	16.2^{a}
	(0.44-2.31)	(8.33-52)	(14.4-17.3)
Bac	1.06	48.23	13.2^{ab}
	(0.25-2.54)	(4.38-48.23)	(11.1-13.4)
Ap	1.45	20.63	13.1^{ab}
	(0.74-2.12)	(8.33-40)	(11.7-14.9)
Fung1	1.12	27.50	12.3^{b}
	(0.67-1.9)	(10-45)	(10.9-11.8)
Fung2	0.79	18.37	11.7^{b}
	(0.43-1.5)	(8.21-35.22)	(10.9-12.3)
Biost	1.64	26.50	14.2^{ab}
	(0.39-3.51)	(7.86-54.96)	(12.1-16.3)
Mean:			
2009	2.31^{A}	45.90^{A}	13.3^{A}
2010	0.51^{B}	9.61^{B}	15.0^{A}
2011	0.92^{B}	11.00^{B}	10.1^{B}

FHB symptoms % is percent of spike area with symptoms of FHB; FR is frequency of occurrence; values followed by the same letter (separately for each feature) do not differ significantly at $p < 0.01$; †See Table 1

Table 3. The mean number (Log (CFU+1) per 1 gram grain) of *Fusarium* fungal communities colonizing the grain of winter wheat in conditions of experiment in 2009-11 (min-max values are given within brackets).

Treatment‡	Term	Percentage of *Fusarium* colonies	*Fusarium* spp.†	*F. poae*	*F. culmorum*	*F. avenaceum*	*F. graminearum*
Epiphytes							
Control	H	78.37 (54.2-90.9)	2.50 ^{ab} (2.35-2.64)	1.58 (0.5-2.47)	0.74 (0-1.73)	1.10 (0-2.3)	0.17 (0-0.5)
	S	41.77 (4.8-77.4)	2.41 ^{abc} (1.15-3.33)	1.23 (0-3.2)	0.91 (0-2.73)	0.17 (0-0.5)	0.19 (0-0.58)
Bac	H	40.97 (31-51.9)	1.83 ^{dc} (0.65-2.5)	0.72 (0-1.65)	0.95 (0-2.23)	0.00 -	0.00 -
	S	32.27 (0-93.5)	1.04 ^{e} (0-2.55)	0.85 (0-2.55)	0.17 (0-0.5)	0.00 -	0.17 (0-0.5)
Ap	H	83.20 (69.6-100)	2.80 ^{a} (2.6-2.99)	1.25 (0.58-2.6)	1.53 (0-2.94)	0.00 -	0.00 -
	S	44.63 (7.7-91.2)	2.18 ^{bcd} (1.2-3.32)	0.94 (0-2.81)	0.36 (0-0.58)	0.00 -	0.37 (0-0.62)
Fung1	H	82.77 (59.3-100)	1.96 ^{cd} (1.08-2.78)	1.57 (0.58-2.55)	0.73 (0-1.7)	0.77 (0-1.7)	0.00 -
	S	30.10 (0-67.3)	1.90 ^{cd} (0-3.25)	1.84 (0-3.24)	0.00 -	0.17 (0-0.5)	0.19 (0-0.58)
Fung2	H	47.57 (0-84.4)	1.78 ^{d} (0-2.82)	0.95 (0-2.35)	1.09 (0-2.78)	0.00 -	0.00 -
	S	24.70 (17.3-28.5)	2.54 ^{ab} (2.25-2.87)	1.30 (0-2.75)	0.84 (0-2.51)	0.19 (0-0.58)	0.17 (0-0.5)
Biost	H	71.40 (24.2-100)	2.04 ^{bcd} (1.68-2.62)	0.82 (0.58-1.24)	1.16 (0.5-1.73)	0.00 -	0.00 -
	S	11.33 (0-23.3)	0.99 ^{e} (0-2.36)	0.37 (0-0.62)	0.00 -	0.19 (0-0.58)	0.17 (0-0.5)
Mean	H	67.38^{i}	2.15^{i}	1.15	1.03^{a}	0.31	0.03
	S	30.80$^{** x}$	1.84^{x}	1.09^{x}	0.38*	0.12^{x}	0.21$^{** x}$
Endophytes							
Control	H	21.80 (17.1-24.3)	2.26 ^{b} (2-2.57)	0.55 (0-1.65)	0.36 (0-0.58)	0.82 (0-2.47)	0.00 -
	S	23.83 (5-52)	1.64 ^{c} (0-2.5)	0.00 -	1.57 (0-2.43)	0.00 -	0.00 -
Bac	H	20.20 (8.3-27)	1.73^{d} (1.08-2.62)	1.03 (0-2.6)	0.17 (0-0.5)	0.00 -	0.17 (0-0.5)
	S	0.00 -	0.00 ^{e} -	0.00 -	0.00 -	0.00 -	0.00 -
Ap	H	24.20 (9-32.1)	1.49 ^{c} (0-2.64)	0.38 (0-1.15)	0.36 (0-1.08)	1.07 (0-2.64)	0.00 -
	S	11.37 (3-27.1)	0.97 ^{e} (0-2.3)	0.19 (0-0.58)	0.00 -	0.00 -	0.00 -
Fung1	H	12.00 (9-15)	1.76 ^{bc} (1.15-2.33)	1.69 (1.08-2.25)	0.00 -	0.19 (0-0.58)	0.00 -
	S	0.00 -	0.00 ^{e} -	0.00 -	0.00 -	0.00 -	0.00 -
Fung2	H	33.17 (5-64)	0.78 ^{e} (0-1.83)	0.72 (0-1.65)	0.39 (0-0.58)	0.36 (0-1.08)	0.38 (0-1.15)
	S	0.00 -	0.00 ^{e} -	0.00 -	0.00 -	0.00 -	0.00 -

