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Original Research

Yeasts Isolated from Wheat Grain Can Suppress Fusarium Head Blight and Decrease Trichothecene Concentrations in Bread Wheat and Durum Wheat Grain

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Abstract

Fusarium head blight (FHB) is the most dangerous disease in all regions where bread wheat and durum wheat are grown, mostly due to grain contamination with trichothecenes produced by fungi of the genus *Fusarium*. Biological control of FHB with the use of yeast suspensions could pose a viable alternative to fungicides. The aim of this study was to perform *in vitro* selection of yeast isolates capable of inhibiting the development of FHB and reducing the concentrations of type A and type B trichothecenes in grain. In a field experiment, the inoculation of durum wheat spikes with *Fusarium culmorum* decreased grain yield by 9.13% and led to the highest accumulation of deoxynivalenol (DON) in grain at 11.704 mg kg^{-1}. Isolates *Candida sake* Cs58, *Rhodotorula glutinis* Rg64 and *Debaryomyces hansenii* Dh53 most effectively inhibited the decline in durum wheat yield. Biological treatments induced a 10-fold decrease in the DON content of inoculated grain. Spike inoculation with *F. culmorum* decreased bread wheat yield by 19.88%. The isolate *Aureobasidium pullulans* Ap24 was most effective in decreasing FHB symptoms, whereas the isolate *D. hansenii* Dh53 reduced DON concentration by 11.33-fold in inoculated grain and decreased nivalenol concentration by 18.12-fold in the grain of non-inoculated bread wheat.

Keywords: *Fusarium culmorum,* biological protection, *Aureobasidium, Debaryomyces*

Introduction

Durum wheat (*Triticum turgidum* L. spp. *durum*) and bread wheat (*Triticum aestivum* L. spp. *aestivum*) are important small grain cereals that are produced for human consumption. In 2017, the ten leading wheat producing countries were China, India, Russia, the USA, France, Australia, Canada, Pakistan, Ukraine and Germany. In Poland, bread wheat is grown on 2.38 million hectares and durum wheat is cultivated in southern Poland on around 2,200 hectares [1]. Tetraploid durum wheat and hexaploid bread wheat are

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susceptible to colonization by *Fusarium* fungi causing Fusarium head blight (FHB). However, due to their different origin, wheat cultivar differ in their resistance against initial infection (type I) and resistance against fungal spread within a spike (type II) [2]. The severity of FHB epidemics is affected not only by wheat cultivar and weather conditions but also by the structure of the *Fusarium* species complex. *Fusarium graminearum* sensu stricto is the most common species. Other *Fusarium* species have also been frequently reported, including *F. avenaceum, F. culmorum, F. poae, F. langsethiae* and *F. sporotrichioides* [3, 4].

Fusarium head blight (FHB) significantly decreases grain yields and lowers the quality of grain through contamination with mycotoxins, including trichothecenes [5]. Type B trichothecenes (deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON) and nivalenol (NIV) are produced mainly by *F. graminearum* and *F. culmorum* and are among the most frequently identified mycotoxins in cereal grain. In Central Europe, DON concentration in durum wheat grain was determined in the range of 116.94 μg kg^{-1} to 10.88 μg/g, subject to cultivar and weather conditions [6, 7]. In the discussed region, the average DON content of *T. aestivum* grain ranged from 140.2 to 210 μg kg^{-1} in non-inoculated grain [8], and the highest concentration of DON was determined at 11650 μg kg^{-1} in grain inoculated with *F. culmorum* [9]. Apart from their adverse effects on plants [10], type B trichothecenes disrupt gut homeostasis, and compromise neuroendocrine function and immunity of animals [Table 1, 10-13]. The less frequently detected T-2 toxin, a type A trichothecene, is produced by, among others, *F. sporotrichioides* and *F. poae* [14]. Type A trichothecenes also include scirpentriol (STO), T-2 tetraol, T-2 triol, diacetoxyscirpenol (DAS) and HT-2. In a study by Stuper and Perkowski [15], STO was the most frequently identified type A trichothecene colonizing bread wheat grain in a concentration range of 0.001-0.042 mg kg^{-1}. Relating toxicity of individual trichothecenes based on any of these classification systems is not always straightforward [Table 1, 16]. The synergistic effects of several trichothecenes are greater than those exerted by individual trichothecenes [17, 18]. Type A trichothecenes such as DAS and T-2 toxin are generally more cytotoxic than type B trichothecenes such as DON [19]. Animal toxicity increases with increasing oxygenation of EPT [20]. A test of *Fusarium* trichothecenes on the model plant system *Chlamydomonas* revealed that both type A and type B C-3 acetylated trichothecenes were much less toxic than the corresponding C-3 hydroxyl trichothecenes [21].

