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Variations in grain lipophilic phytochemicals, proteins and resistance to *Fusarium* spp. growth during grain storage as affected by biological plant protection with *Aureobasidium pullulans* (de Bary)

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ABSTRACT

Modern agriculture relies on an integrated approach, where chemical treatment is reduced to a minimum and replaced by biological control that involves the use of active microorganisms. The effect of the antagonistic yeast-like fungus *Aureobasidium pullulans* on proteins and bioactive compounds (alkylresorcinols, sterols, tocols and carotenoids) in winter wheat grain and on the colonization of wheat kernels by fungal microbiota, mainly *Fusarium* spp. pathogens, was investigated. Biological treatment contributed to a slight increase contents of tocols, alkylresorcinols and sterols in grain. At the same time, the variation of wheat grain proteins was low and not significant. Application of *A. pullulans* enhanced the natural yeast colonization after six months of grain storage and inhibited growth of *F. culmorum* pathogens penetrating wheat kernel. This study demonstrated that an integrated approach of wheat grain protection with the use of the yeast-like fungus *A. pullulans* reduced kernel colonization by *Fusarium* spp. pathogens and increased the content of nutritionally beneficial phytochemicals in wheat grain without a loss of gluten proteins responsible for baking value.

1. Introduction

The grain of winter wheat (*Triticum aestivum* L.) is commonly used for breadmaking. Its baking value is mostly dependent on the content and properties of gluten proteins (Hurkman et al., 2013), whereas the nutritional and prophylactic value is affected by the content and composition of dietary fiber as well as a large group of bioactive compounds such as polyphenols, alkylresorcinols, sterols, tocols, carotenoids and others (Konopka et al., 2012; Shewry and Ward, 2012). Environmental factors (fertilizers, method of plant protection, weather conditions) can change the wheat grain composition and physical properties. Response of wheat to stress conditions, varies across genotypes. Some genotypes tolerate various types of stresses, whereas others are more sensitive. These differences can be attributed to the accumulation of specific proteins, especially enzymes and inhibitors, which are active against pests and pathogens (Dupont and Altenbach, 2003; Hurkman et al., 2013) or are responsible for plant resistance to abiotic stress (Skylas et al., 2002). Plant resistance/defense strategy utilizes also secondary metabolites, which are synthesized in secondary metabolic pathways (De Coninck et al., 2015). Grain inoculated with *Fusarium culmorum* accumulated approx. 40-fold higher content of total flavonoids than control sample, with the highest increase of quercetin (73-fold) and luteolin (65-fold) content (Buśko et al., 2014). The protective agents against the development of Fusarium head blight (FHB) symptoms may be also 5-n-alkylresorcinols (Ciccoritti et al., 2015). Participation of phenolic compounds in a defense strategy of plants explains why they are highly susceptible (variable in content and composition) to the effect of the environment (Shewry and Ward, 2012). Environment also determines the content of other groups of plant secondary metabolites, which are also essential components of plant defense against stressful conditions (Fratianni et al., 2013).

Modern agriculture uses the different biological methods or integrates them with standard pesticide treatments (Ferron and Deguine, 2009). Eco-friendly crop protection includes biological methods based on specific microorganisms (Palazzini et al., 2013; Wachowska et al., 2013a) or plant-derived natural compounds (Ciccoritti et al., 2015). *Aureobasidium pullulans* (de Bary) Arnaud is a saprotrophic, polymorphic fungus (Gniewosz and Duszkiewicz-Reinhard, 2008) that can be used for wheat protection (Wachowska et al., 2013b). It is commonly found on the surface and in the tissues of cereal kernels where suppress the growth of phytopathogens (Wachowska and Głowacka, 2014). Antagonistic effect of *A. pullulans* may be related to production of extracellular enzymes (Castoria et al., 2001; Ma et al., 2007) as well as grain phytochemicals. However, the influence of the microorganisms used as plant protection agents on chemistry of cereal grain is poorly understood.

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The objective of this study was to determine the suppressive effect of *A. pullulans* on *Fusarium* spp. pathogens colonizing winter wheat grain under field conditions and during storage, and to evaluate variations in gluten content and antioxidant capacity under the applied biological treatment.