Table 3. Continued.

Biost	H	7.33 (2-15)	0.72^{e} (0.5-1.08)	0.36 (0-1.08)	0.19 (0-0.58)	0.00 -	0.00 -
	S	0.00 -	0.00^{e} -	0.00 -	0.00 -	0.00 -	0.00 -
Mean	H	19.78^{j}	1.46^{j}	0.79	0.25^{b}	0.41	0.09
	S	5.87^{*y}	0.44^{**y}	0.03^{*y}	0.26	0.00^{y}	0.00^{y}

H - immediately after harvest, S - after six months of grain storage. † Sum for species *F. poae, F. culmorum, F. avenaceum, F. graminearum, F. tricinctum, F. sporotrichioides, F. solani*, and *F. dimerum*; a-e values signed by the same letter differ not significantly according to SNK test at $p<0.01$; i, j; x,y – difference between endophytes and epiphytes significant at $p<0.01$,*, **- difference between H and S significant at $p<0.05$ and $p<0.01$, respectively; ‡ See Table 1

respectively, compared with the communities of epiphytic fungi (Table 3). In all years of the study, endophytic *Fusarium* pathogens were not isolated from wheat grain treated with fungicides (Fung1, Fung2), Asahi SL (Biost), or the bacterial (Bac) suspension after six months of storage. Immediately after harvest, fungi of the genus *Fusarium* (mainly *F. poae*) were 34.1% less abundant in kernels protected with a suspension of *A. pullulans* than in unprotected kernels, and after six months of storage the difference reached 40.9%, mainly in the abundance of *F. culmorum*.

Concentrations of Fungal Metabolites in Grain

The average content of ERG in FDK (4,855 μg kg^{-1}) was more than two-fold higher than in healthy-looking kernels (HLK, 2,273 μg kg^{-1}) throughout the experiment (Table 4). The greatest ERG concentrations in HLK and FDK were reported in grain treated with *A. pullulans* (at 2,912 and 5,914 μg kg^{-1}, respectively), and the lowest ERG levels were found in HLK-treated grain treated with Fung1 fungicides (at 1,569 and 3,730 μg kg^{-1}, respectively). Asahi SL and fungicides containing propiconazole or fluoxastrobin + prothioconazole (Fung1) reduced ERG concentrations in HLK by 24.1 and 35.7%, respectively. The above can be attributed to the presence of toxin-producing fungi of the genus *Fusarium*, and the Pearson correlation coefficient between ERG and DON for HLK and FDK was determined at 0.404 (not significant) and 0.658 (significant at $p<0.05$), respectively. In biological control treatments with *A. pullulans,* greater ERG concentrations resulted mainly from the presence of ERG in the fungal cell wall. The presence of NIV, ZEA, BEA, ENNs, and MON was detected in 44.4, 27.8, 5.6, 11.1, and 38.9% in 18 tested HLK samples, respectively. Fumonisins were present in all analyzed samples, and their concentrations ranged from 4.9 to 29.1 μg kg^{-1}. DON concentrations were determined in the range of 3.4 to 34.0 μg kg^{-1} in 94.5% of tested HLK samples, and the presence of this mycotoxin was not observed only in grain treated with Asahi SL in 2011.