In line with the integrated pest management strategy introduced by the European Union [22], crop producers should attempt to control *Fusarium* fungi with the use of alternatives, including biological, methods [23-26]. Alternative approaches can be used in combination with fungicides which are not always effective in controlling FHB due to the rapid spread of the disease and variations in the sensitivity of *Fusarium* species to these chemical control agents [27]. Safe mycotoxin levels have been set for wheat grain to protect the consumers' health. According to Commission Regulation (EC) No. 1881/2006 of 19 December 2006 and Commission Recommendation of 27 March 2013 [28, 29], the maximum concentrations of DON in unprocessed wheat grain are set at 1250 μg kg^{-1} for *T. aestivum* and 1750 μg kg^{-1} for *T. durum*, and the total content of T-2 toxin and HT-2 toxin may not exceed 100 μg kg^{-1} [30].

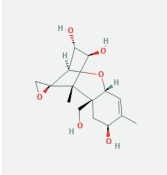
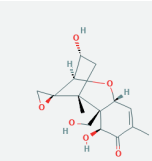
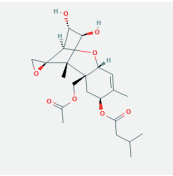
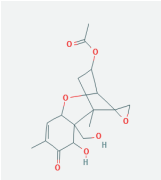
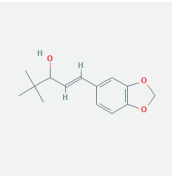
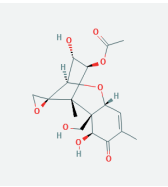
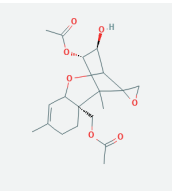
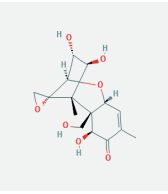
Yeasts are ubiquitous on cereal kernels, and they far more abundant fungi of the genus *Fusarium* [31, 32]. The role played by yeasts in agroecosystems and their usefulness for inhibiting the development of *Fusarium* pathogens through antibiosis and competition during the growing season have been widely researched [32-35]. Yeasts are also capable of biotransforming trichothecenes into less toxic compounds [36]. Yeasts can potentially be used for reducing trichothecene contamination of wheat grain. The aim of this study was to perform *in vitro* selection of yeast isolates obtained from wheat grain, capable of inhibiting the development of *Fusarium culmorum: Aureobasidium pullulans*, *Candida sake*, *C. albicans*, *Debaryomyces hansenii*, *Metschnikowia pulcherrima* and *Rhodotorula glutinis*. Active yeast isolates were evaluated under field conditions for their ability to alleviate the symptoms of FHB in bread wheat and durum wheat spikes inoculated with *F. culmorum*. Wheat spikes were analyzed to determine: (1) the severity of FHB symptoms, (2) grain yield, (3) grain contamination with fungi of the genus *Fusarium*, (4) content of type A and B trichothecenes in grain. The application of yeast in biological protection treatments against FHB in bread wheat has been previously studied by, among others, Schisler et al. [25, 26] and Wachowska et al. [37]. The effectiveness of biological protection methods against *Fusarium* pathogens in durum wheat has been evaluated by very few studies [7].

Experimental

Yeast Isolation

Yeasts were isolated from the grain of winter wheat cv. Bogatka according to a previously described method [37]. Yeasts were washed off from grain by shaking randomly selected kernels in 250 cm^3 flasks containing 90 cm^3 of sterile water (Elpin+ 378 S Shaker Table, Poland). The resulting fungal suspensions were transferred to Petri dishes. Selective Martin's medium [38] cooled to 42ºC was poured into the plates. Pure yeast cultures grown on potato dextrose agar (PDA, Merck, Poland) were cultured at 24ºC for 48 h (En 120 incubator, Poland).