2. Material and methods

2.1. Field experiment

A field-plot experiment was conducted in north-eastern Poland (50°43'N, 20°26'E), in a randomized block design with four replications. Winter wheat (*T. aestivum*, cv. Bogatka) was sown in 20 m^2 plots. Standard fertilization regimes were as follows: nitrogen (N) and potassium (K_2O) at 100 kg/ha and phosphorus (P_2O_5) at 60 kg/ha. Plots were divided based on protection protocols of plants:

1) Plots with plants sprayed with CFU suspension of *A. pullulans* isolates at the stem elongation (first node) stage (BBCH 31) and the heading stage (BBCH 55).
2) Control plots with plants sprayed with water at BBCH 31 and BBCH 55.

Isolates of *A. pullulans* were obtained and indentified according to procedure described by Wachowska et al. (2013b). They were cultured on potato glucose agar (PDA) (Merck) for seven days at 24 °C, next they were washed off from the medium with sterile water and transferred into 1 L flasks to produce fungal cell suspensions with the concentration of 10^8 cells per 1 cm^3 of water. The suspension was diluted with water and sprayed onto plants. Finally, on average 5000 *A. pullulans* cells were applied per one plant. Moment of application corresponded to BBCH for wheat (Meier, 2003).

2.2. Biometric and biochemical measurements

Selected morphological traits were evaluated at the over-ripe stage (BBCH 92) of grain. One hundred plants were randomly sampled from each treatment for analysis. The evaluated parameters were thousand kernel weight and spike density (number of spikelets per 10 cm of the rachis). Harvested grain was dried to approx. 14%, manually cleaned from broken kernels and ground in a type A10 IKA Labortechnic mill. Flour (with particles below 300 μm) was used to chemicals analyses.

2.3. Determination of sterols

The content of sterols was determined by GC/MS method as described by Roszkowska et al. (2015) with modifications. Before analysis, samples were hydrolyzed under acidic conditions according to the procedure proposed by Ryan et al. (2007). Each dry extract was re-dissolved in 4.5 mL of ethanol and 0.2 mL of a 5α-cholestane solution (0.4 mg/g) as an internal standard was added, and the mixtures were saponified by adding 0.5 mL of a 10 M KOH solution in methanol at a temperature 70 °C for 30 min. The mixtures were transferred to separatory funnels containing 10 mL of deionized water, and unsaponifiables were extracted twice with 10 mL of diethyl-ether. The collected ether layers were washed twice with 2 mL of 0.5 M KOH and four times with deionized water. The ether fractions were filtered through anhydrous sodium sulfate and evaporated in a vacuum evaporator (BÜCHI R-200 type, Flawil, Switzerland) at 45 °C. The dry extracts were re-dissolved in 1.5 mL of hexane, transferred into vials and evaporated under a nitrogen. The residues were re-dissolved in 100 μL of pyridine and 100 μL of N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) and were heated at 60 °C for 1 h. Finally, 0.5 mL of heptane was added, and the mixtures were analyzed using the GC–MS QP2010 PLUS manufactured by Shimadzu (Kyoto, Japan). Sterols were separated on a ZB-5 ms capillary column (30 m × 0.25 mm × 0.25 μm) (Phenomenex, Torrance, CA, USA) using helium with a 0.9 mL/min flow rate. The temperatures were as follows: injector—230 °C, column—70 °C increased to 230 °C at 15 °C/min, and to 310 °C at 3 °C/min and maintained for 10 min, GC–MS interface—240 °C, ion source—220 °C. Electron energy was set as 70 eV. The total ion current (TIC) mode was used for quantification (100–600 m/z range). Sterols were identified by comparison with the mass spectral library. Sterol content was determined based on the concentration of internal standard and expressed as μg of 5α-cholestane per 1 g of grain.

2.4. Determination of alkylresorcinols

The content of alkylresorcinols was determined according to the method described by Sampietro et al. (2009). Alkylresorcinols were extracted from 1 g of ground samples with 30 mL of acetone for 48 h at 20 °C. The mixtures were centrifuged and acetone extracts were evaporated in a vacuum evaporator (BÜCHI R-200 type). Solid residues were re-dissolved in 1 mL of methanol. Subsequently, color reaction was performed by adding 2 mL of 0.05% Fast Blue RR reagent (4-Benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi (zinc chloride) salt) diluted with methanol 1: 5) and 10 μL of 10% K_2CO_3 solution to 100 μL of each extract. Absorbance measurements were carried out after 20 min at 480 nm using a UNICAM UV/Vis UV2 spectrophotometer (ATI Unicam, Cambridge, United Kingdom). The content of alkylresorcinols was calculated using a standard curve prepared for olivetol. The composition of alkylresorcinols was determined with the use of the GC–MS QP2010 PLUS manufactured by Shimadzu. The conditions of derivatization, separation and identification were identical as those in sterol analysis.