All protective treatments significantly reduced average DON concentrations in HLK. Bacterial (Bac) and mycological (Ap) control agents, as well as fungicides containing propiconazole, fluoxastrobin and prothioconazole (Fung1), and carbedazim, flusilazole and epoxiconazole (Fung2) decreased mean DON concentrations from 17.2 to 8.5, 13.9, 9.3, and 7.0 μg kg^{-1} in HLK samples, respectively. NIV was not detected in HLK from plants protected with the bacterial suspension (Bac), and the suspension's ability to reduce toxin levels in FDK grain was estimated at 46.2%. NIV concentrations in HLK were not reduced by the remaining treatments, and NIV levels in FDK grain protected with Fung2 or Biost were lower than in unprotected grain.

The applied control agents, excluding Bac, significantly reduced ZEA concentrations only in FDK. BEA and ENNs were identified sporadically in HLK samples and in all FDK samples. All biological and fungicidal control agents reduced BEA levels in FDK and ENNs in HLK. None of the applied agents was successful in inducing a significant reduction in fumonisin levels in FDK or HLK. Except for Asahi SL (Biost), all treatments decreased MON concentrations in HLK.

The applied control agents modified the concentrations of the analyzed metabolites in wheat grain, as demonstrated by PCA results (Figs 2-3). The differences in the mycotoxin profiles of HLK harvested from various treatments were in a narrower range than in FDK (more evident in Table 4). In HLK, both fungicide treatments (Fung1 and Fung2) had similar effects and reduced the concentrations of the evaluated mycotoxins in grain (excluding ZEA in 2011) (Fig. 2). Grain from spikes protected with *A. pullulans* was characterized by somewhat increased levels of fumonisins (only in 2011), mostly ERG, in comparison with control. The greatest concentrations of all mycotoxins were observed in control samples, whereas the levels of mycotoxins, in particular DON, were reduced in grain treated with Asahi SL and the bacterial suspension. PCA results demonstrate that *A. pullulans* was effective in lowering the concentrations of *Fusarium* toxins in FDK (Fig. 3). The applied isolates did not dramatically reduce the concentrations of toxic metabolites in grain showing symptoms of FHB, but their profiles differed completely

Table 4. Mean concentrations of fungal metabolites (μg kg^{-1}) in winter wheat grain in conditions of experiment in 2009-11 (min-max values are inside brackets).