Table 1. Characteristics of type A and B trichothecenes.

Trichothecene A compound	Chemical structure	Activity	Trichothecene B compound	Chemical structure	Activity
T-2 Tetraol		Potential endocrine disrupting compound [13]	DON		Phytotoxic, affect astrointestinal homeostasis, growth, neuroendocrine function, and immunity of animals [10]
HT-2-toxin		Potential immunosuppressive agent [11]	3ADON		Gastrointestinal effects: ulceration or bleeding from small intestine, hypermotility, diarrhea [19]
STO		Hepatotoxicity [75]	FUS-X		Potential immunosuppressive agent [11]
DAS		Hepatotoxicity [76]	NIV		Potential nematicide [12]

STO scirpentriol, DAS - diacetoxyscirpenol, DON - deoxynivalenol, 3ADON - 3-acetyl-deoxynivalenol, FUS-X - fusarenon X, NIV - nivalenol.

Yeast Identification

Selected isolates were identified based on their morphological features and the shape and size of budding cells, pseudofilaments and chlamydospores [39]. Yeast DNA was extracted with the DNA Genomic Mini AX Yeast Kit (A&A Biotechnology, Poland). The fragment containing ITS 1, 5.8S and ITS 2 rDNA was amplified with specific ITS5 primers (F) GTATCGGACGGAGATCCAGC and ITS4 primers (R) TTGCTCAGTGCATTG TCGG [26] in the FailSafe PCR system (Epicentre, Poland). The electrophoresis of amplification products was carried out in 1% agarose gel (Prona, Poland) with the addition of Midori Green (ABO, Poland) in TBE buffer (Sigma, Poland). PCR products were sequenced by the Institute of Biophysics and Biochemistry of the Polish Academy of Sciences in Warsaw. Sequence similarity was determined in the BLAST program in the NCBI database [40].

Isolate Accession Numbers

The ITS1, 5.8S and ITS2 rDNA sequences of 20 isolates were deposited in GenBank under the following accession numbers: *Aureobasidium pullulans* - KX444657, KX44458, KX444670, KX424381-KX424384, *Candida sake* - KX444660, *C. albicans* - KX444661, *Debaryomyces hansenii* - KX444669, KX444668, *Metschnikowia pulcherrima* - KX424389, *Rhodotorula glutinis* - KX424385-KX424388, KX444653-KX444655.

In vitro Selection of Yeast Isolates Inhibiting the Development of *Fusarium culmorum* Colonies

The activity of yeast isolates was evaluated on Petri plates filled with PDA to select isolates capable of inhibiting the growth of *Fusarium* fungi (Merck, Poland) (Table 2). Agar discs with a diameter of 5 mm, overgrown with *F. culmorum* filaments, were placed in the center of Petri plates with a diameter of 9 cm. Isolates of *A. pullulans*, *C. sake*, *C. albicans*, *D. hansenii*, *M. pulcherrima* and *Rh. glutinis* cultured for 48 hours were placed on Petri plates at a distance of 2 cm from agar discs. After four days of incubation at 24ºC (En 120 incubator, Poland), the isolates were photographed (Sony Alpha DSLR-A330, Japan), and the images were transferred to a PC (Integrit, Poland). The ellipticity index of a pathogenic colony was calculated by dividing the smaller diameter by the larger diameter. The area of the pathogenic colony was measured in the ImageJ 1.49 program [41]. Yeast isolates which

Table 2. Characteristic features of epiphytes and endophytes isolated from wheat and used for screening tests by dual culture with *Fusarium culmorum*.