2.5. Determination of tocols

Tocol extracts were prepared according to Engelsen and Hansen (2008). 10 mL hexane was added to 0.5 g ground samples placed in brown glass flasks. Extraction was carried out in an ultrasound bath for 15 min. The supernatants were quantitatively transferred to a flask and evaporated to dryness in a vacuum evaporator (BÜCHI R-200 type) at 45 °C. The extracts were re-dissolved in 2 mL of n-hexane, the solution was subsequently centrifuged (25,000 *g* for 10 min) in a 5417R-type Eppendorf centrifuge (Eppendorf AG, Hamburg, Germany) and transferred to vials. Tocol content was determined according to the method described by Czaplicki et al. (2011) using an Agilent Technologies 1200 series RP-HPLC system (Santa Clara, CA, USA), equipped with a fluorescence detector from the same manufacturer. Tocols were separated on a LiChrospher Si60 column (250 mm × 4 mm × 5 μm) (Merck, Darmstadt, Germany). The mobile phase was a 0.7% iso-propanol solution in n-hexane (v/v), and the flow rate was 0.7 mL/min. The fluorescence detector was set at 296 nm of excitation and 330 nm of emission. Tocol content was calculated using external calibration curves, and was expressed as μg per 1 g of grain.

2.6. Determination of carotenoids

The content of carotenoids was determined according to Konopka et al. (2004) A 10 mL mixture of hexane, acetone, absolute ethanol and toluene (10:7:6:7 v/v/v/v), 2 mL of 40% KOH in methanol and 1 mL of 0.1% BHT in ethanol were added to 10 g of samples placed in 100 mL glass flasks. The solutions were vigorously shaken and left in the dark at room temperature for 16 h. After saponification, 30 mL of 10% Na_2SO_4 was added to each flask, and carotenoids were extracted four times with 10 mL of hexane. The collected extracts were evaporated to dryness at 40 °C in a vacuum evaporator (BÜCHI R-200 type). Finally, the extracts were re-dissolved in 1 mL of a methanol and dichloromethane mixture (45:55, v/v) and centrifuged (25,000 *g* for 10 min) in a 5417R-type Eppendorf centrifuge. An Agilent Technologies 1200

series HPLC system equipped with a photodiode array detector from the same manufacturer was used. Carotenoids were separated on a YMC C30 column (3 μm, 150 mm × 4.6 mm, YMC Europe GmbH, Germany) at 30 °C. The chromatogram was recorded at a wavelength of 450 nm. The mobile phase was methanol and methyltert-butyl ether (89:11 v/v) at a constant flow rate of 1 mL/min. Carotenoids were identified based on their characteristic spectra and a comparison of their retention times with those of known standard solutions. Their content was calculated using a calibration curve of lutein, and was expressed as μg lutein per 1 g of grain.

2.7. Determination of antioxidant capacity

The antioxidant capacity of grain samples was determined by the DPPH Radical Scavenging Assay, according to Yang et al. (2014) with some modifications. Grain extracts were prepared after alkaline hydrolysis of 0.2 g of samples with 8 mL of 2 M NaOH for 4 h at room temperature. The mixtures were continuously mixed with a magnetic stirrer, then neutralized with 6 M HCl and evaporated to dryness. Residues were extracted twice by adding 10 mL of methanol and centrifuged (a 5417R-type Eppendorf centrifuge). The collected extracts were filled up to 25 mL. Finally, 2 mL of a DPPH solution (0.2 mmol/L in methanol) was added to 0.5 mL of grain extract. The mixture was shaken and incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm against methanol. The DPPH radical scavenging rate (%) was calculated according to the formula: [(absorbance of control sample − absorbance of grain sample)/(absorbance of control sample)] × 100. The antioxidant capacity was determined based on a curve of % DPPH• scavenging activity of different Trolox concentrations in methanol and expressed as μmol Trolox per 1 g of grain.

2.8. Determination of proteins

The content and composition of proteins were determined by RP-HPLC as described by Konopka et al. (2007). The assays were performed using a Hewlett Packard 1050 series system. Protein fractions (albumins, globulins, Ω, α/β, γ gliadins and HMW (high molecular weight) and LMW (low molecular weight) glutenins) were detected at a 210 nm wavelength and were identified based on their UV spectra and retention times. The total content of extracted protein was quantified based on Bio-Rad Bradford Protein Assay procedures using bovine serum albumin (Bio-Rad) for an albumins and globulins assay and gliadin standard (Sigma-Aldrich) for gluten proteins assay. Content of each subunit was calculated as its area share to total area of fraction. Protein content was expressed as g/100 g.