Treatment	ERG	DON	NIV	ZEA	BEA	ENNs	FBs	MON	Sum of mycotoxins
	HLK								
Control	2,440^{b}	17.1^{a}	2.77^{b}	1.46	<LOD	3.20^{a}	16.5	4.14^{a}	45.17
	(1,913-3,402)	(3.8-34.0)	(<LOD -3.91)	(<LOD -3.38)		(<LOD -7.59)	(9.0-25.6)	(<LOD -8.36)	
Bac	2445^{b}	8.5^{c}	<LOD^{c}	1.70	<LOD	<LOD^{c}	15.1	1.51^{b}	26.81
	(1,089-3,948)	(5.7-11.1)		(<LOD -4.1)			(4.9-20.9)	(<LOD -3.52)	
Ap	2,912^{a}	13.9^{b}	4.67^{a}	<LOD	<LOD	<LOD^{c}	17.8	2.02^{b}	38.39
	(2,071-3,468)	(9.9-18.8)	(<LOD -8.81)				(9.6-29.1)	(<LOD -5.07)	
Fung1	1,569^{d}	9.3^{c}	3.36^{b}	1.79	<LOD	<LOD^{c}	7.4	1.57^{b}	23.42
	(601-3,050)	(3.4-18.2)	(<LOD -8.08)	(<LOD -4.36)			(5. 6-10.6)	(<LOD -3.71)	
Fung2	2,419^{b}	7.0^{c}	3.51^{b}	1.46	<LOD	<LOD^{c}	12.2	0.50^{c}	24.67
	(1,071-4,159)	(3.8-9.4)	(<LOD -5.23)	(<LOD -3.37)			(5.3-17.4)	(<LOD)	
Biost	1,854^{c}	8.2^{c}	1.97^{b}	1.49	2.15	1.96^{b}	12.5	4.09^{a}	32.36
	(1,736-2,085)	(<LOD-19.2)	(<LOD-3.91)	(<LOD -3.48)	(1.5-3.46)	(<LOD -3.88)	(10.2-15.4)	(<LOD -7.76)	
Mean 2009-11	2,273***	10.7***	3.25***	1.58***	2.15***	2.58***	13.6***	2.30***	36.16
	FDK								
Control	3,826^{e}	1,236^{a}	221^{a}	44.5^{a}	36.9^{a}	42.3	353	38.4	1,972.1
	(2,588-5,409)	(208-2,419)	(84-436)	(38.1-54.2)	(31.2-40.5)	(31.5-55.8)	(201-512)	(27.1-59.9)	
Bac	5,548^{b}	1,286^{a}	119^{c}	41.4^{a}	26.7^{c}	34.9	399	42.7	1,949.7
	(3,399-8,356)	(153-2,169)	(93-141)	(19.1-59.6)	(19.6-32.2)	(17.4-44.1)	(161-519)	(25.9-55.7)	
Ap	5,914^{a}	1,169^{b}	225^{a}	31.7^{b}	13.6^{d}	23.4	236	52.1	1,750.8
	(4,063-8,992)	(139-2,065)	(106-323)	(17.3-46.8)	(4.9-30.8)	(9.9-38.2)	(213-265)	(23.6-85.3)	
Fung1	3,730^{e}	959^{b}	241^{a}	32.7^{b}	11.3^{d}	20.4	222	27.8	1,514.2
	(969-7,494)	(139-2,069)	(73-458)	(21.3-55.2)	(4.6-24.5)	(14.4-24.6)	(138-300)	(10.9-41.9)	
Fung2	5,144^{c}	615^{d}	189^{b}	30.1^{b}	33.4^{b}	28.5	274	23.0	1,193.0
	(2,786-9,699)	(169-1,221)	(124-306)	(18.4-42)	(24-38.4)	(19.4-43.8)	(123-381)	(14.9-28.8)	
Biost	4,965^{d}	882^{c}	142^{b}	30.8^{b}	35.4^{ab}	42.8	344	49.9	1,526.9
	(2,754-7,867)	(117-1515)	(61-271)	(12.9-46.1)	(26.2-50.2)	(31.5-59.5)	(186-428)	(19.5-79.4)	
Mean 2009-11	4,855	1,025	190	35.2	26.2	32.1	305	39.0	1,652.0

Means signed by the same letter differ not significantly within columns (separately for HLK and FDK) according to SNK test at $p<0.01$. HLK – healthy looking kernels, FDK – Fusarium-damaged kernels, LOD – limit of detection, ERG – ergosterol, DON – deoxynivalenol, NIV – nivalenol, ZEA – zearalenone, BEA – beauvericin, ENNs – enniatins, FBs – fumonisins (FB1-FB3), and MON – moniliformine. If the toxin concentration in a sample was below the LOD, then the half of the LOD was used for calculation. *** - by comparison of mean values for HLK and FDK the test t probability $p<0.001$. For other designations see Table 1.

