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Aureobasidium pullulans Used as a Biological Control Agent under Field Conditions Affects the Microbial Quality of Winter Wheat Grain

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***Abstract*—**The biological activity of *A. pullulans* isolates against species of the genus *Fusarium,* bacteria of the genus *Azotobacter* and pseudomonads colonizing wheat kernels was evaluated. A field experiment was carried out in 2009-2011, in north-eastern Poland. Winter wheat (cv. Bogatka) plants were sprayed with a cell suspension of *A. pullulans* at a density of 10^6 - 10^8 per cm^3 water at the stem elongation stage and the heading stage. Untreated plants served as control. The abundance of epiphytic yeasts, bacteria of the genus *Azotobacter,* pseudomonads and *Fusarium* pathogens on wheat grain was estimated at harvest and after six months' storage. The average size of yeast communities was significantly greater on wheat kernels treated with a cell suspension of *A. pullulans,* compared with control samples. In 2010-2011, biological control reduced the abundance of some species of the genus *Fusarium.*

Keywords*—Aureobasidium pullulans,* winter wheat grain, *Fusarium,* bacteria.

I. Introduction

AUREOBASIDIUM PULLULANS is a saprotrophic, polymorphic yeast-like fungus that produces hyphae, chlamydospores and blastospores [6]. *A. pullulans* is commonly found on the surface and in the tissues of crops. It produces lytic enzymes such as chitinase and beta-1,3-glucanase [15], which suppress the growth of phytopathogens [13]. Particular attention should be paid to the inhibitory effect of *A. pullulans* on mycotoxin-producing *Fusarium* species that colonize winter wheat grain [14], [16]. Changes induced by *A. pullulans* in communities of non-pathogenic yeasts and bacteria can affect wheat flour strength [5] and the sowing value of seeds that is determined, among others, by the abundance of nitrogen-fixing bacteria [2], [8]. The objective of this study was to determine the effect of the antagonistic species *A. pullulans* on the abundance of yeasts, bacteria of the genus *Azotobacter,* pseudomonads and filamentous fungi, including *Fusarium* species, colonizing winter wheat grain under field conditions.

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II. Methods

A. Field Experiment

A field-plot experiment was conducted in 2009-2011 in north-eastern Poland, in a randomized block design with four replications Winter wheat (*Triticum aestivum* L., cv. Bogatka) was sown in $20m^2$ plots. Wheat plants were protected biologically with a mixture of *A. pullulans* isolates, at the stem elongation stage (BBCH 31) and the heading stage (BBCH 55). The isolates were identified by biochemical and molecular (PCR-ITS) methods. The microorganisms, isolated from the grain of winter wheat cv. Tonacja, were cultured on potato glucose agar (Merck) for seven days at 24°C. After incubation, they were rinsed from the medium with sterile water and transferred into 1l flasks to produce fungal cell suspensions with density of 10^6 - 10^8 cells per cm^3 water, which were sprayed onto plants. Control plants were sprayed with water.

B. Abundance of Microorganisms

The abundance of microorganisms colonizing wheat kernels was estimated at harvest and after six months' storage at 11°C. 10g grain samples were placed in 250ml flasks filled with 90 ml of sterile water. The flasks were shaken for 60 minutes on the shaker table type 358 S at 180rpm, to wash off microorganisms from kernel surfaces. Microbial suspensions were serially diluted, two-fold or three-fold, and 0.1ml samples were placed in Petri dishes using a pipette. Selective Martin's medium [10], King's B medium [7] or nitrogen-free agar medium [11] cooled to 42°C were poured into the dishes. Microorganisms were incubated in the dark at 24°C for seven days. Yeast and bacterial colony forming units were counted on Petri dishes. The colonies of filamentous fungi were identified to the genus or species level based on their sporulation characteristics [4], [9].

C. Statistic Methods

The significance of differences between average microbial counts was estimated by the Newman-Keuls test using Statistica 9.0 software (ANOVA). The abundance of microorganisms was determined in view of the applied dilutions, and the data were log transformed (CFU+1). The community structure of filamentous fungi was expressed as the number of colonies of a given species or genus grown on Martin's medium and the number of identified species. The

biodiversity of filamentous fungi was estimated using the Margalef's index, which indicates the number of species – 1/ln (abundance of fungal communities).