2.9. Characteristics of fungal microbiota on wheat kernels

The abundance of yeasts colonizing wheat kernels was estimated at harvest and after six months of storage at 11 °C in paper bags at low humidity. Yeast abundance was analyzed on the surface of kernels and in kernel tissues (Laca et al., 2006). 10 g grain samples were placed in 250 mL flasks filled with 90 mL of sterile water. The flasks were shaken for 60 min on the 358 S shaker table (ElpinPlus, Poland) at 180 rpm to wash off microorganisms from kernel surface. To isolate endophytic yeasts, 10 g grain samples were disinfected with 1% sodium hypochlorite solution and ground. The resulting colony forming unit (CFU) suspension was diluted three-fold and 0.1 mL samples were transferred onto Petri dishes. Selective Martin's medium (Martin, 1950) cooled to 42 °C was poured into the dishes. The microorganisms were incubated in the dark at 24 °C for seven days. The number of yeast colonies in plates was counted, and yeasts were identified under a light microscope (Nikon E 200). Kernels disinfected and not disinfected with 1% hypochlorite solution and afterwards washed with distilled water were placed on Petri dishes containing potato dextrose agar (Merck) to analyze the abundance and species composition of filamentous fungi. Fungal colonies of the genus *Fusarium* were passaged on SNA medium. Filamentous fungi were identified to the genus or species level using a light microscope (Nikon E 200) based on their sporulation characteristics (Ellis, 1971; Leslie et al., 2006).

2.10. Statistical analysis

The significance of differences between mean values was estimated by analysis of variance (ANOVA), and the mean values were compared by the multiple Student–Newman–Keuls (SNK) test using Statistica 10 software (StatSoft Inc., 2011). The abundance of the analyzed microorganisms was determined in view of the applied dilutions, and the data were log transformed (CFU + 1). The structure of filamentous fungi communities was expressed as the number of colonies of a given species or genus grown on PDA.

3. Results and discussion

3.1. Lipophilic phytochemicals in control wheat grain

The content of lipophilic compounds in wheat grain samples is presented in Table 1. Sterols were the predominant compounds in extract, with an average content of 693 μg/g. This value lies within the range of grain tested in Healthgrain project (www.healtgrain.org) that showed that the total sterol content of wheat kernels may range from around 650 to 1200 μg/g, with 840 μg/g on average, depending on the genotype and environmental factors (Shewry et al., 2012; Shewry and Ward, 2012). In all cited studies, the predominant sterol was β-sitosterol that accounted for approximately 60% of total phytosterols in wheat grain. In our study β-sitosterol accounted for ca. 77% of total phytosterols, followed by campesterol (ca. 19%) and stigmasterol (ca. 4%). The higher

Table 1
The content of selected lipophilic bioactive compounds in the analyzed wheat grain samples.

Compounds	Control sample		*A. pullulans* sample	
	average	% of fraction	average	% of fraction
Total sterols (μg/g)	693	–	703	–
Campesterol	134	19.3	134	19.1
Stigmasterol	26	3.8	29	4.1
β-sitosterol	533	76.9	540	76.8
Total alkylresorcinols (AR) (μg/g)	429[a]	–	448[b]	–
AR C17:0	22	5.1	23	5.1
AR C19:0	135	31.5	133	29.7
AR C21:0	170	39.6	177	39.5
AR C23:0	53	12.4	58	12.9
AR—unidentified	49[a]	11.4	57[b]	12.7
Total tocols (μg/g)	50.4[a]	–	53.0[b]	–
α—Toc	16.9	33.5	17.2	32.5
β—Toc	4.3	8.5	4.7	8.9
γ—Toc	2.1	4.2	3	5.7
δ—Toc	0.2	0.4	0.2	0.4
α—Toc3	8.9	17.7	8.8	16.6
β—Toc3	16.2[a]	32.1	17.3[b]	32.6
γ—Toc3	1.8	3.6	1.9	3.6
Total carotenoids(μg/g)	2.78[b]	–	2.36[a]	–
Lutein	2.52[b]	90.6	2.14[a]	90.7
Zeaxanthin	0.14	5.0	0.12	5.1
β-carotene	0.12	4.3	0.1	4.2
Total lipophilic compounds (μg/g)	1176[a]	–	1206[b]	–
Antioxidant capacity in the DPPH assay (μmol TE/g)	2.96[a]	–	3.38[b]	–