Isolate	Origin [A]	Species	Dual culture inhibition			
			Ellipticity index (±SE)	Isolate activity [B]	% inhibition of colony growth (±SE)	Isolate activity [C]
Ca 6a	En/NC	*Candida albicans*	0.99^{a}(±0.010)	NA	34.05^{b-e}(±3.556)	NR
Ca 6	En/NC	*Candida albicans*	0.79^{a-o}(±0.113)	NA	32.68^{cde}(±2.516)	NR
Ca 4	En/NC	*Candida albicans*	0.57^{rs}(±0.023)	NA	76.94^{abc}(±4.267)	NR
Ca 5	En/NC	*Candida albicans*	0.67^{l-s}(±0.162)	A	80.95^{abc}(±8.717)	R
Ca 3	En/NC	*Candida albicans*	0.83^{a-m}(±0.042)	NA	85.98^{abc}(±1.680)	R
Ca 2	En/NC	*Candida albicans*	0.84^{a-n}(±0.025)	NA	86.52^{abc}(±2.740)	R
Ca 1	En/NC	*Candida albicans*	0.78^{b-p}(±0.034)	NA	88.00^{abc}(±0.537)	R
Mp 13	EP/E	*Metschnikowia pulcherrima*	0.76^{c-s}(±0.070)	NA	90.36^{a}(±0.055)	Z
Mp 12	EP/E	*Metschnikowia pulcherrima*	0.82^{a-n}(±0.071)	NA	89.89^{a}(±0.468)	R
Mp 11	EP/E	*Metschnikowia pulcherrima*	0.68^{k-s}(±0.065)	NA	87.19^{ab}(±2.231)	R
Mp 10	EP/E	*Metschnikowia pulcherrima*	0.62^{o-s}(±0.085)	A	82.00^{abc}(±6.762)	R
Mp 9	EP/E	*Metschnikowia pulcherrima*	0.74^{e-s}(±0.025)	NA	87.88^{ab}(±0.061)	R
Mp 8	En/I	*Metschnikowia pulcherrima*	0.68^{l-s}(±0.056)	A	88.27^{a}(±2.919)	R
Mp 7	En/I	*Metschnikowia pulcherrima*	0.73^{g-s}(±0.040)	NA	89.46^{a}(±1.032)	R
Ca 14	En/I	*Candida albicans*	0.82^{a-n}(±0.029)	NA	46.19^{a-e}(±19.597)	NR
Ca 15	En/I	*Candida albicans*	0.94^{a-f}(±0.032)	NA	44.37^{a-e}(±17.750)	NR
Mp 21	En/I	*Metschnikowia pulcherrima*	0.79^{a-p}(±0.066)	NA	91.18^{a}(±0.434)	Z
Mp 20	En/I	*Metschnikowia pulcherrima*	0.82^{a-n}(±0.112)	NA	83.89^{abc}(±3.725)	R
Mp 19	En/I	*Metschnikowia pulcherrima*	0.71^{i-s}(±0.140)	NA	76.19^{abc}(±6.080)	NR
Mp 18	En/I	*Metschnikowia pulcherrima*	0.74^{c-s}(±0.006)	NA	84.34^{abc}(±2.272)	R
Mp 17	En/I	*Metschnikowia pulcherrima*	0.84^{a-n}(±0.059)	NA	87.87^{ab}(±1.749)	R
Mp 16	En/I	*Metschnikowia pulcherrima*	0.94^{a-f}(±0.028)	NA	85.91^{abc}(±0.426)	R
Ca 39	En/I	*Candida albicans*	0.93^{a-g}(±0.058)	NA	18.47^{de}(±5.095)	NR
Ca 40	En/I	*Candida albicans*	0.59^{prs}(±0.185)	NA	54.07^{a-e}(±6.128)	NR
Ca 41	En/I	*Candida albicans*	0.84^{a-n}(±0.043)	NA	57.94^{a-e}(±1.322)	NR
Ca 42	En/I	*Candida albicans*	0.95^{abc}(±0.029)	NA	67.96^{a-e}(±0.358)	NR
Ca 43	En/I	*Candida albicans*	0.83^{a-n}(±0.004)	NA	80.26^{abc}(±13.723)	R
Cs 37	En/I	*Candida sake*	0.93^{a-f}(±0.003)	NA	17.02^{e}(±10.673)	NR
Cs 38	En/I	*Candida sake*	0.94^{a-e}(±0.036)	NA	63.50^{a-e}(±1.377)	NR
Ca 39a	En/I	*Candida albicans*	0.74^{f-s}(±0.165)	NA	74.74^{abc}(±13.303)	NR
Ca 40a	En/I	*Candida albicans*	0.95^{a-e}(±0.011)	NA	68.60^{a-e}(±1.652)	NR
Ap 36	En/I	*Aureobasidium pullulans*	0.97^{ab}(±0.005)	NA	63.01^{a-e}(±10.934)	NR
Cs 42a	En/I	*Candida sake*	0.90^{a-d}(±0.063)	NA	53.73^{a-e}(±13.221)	NR
Ap 22	En/B	*Aureobasidium pullulans*	0.81^{a-o}(±0.047)	NA	90.26^{a}(±0.241)	Z
Ap 23	En/B	*Aureobasidium pullulans*	0.74^{d-s}(±0.037)	NA	88.87^{ab}(±0.110)	R
Ap 24	En/B	*Aureobasidium pullulans*	0.79^{a-o}(±0.003)	NA	90.45^{a}(±0.364)	Z
Ap 25	En/B	*Aureobasidium pullulans*	0.89^{a-k}(±0.068)	NA	91.76^{a}(±0.633)	Z

