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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