Sterols are expressed as μg of 5α-cholestane per g of grain; alkylresorcinols are expressed as μg of olivetol per g of grain; carotenoids are expressed as μg of lutein per g of grain; and tocols are expressed as μg of homologs (α-, β-, γ-, δ-) of tocopherol per g of grain (tocotrienols are calculated as tocopherols).
Means denoted by different superscript letters within features (content of compound) differ significantly at $P < 0.05$. All values calculated on a dry matter basis.

share of β-sitosterol may be genotype-related, but it is also possible that the phytosterol content was slightly underestimated in our study since the presence of minor, unidentified sterol-like compounds, was ignored. Alkylresorcinols were the second largest group of lipophilic compounds in the analyzed wheat grain with value of 429 μg/g. Alkylresorcinol concentrations may be vary over a wide range of 100 to 900 μg/g in different wheat grain samples and extraction protocols (Ciccoritti et al., 2015; Kulawinek et al., 2008; Magnucka et al., 2014). For example, the lowest alkylresorcinol content was noted in the grain of durum wheat extracted with cyclohexane, and the highest—in selected common wheat genotypes. The share of main homologs (C21:0—ca. 40% and C19:0—ca. 30%) was typical for wheat grain samples (Kulawinek et al., 2008). The ratio between the alkylresorcinol homologs C17:0 and C21:0 was 0.13. Kulawinek et al. (2008) demonstrated that the above ratio in wheat samples is close to 0.2. However, in a study by Ciccoritti et al. (2015) this parameter was below 0.03, and its values can be varied depending on the extraction solvent used. This shows that the characteristics of alkylresorcinols in the cultivar used in the study were similar to those of other wheat grain samples. In our study, the total tocol content of control grain samples reached 50.4 μg/g, with the highest share of two major homologs, α-Toc and β-Toc3 (ca. 33% each). The third most important tocol homolog was α-Toc3 (17%). According to previous studies, the grain of common wheat contains from 10 to 80 μg/g of tocols, 50 μg/g on average (Konopka et al., 2012; Okarter et al., 2010; Shewry and Ward, 2012). A considerably higher tocol content was determined in einkorn wheat (up to 116 μg/g) (Hidalgo et al., 2006). In the majority of studies investigating common and emmer wheat, α-Toc and β-Toc3 were the major homologs, and β-Toc3 predominated in einkorn wheat (Hidalgo et al., 2006; Lachman et al., 2013). Similarly to the previously discussed compounds, also the content and composition of tocols were typical of wheat grain samples. In our experiment, carotenoids had the lowest share of the total lipophilic phytochemicals identified in wheat grain (2.78 μg/g in the control sample). The major homolog was lutein, which had a 91% share of total carotenoids. Zeaxanthin and β-carotene were found in low quantities (5% and 4%, respectively). Only trace amounts of other carotenoids were found, and they were not included in the analysis. Similar concentrations and composition of carotenoids in wheat grain were determined in previous studies.(Hidalgo et al., 2006; Konopka et al., 2004; Lachman et al., 2013; Li et al., 2013). However, Okarter et al. (2010) reported that lutein accounted for only 50–70% of total carotenoids in wheat, followed by zeaxanthin (13–20%), β-cryptoxanthin (5–12%) and β-carotene (14–19%).

3.2. Variation in the concentrations of lipophilic phytochemicals in wheat grain and antioxidant capacity in response to the application of A. pullulans

A comparison of control and *A. pullulans*-treated samples showed that the content of total lipophilic phytochemicals was only slightly higher in biologically protected grain (in summary by 2.6%). A detailed analysis of the identified compounds revealed that *A. pullulans* treatment increased the content of tocols (by 5.2%), alkylresorcinols (by 4.4%) and sterols (by 1.3%—increase not significant), whereas carotenoid concentrations decreased by approximately 15%. Within phytochemical fractions, a decrease was noted in the relative ratio of AR 19:0 (from 31.5% to 29.7%) and α-Toc3 (from 17.7% to 16.6%), whereas the share of unidentified AR-homologs and γ-Toc increased (from 11.4% to 12.7% and from 4.2% to 5.7%, respectively). Although the heritability of tocol, phytosterol and alkylresorcinol content in 26 lines of wheat was noted as above 57%, the environment-dependent variation is also significant (Shewry et al., 2012; Shewry and Ward, 2012). For example Chen et al. (2009) determined that under rain-fed conditions wheat grain accumulates less phytosterols. The noted increase in the concentrations of tocols and alkylresorcinols under the influence of *A. pullulans* treatment improved the nutritional value of grain, and could be regarded as a beneficial effect of the novel plant protection technology tested in this study.