III. Results

The microbial communities isolated from wheat kernels were typical of the analyzed ecological niche, and they were dominated by pseudomonads, yeasts and *Azotobacter* bacteria (Tables I-III). In all years of the study, the communities of yeasts, *Azotobacter* bacteria and pseudomonads were significantly more abundant after six months of grain storage than immediately after harvest (Tables I-III). In 2009-2011, the population size of yeasts isolated at harvest from the surface of wheat kernels treated with a cell suspension of *A. pullulans* was by 28.80%, 12.72% and 3.82% larger, respectively, compared with the control treatment (Table I). In the first two years, the stimulating effect of the biological control agent was also observed after six months' storage (19.22% and 9.40%, respectively).

There was a significant positive correlation between the abundance of epiphytic yeasts, and bacteria of the genus *Azotobacter* and pseudomonads (r = 0.770 and r = 0.595, respectively). The stimulating effect of the biological control agent was affected by weather conditions. Only in 2009, the community of pseudomonads was more abundant in the *A. pullulans* treatment than in the control treatment on both dates of analysis (Table II). The stimulating effect of biological control on *Azotobacter* bacteria was noted only in 2010 (Table III).

TABLE I
EPIPHYTICYEASTS FUNGI FROM THE SURFACE OF WINTER WHEAT GRAIN

Treatment	Sampling time	Log(CFU + 1) per g grain					
		2009		2010		2011	
Control	After harvest	2.50	a	2.75	a	3.09	b
	After six months' storage	2.81	a	3.51	d	4.53	g
A. pullulans	After harvest	3.22	c	3.10	b	3.21	c
	After six months' storage	3.35	d	3.84	e	4.14	f

Values followed by the same letters do not differ significantly for microorganisms groups according to the Newman-Keuls test ($p<0.001$).

Under field conditions, communities of epiphytic yeasts had an inhibitory effect on selected pathogens. A weak negative correlation was observed between the abundance of yeasts colonizing the surface of winter wheat kernels and the abundance of epiphytic filamentous fungi of the genus *Fusarium* (r = - 0.205). In 2009-2011, the above pathogens dominated in the community of filamentous fungi, accounting for 58.07% of all identified colonies (Table IV). The predominant species was *F. poae, F. culmorum* and *F. sporotrichiodes* were also relatively abundant, accounting for 23.51, 12.81% and 15.56% of all isolates (Fig. 1).

TABLE II
EPIPHYTIC PSEUDOMONADS BACTERIA FROM THE SURFACE OF WINTER WHEAT GRAIN

Treatment	Sampling time	Log(CFU + 1) per g grain					
		2009		2010		2011	
Control	After harvest	5.04	c	4.86	bc	5.83	e
	After six months' storage	4.28	a	4.66	b	6.23	f
A. pullulans	After harvest	5.40	d	4.08	a	4.81	bc
	After six months' storage	4.55	b	4.77	bc	6.46	f

Values followed by the same letters do not differ significantly for microorganisms groups according to the Newman-Keuls test ($p<0.001$).

TABLE III
EPIPHYTIC *AZOTOBACTER* BACTERIA FROM THE SURFACE OF WINTER WHEAT GRAIN

Treatment	Sampling time	Log(CFU + 1) per g grain					
		2009		2010		2011	
Control	After harvest	2.84	a	2.85	a	3.71	b
	After six months' storage	3.11	a	4.01	bc	4.68	cd
A. pullulans	After harvest	3.01	a	2.97	a	3.78	b
	After six months' storage	3.00	a	4.09	c	4.68	cd

Values followed by the same letters do not differ significantly for microorganisms groups according to the Newman-Keuls test ($p<0.001$).