Table 2. Continued.

Isolate	Origin [A]	*Species*	Dual culture inhibition			
			Ellipticity index (±SE)	Isolate activity [B]	% inhibition of colony growth (±SE)	Isolate activity [C]
Ap 26	En/B	*Aureobasidium pullulans*	0.85^{a-m}(±0.021)	NA	91.37^{a}(±1.693)	Z
Ap 27	En/B	*Aureobasidium pullulans*	0.72^{h-s}(±0.112)	NA	85.50^{abc}(±0.378)	R
Ap 29	En/B	*Aureobasidium pullulans*	0.78^{a-p}(±0.009)	NA	88.56^{a}(±1.198)	R
Ap 30	En/B	*Aureobasidium pullulans*	0.79^{a-o}(±0.032)	NA	87.62^{ab}(±2.795)	R
Dh 33	En/B	*Debaryomyces hansenii*	0.69^{j-s}(±0.143)	A	81.66^{abc}(±7.230)	R
Ap 34	En/B	*Aureobasidium pullulans*	0.71^{i-s}(±0.012)	NA	87.67^{bc}(±3.649)	R
Ap 35	En/B	*Aureobasidium pullulans*	0.71^{i-s}(±0.087)	NA	85.12^{abc}(±4.957)	R
Dh 50	EP/NC	*Debaryomyces hansenii*	0.65^{m-s}(±0.042)	A	62.87^{a-e}(±16.856)	NR
Dh 51	EP/NC	*Debaryomyces hansenii*	0.56^{s}(±0.076)	A	65.08^{a-e}(±9.158)	NR
Dh 52	EP/NC	*Debaryomyces hansenii*	0.70^{i-s}(±0.003)	NA	83.54^{abc}(±2.327)	R
Dh 53	EP/E	*Debaryomyces hansenii*	0.56^{s}(±0.076)	A	76.13^{abc}(±11.313)	NR
Dh 54	EP/E	*Debaryomyces hansenii*	0.74^{d-s}(±0.184)	NA	71.47^{abc}(±7.065)	NR
Dh 55	EP/E	*Debaryomyces hansenii*	0.79^{a-o}(±0.024)	NA	80.91^{abc}(±0.578)	R
Dh 56	EP/E	*Debaryomyces hansenii*	0.77^{a-r}(±0.065^{j})	NA	81.71^{abc}(±1.542)	R
Dh 57	EP/E	*Debaryomyces hansenii*	0.77^{c-r}(±0.145)	NA	82.37^{abc}(±2.203)	R
Cs 58	EP/E	*Candida sake*	0.70^{j-s}(±0.035)	NA	80.45^{abc}(±1.611)	R
Cs 59	EP/E	*Candida sake*	0.73^{g-s}(±0.038)	NA	81.50^{abc}(±3.319)	R
Ap 60	EP/I	*Aureobasidium pullulans*	0.74^{e-s}(±0.001)	NA	88.82^{a}(±1.356)	R
Ap 61	EP/I	*Aureobasidium pullulans*	0.76^{c-s}(±0.025)	NA	89.91^{a}(±1.611)	R
Ap 62	EP/I	*Aureobasidium pullulans*	0.75^{c-s}(±0.024)	NA	89.22^{a}(±2.974)	R
Dh 63	EP/I	*Debaryomyces hansenii*	0.57^{rs}(±0.036)	A	65.82^{a-e}(±4.214)	NR
Rg 64	EP/I	*Rhodotorula glutinis*	0.84^{a-n}(±0.147)	NA	46.81^{a-e}(±27.406)	NR
Rg 65	EP/I	*Rhodotorula glutinis*	0.70^{i-s}(±0.011)	NA	80.59^{a-e}(±25.677)	R
Cs 66	EP/I	*Candida sake*	0.87^{a-l}(±0.019)	NA	83.87^{abc}(±1.666)	R
Dh 67	EP/I	*Debaryomyces hansenii*	0.92^{a-g}(±0.002)	NA	80.65^{abc}(±1.032)	R