The variations in the phytochemical content of control and *A. pullulans*-treated samples resulted in differences of antioxidant capacity of methanol extracts from grain hydrolyzate. The value determined in the DPPH assay was approximately 14% higher in biologically treated grain (2.96 vs. 3.38 μmol TE/g) and may be related to the higher total content of lipophilic compounds. Reference data about the antioxidant capacity of wheat grain are not consistent and present results from 1 to 40 μmol TE/g, depending on the type of antioxidant, cultivar, extraction method and the type of the antioxidant activity assay used. Konopka et al. (2012) demonstrated that the total Trolox equivalent antioxidant capacity (TEAC) of wheat flour varied from 4 to 7 μmol TE/g between dichloromethane and 80% methanol extracts.

3.3. Variation in the protein content of wheat grain

The chromatographic conditions used in our study resulted in approximately 100 protein peaks being resolved. The presence of around 60 peaks in the chromatograms of albumins and globulins (Fig. 1A–B), as well as about 40 peaks of gluten proteins (Fig. 1C–D) was observed. Although, the separation efficiency of RP-HPLC is relatively low for highly advanced studies, this technique is still considered as a fast and low-cost for screening purposes. For example, similar separation conditions of chromatography coupled with chemometrics, has recently been proposed as a tool for process monitoring in industrial bread-making (Li et al., 2013).

The protein content of control wheat grain was determined at 11.44% on a dry matter basis (Table 2), of which albumins represented 13.29%, globulins—6.91%, gliadins—38.11% and glutenins—41.70%. The proportion between gliadins and glutenins (Gli to Glu ratio) was determined at 0.91 and a similar value was reported for other wheat samples (Gil-Humanes et al., 2012; Konopka et al., 2007). However, the Gli to Glu ratio may be substantially greater, ranging from 1.68 to 2.71, and its highest values were noted for the grain from water-stressed plants (Park et al., 2014). Biological protection contributed to a slight decrease (in summary by 2.9%) in the total grain protein content, triggered by a equivalent decrease of gluten proteins and albumins, accompanied by 6% increase of globulins. A detailed analysis of globulin chromatograms (Fig. 1B) revealed that application of *A. pullulans* increased only the area of peaks with retention times up to 12 min. The practically constant Gli to Glu ratio, with variation from 0.91 to 0.92, pointed to prevalence of elastic (glutenin-related) above viscous (gliadin-related) properties of the gluten matrix. No newly-formed peaks in biologically treated samples were detected. It may be summarized that grain modification under the impact of *A. pullulans* led to only minor variation of grain proteome.

The application of *A. pullulans* slightly improved the grain filling, marked as 1000 kernel weight (Table 2). The observed increase (3.8%) was higher than that reported by Schisler et al. (2006) in a study of wheat cv. Freedom treated with *A. pullulans* and lower than that noted by Khan et al. (2004) in a study of *Cryptococcus flavescens*. This indicates that variations in 1000 kernel weight are closely related to the genotype of winter wheat, the type of antagonistic microorganisms used in the study and genotype-environment interactions.

3.4. Fungal microbiota on wheat kernels

The field application of *A. pullulans* influenced the microbiota of grain. This treatment increased the number of yeast communities on the surface of freshly harvested grain by 9.4%, and enhanced their natural tendency to proliferate during storage (Table 3). The number of yeasts penetrating the tissue of disinfected kernels was on the same level in both types of grain samples. The abundance of yeasts colonizing the surface of kernels was slightly related to abundance of *Fusarium* ssp. that accounted for 18.5% and 15.9% of epiphytic and endophytic grain microbiota, respectively. The predominant