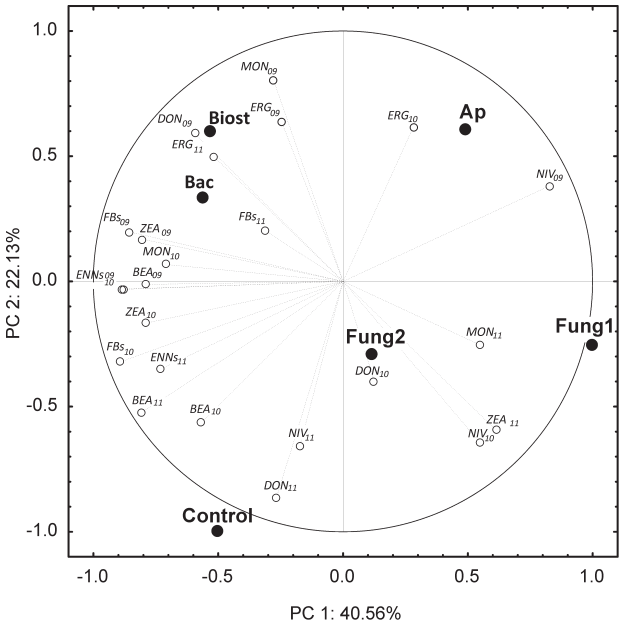

Fig. 2. Biplot presenting the PCA results for all analyzed fungal metabolites in HLK. Graph presents the distributions of experimental objects in the space of two principal components (PCs) for all analyzed metabolites within three years of experiments, and a projection of variables on the PC surface. Subscripts represent the year of experiment (2009-11), Bac, Ap, Fung1, Fung2, and Biost (see Table 1).

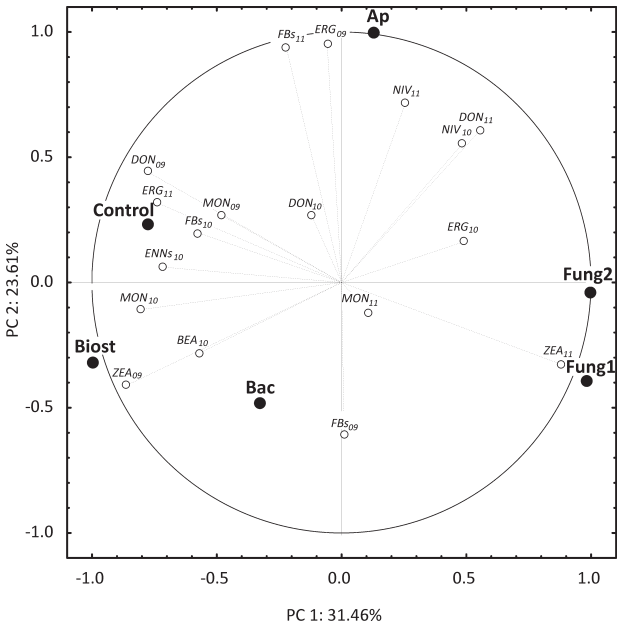

Fig. 3. Biplot presenting the PCA results for all analyzed fungal metabolites in FDK. Graph presents the distributions of experimental objects in the space of two principal components (PCs) for all analyzed metabolites within three years of experiments, and a projection of variables on the PC surface. Subscripts represent the year of experiment (2009-11), Bac, Ap, Fung1, Fung2, and Biost (see Table 1).

from control grain and grain treated with Asahi SL and bacteria (Fig. 3).

Discussion

This paper presents the results of a study that investigated the effectiveness of biological control agents, a plant biostimulator, and fungicides in protecting winter wheat against *Fusarium* spp. and fusariotoxins. In our study, DON was detected in nearly all grain samples, but the maximum DON level for unprocessed grain (1,250 μg kg^{-1}) set in the EU [22] was exceeded in all FDK samples, in particular in 2010, which was characterized by a prevalence of *F. culmorum*, the main producer of DON. In one case (in 2009), significant concentrations of DON were observed in grain despite an absence of *Fusarium* fungi on the surface of kernels and low pathogen concentrations in kernel tissues. In studies by Xu et al. [23] and Covarelli et al. [24], DON was frequently detected in grain despite an absence of *Fusarium* species capable of producing that toxin. The cited authors attributed their findings to high levels of DON mobility in plant tissues. In cereal plants infected with *F. culmorum*, DON can be moved from the stem bases to spikes that do not display symptoms of FHB [24]. The above mechanism poses a considerable problem in modeling the spread of FHB and DON concentrations in grain. In our study, fungicides containing propiconazole, fluoxastrobin, and prothioconazole were most effective in reducing ERG concentrations and the counts of endophytic *Fusarium* fungi in kernels, but they were less effective in lowering DON levels than carbedazim, flusilazole, and epoxiconazole. This apparent contradiction can be at least partially attributed to the high transfer rate of DON between the stem bases infected with *Fusarium* spp. and spikes [24]. It should be noted that high ERG concentrations in the grain of spikes protected with *A. pullulans* can be attributed to the metabolite's presence in the cell wall of this yeast-like fungus [25].