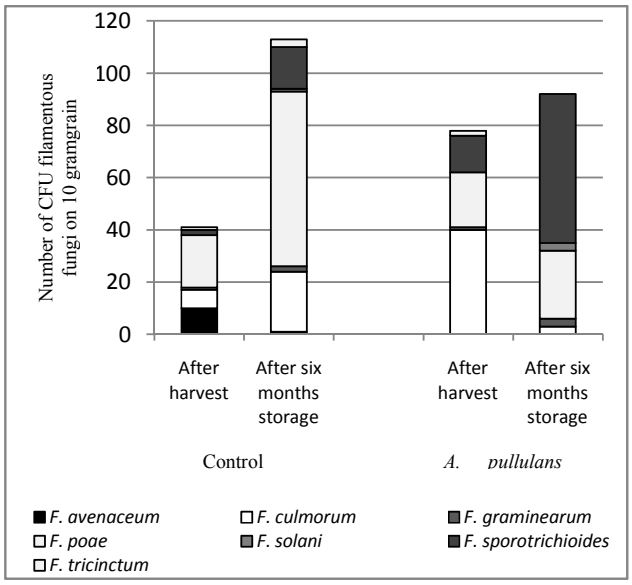

Fig. 1 Filamentous fungi from the surface of winter wheat grain

The community of *Fusarium* species comprised also *F. graminearum, F. tricinctum, F. solani* and *F. concolor*, but their abundance did not exceed 1.5% of all colonies. The abundance of the above pathogens on the surface of wheat kernels harvested in 2010 and 2011 reduced during grain storage (Table IV), and the noted decrease was particularly high in the treatment involving biological control with *A. pullulans* (at 82.05% and 68.75%, respectively), compared with the control treatment (Table IV). However, *A. pullulans* inhibited only the growth of *F. avenaceum*, *F. culmorum* and *F. poae* (Fig. 1). In all years of the study at harvest and in 2009 on both dates of analysis, the biological control agent did not suppress the growth of *Fusarium* pathogens (Table IV, Fig. 1).

TABLE IV
EPIPHYTIC FILAMENTOUS FUNGI FROM THE SURFACE OF WINTER WHEAT GRAIN

Species of fungi	Control						*A. pullulans*						Total
	2009		2010		2011		2009		2010		2011		
	A	B	A	B	A	B	A	B	A	B	A	B	
Alternaria alternata				1	1			4	4	3			13
Cladosporium herbarum		18				72						3	93
Fusarium spp.	18	85	10	24	13	5	26	83	39	7	16	5	331
Other saprotrophs	2	6		7	2	15		4	12	9	4	26	87
Non-sporulating colonies			1		1	12			1			31	46
Total colonies	20	109	11	32	17	104	26	91	56	19	20	65	570
Number of species	7	8	4	5	6	7	5	4	6	9	8	6	32
Biodiversity (Margalef index)*	2.00	1.49	1.25	1.15	1.76	1.29	1.23	0.67	1.24	2.72	2.34	1.20	4.89

A- After harvest, B - After six months' storage
* - Calculated using the formula given in the Methods section.

The applied biological control had a significant effect on the biodiversity and abundance of other species of filamentous fungi – it contributed to a 96.67% reduction in the population size of *Cladosporium herbarum* (Table IV), while it did not inhibit the growth of *A. alternata*.

IV. DISCUSSION

A. pullulans applied under field conditions considerably affected the homeostasis between the microorganisms colonizing winter wheat kernels. The species contributed to an increase in the abundance of microorganisms that support plant growth. Bacteria of the genus *Azotobacter* and pseudomonads, analyzed in our study, are regarded as plant growth promoters [2], [8] or as potential antagonists of pathogens [3], [12], [17]. In all years of the study, biological control contributed to an increase in the abundance of yeasts, which most probably modified wheat flour strength. Gliadin content increased and the content of glutenin fractions of high molecular weight (HMW) and low molecular weight (LMW) decreased in winter wheat grain (unpublished data).

In the present study, the abundance of *Fusarium* pathogens in winter wheat grain was 3.44-fold higher than that reported by Berghofer et al. (2003). *A .pullulans* suppressed the growth of *Fusarium* species only on some dates of analysis, and its inhibitory effect was limited to *F. avenaceum*, *F. culmorum* and *F. poae* [1]. Zhang et al. (2007) demonstrated that under greenhouse conditions, the *Cryptococcus flavescens* OH 182.9 isolate applied to protect wheat spikes reduced the severity of *F. graminearum* infections. The cited authors reported that the defense response induced in plants by the analyzed isolate was not the key mechanism responsible for spike protection against infections [17]. However, the current study shows that under field conditions the use of yeasts for biological control of wheat pathogens may be less effective in adverse weather conditions.

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