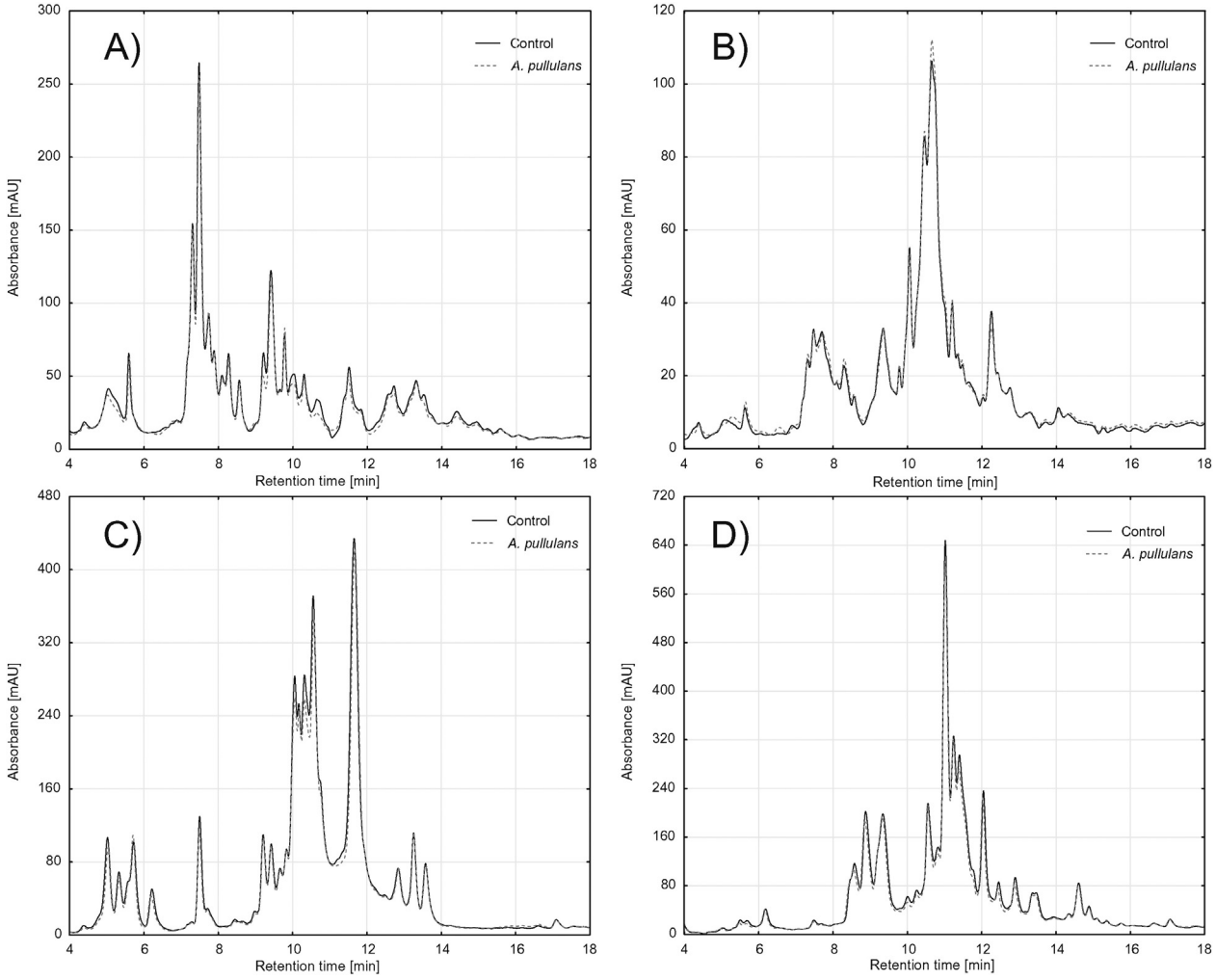

Fig. 1. Chromatograms of albumins (A), globulins (B), gliadins (C) and glutenins (D) of wheat grain in conditions of experiment.

Table 2
The selected biometric characteristics of wheat and protein content and composition of studied grain.