In the present study, ZEA and FB concentrations did not exceed the maximum values for small grain cereals (100 μg kg^{-1}) or maize (2,000 μg kg^{-1}) [22]. Our results suggest that the above toxins pose a less serious threat for wheat grain than trichothecenes, and similar conclusions were formulated by Birck et al. [26].

In our study, the bacterial suspension was effective in reducing FHB symptoms only in selected years. Schisler et al. [9] reported a 67% reduction in FHB symptoms in field conditions following the application of *Arthrobacter* sp. OH 221.3 bacteria that degrade choline, a compound that promotes the growth of *F. graminearum*. In the work of Nourozian et al. [8], only one of the four tested bacterial isolates of the genus *Streptomyces* alleviated FHB symptoms. Our results indicate that the application of *Sphingomonas* sp. isolates decreased contamination levels and kernel penetration by *Fusarium* fungi. The above led to a reduction in DON concentrations in grain, in particular in 2010. Thus, it cannot be ruled out that bacteria isolated

from wheat grain are able to degrade DON. *A. pullulans* proved to be ineffective in mitigating the symptoms of FHB. The effectiveness of the *A. pullulans* AS 55.2 isolate was estimated at 52% by Schisler et al. [10]. According to the above authors, this biological control agent inhibited the proliferation of *Fusarium* fungi in only one wheat cultivar in one of two experimental sites in a field study. However, our results suggest that the application of this yeast-like fungus significantly reduces DON concentrations in grain. The PCA results for kernels with FHB symptoms indicate that plants protected with *A. pullulans* are less likely to be contaminated by toxic metabolites. *A. pullulans* does not significantly inhibit the proliferation of the pathogen on the kernel surface, nor does it inhibit the symptoms of disease, but it prevents *Fusarium* fungal filaments from growing into kernel tissues. It could also contribute to partial biodegradation of selected toxins or reduce the pathogens' ability to produce toxins. The effect of *A. pullulans* on OTA accumulation in grapes was described by De Felice et al. [27], but the underlying mechanism was not explained by the authors. Our study demonstrates for the first time that *A. pullulans* is effective in inhibiting the accumulation of *Fusarium* toxins in winter wheat grain.

The plant biostimulator applied in our study did not eliminate FHB symptoms, but significantly lowered the levels of *Fusarium* contamination and decreased DON concentrations in kernels. Ogórek et al. [28] demonstrated that sodium salts of ortho-nitrophenol, para-nitrophenol, and 5-nitroguaiacolate had a minor inhibitory effect on the development of *Fusarium* fungi *in vitro*. The evaluated plant biostimulator probably modified physiological processes in plants, which stimulated their natural defense mechanisms [29] and contributed to the production of masked mycotoxins [30].

Conclusions

Biological protection of winter wheat involving yeast and bacterial isolates, as described in this study, partially reduced grain contamination with endophytic *Fusarium* pathogens and lowered mycotoxin concentrations in grain. *A. pullulans* effectively reduced a broad spectrum of *Fusarium* toxins. Bacterial isolates were most effective in reducing nivalenol concentrations in grain. Fungicides were the strongest inhibitors of pathogen growth on kernel surface, and they lowered deoxynivalenol levels in grain. The plant biostimulator can be effectively used to control mycotoxin contamination in winter wheat grain by inhibiting kernel tissue penetration by *Fusarium* pathogens and significantly reducing nivalenol levels in grain.

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