Trait	Control sample (C)	*A. pullulans* sample (*Ap*)	(C/*Ap*)-1 (%)
Spike density (number of spikelets per 10 cm length of spike)	22.55	21.60	4.4
Thousand kernel weight (g)	42.77[a]	44.44[b]	−3.8
Albumins (A) (g 100/g)	1.52	1.47	3.4
Globulins (G) (g 100/g)	0.79	0.84	−6.0
Gliadins (GLI) (g 100/g)	4.36[a]	4.22[b]	3.3
Ω	0.48	0.45	6.7
α/β	2.44	2.38	2.5
γ	1.44	1.39	3.6
Glutenins (GLU) (g 100/g)	4.77	4.59	3.9
HMW	1.49	1.43	4.2
LMW	3.28	3.16	3.8
GLI/GLU	0.91	0.92	−0.6
Σ (A + G + GLI + GLU) (g 100/g)	11.44[b]	11.12[a]	2.9
Share of fraction in total protein (%)			
Albumins	13.29	13.22	0.5
Globulins	6.91	7.55	−8.6
Gliadins	38.11	37.95	0.4
Glutenins	41.70	41.28	1.0

Means denoted by different superscript letters within features differ significantly at $P < 0.05$.

epiphytic species was *F. poae* (11.1%), while the major endophytic species was *F. culmorum* (5.4%). The abundance of *Fusarium* fungi determined in the current study was higher than in the grain analyzed by Berghofer et al. (2003) The presence of *F. avenaceum*, *F. sporotrichioides*, and sporadically *F. graminearum*, *F. equiseti* and *F. solani*, was also noted.

The abundance of *Fusarium* pathogens generally decreased during grain storage (Table 3). A significant reduction of abundance of toxin-producing endophytic fungi was observed in wheat grain treated with *A. pullulans*, and it was determined 30.5% after six months of storage in comparison with the control. *A. pullulans* inhibited the penetration of wheat kernels by fungi of the genus *Fusarium* only on selected experimental dates, and its inhibitory effect was limited to *F. culmorum*. The results of other studies also demonstrated that in field cultivation, the inhibitory effect of *A. pullulans* is generally weak and affected by weather conditions (Khan et al., 2004). *A. pullulans* isolates inhibited the proliferation of *F. culmorum* by competing for iron and creating siderophores (Wang et al., 2009). The results reported by Zhang et al. (2007), who investigated the ability of *C. flavescens* OH 182.9 isolates to inhibit spike infections caused by *F. graminearum*, validate the observation that the defense response induced in plants by yeasts is not the key mechanism responsible for wheat protection against pathogens.

4. Conclusions

Results of study indicate that wheat grain from plants protected with *A. pullulans* is highly similar in composition to control grain. Biological

Table 3
Average abundance of yeasts, *Fusarium* spp. and *A. alternata* colonizing winter wheat grain.

Microorganisms	Evaluation date[†]	Epiphytic		Endophytic	
		Control sample	*A. pullulans* sample	Control sample	*A. pullulans* sample
		Log(CFU + 1) per 10 g of grain			
Yeasts	H	2.75[c]	3.10[b]	2.40[d]	2.09[d]
	S	3.51[a]	3.84[a]	2.08[d]	2.17[d]
	CFUs in 72 kernels				
Total *Fusarium* spp. species[‡] [number of colonies]	H	7.33[b] *Fa* [1], *Fc* [5], *Fp* [6], *Fso* [1]	6.00[b] *Fa* [3], *Fc* [1], *Fp* [5], *Fs* [5]	9.00[b] *Fa* [1], *Fc* [4], *Fp* [11], *Fs* [1]	4.66[c] *Fa* [2], *Fc* [3], *Fg* [1], *Fp* [5], *Fs* [2]
	S	7.33[b] *Fc* [1], *Fp* [9], *Fs* [2]	5.33[b] *Fa* [1], *Fc* [13], *Fs* [2]	12.00[a] *Fa* [2], *Fc* [18], *Fp* [10], *Fs* [1]	3.66[c] *Fe* [1], *Fp* [2], Fs [4]
A. alternata	H	2.00[c]	2.00[c]	7.00[b]	3.33[c]
	S	12.00[a]	11.00[a]	8.33[a]	11.66[a]

[†] H—immediately after harvest, S—after six months' storage.
[‡] Abbreviations: *Fa—F. avenaceum*, *Fc—F. culmorum*, *Fe—F. equiseti*, *Fg—F. graminearum*, *Fp—F. poae*, *Fs—F. sporotrichioides*, *Fso—F. solani*; Means denoted by the same superscript letters within features not differ significantly at $P < 0.01$.

treatment contributed only to a slight increase of grain tocols, alkylresorcinols and sterols. This effect can be assumed as beneficial from a nutrition point of view. The variation in the content and composition of proteins was low, and indicates that wheat grain baking value which is mostly dependent on gluten proteins remained unchanged. The applied biological control treatment increased the abundance of epiphytic yeasts on wheat kernels, which inhibited the growth of endophytic *Fusarium* fungi.

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