Cs 68	EP/I	*Candida sake*	0.87^{a-l}(±0.092)	NA	85.78^{abc}(±0.577)	R
Ap 70	EP/I	*Aureobasidium pullulans*	0.64$^{n-s(}$±0.027)	A	75.67^{abc}(±8.125^{j})	NR
Dh 69	EP/I	*Debaryomyces hansenii*	0.76^{c-s}(±0.056)	NA	89.28^{a}(±2.534)	R
Dh 71	EP/I	*Debaryomyces hansenii*	0.67^{l-s}(±0.042)	A	73.85^{abc}(±5.247)	NR
Ap 73	EP/B	*Aureobasidium pullulans*	0.91^{a-i}(±0.056)	NA	90.13^{a}(±1.315)	Z
Ap 74	EP/B	*Aureobasidium pullulans*	0.76^{c-s}(±0.178)	A	86.16^{abc}(±3.213)	R
Ap 75	EP/B	*Aureobasidium pullulans*	0.81^{a-n}(±0.026)	NA	90.21^{a}(±1.308)	Z
Ap 76	EP/B	*Aureobasidium pullulans*	0.83^{a-n}(±0.086)	NA	88.32^{a}(±0.661)	R
Ap 77	EP/B	*Aureobasidium pullulans*	0.82^{a-n}(±0.014)	NA	85.37^{abc}(±0.247)	R
Ap 78	EP/B	*Aureobasidium pullulans*	0.79^{a-p}(±0.024)	NA	88.18^{ab}(±0.358)	R
Dh 79	EP/E	*Debaryomyces hansenii*	0.85^{a-m}(±0.045)	NA	80.25^{abc}(±0.082)	R
Dh 80	EP/E	*Debaryomyces hansenii*	0.79^{a-p}(±0.045)	NA	81.47^{abc}(±3.649)	R
Dh 81	EP/E	*Debaryomyces hansenii*	0.74^{f-s}(±0.047)	NA	83.00^{abc}(±2.217)	R

Table 2. Continued.

Ap 82	EP/E	*Aureobasidium pullulans*	$0.68^{l\text{-}s}$(±0.082)	A	81.41^{abc}(±6.410)	R
Ap 83	EP/E	*Aureobasidium pullulans*	$0.77^{a\text{-}p}$(±0.034)	NA	88.00^{abc}(±0.537)	R

[A] - EN - endophytes, EP- epiphytes, NC – grain from unprotected plants, I – grain from plants subjected to intensive fungicide protection, E – grain from plants subjected to extensive fungicide protection, B – grain from plants subjected to biological protection.
[B] A– isolates which induced significant changes in the shape of *Fusarium culmorum* colonies (ellipticity index <= 0.70) in dual cultures were regarded as active; (NA) the remaining isolates were not active.
[C] - Z – isolates which decreased colony area by at least 90%, R – isolates which decreased colony area by 80-90%, NR – isolates which inhibited colony growth by less than 80%.
Values that did not differ significantly in the SNK test ($p<0.001$) are marked with the same letters in columns.
SE-standard error

inhibited the growth of pathogenic fungi by minimum 90% relative to the area of *F. culmorum* colonies growing without yeast isolates and yeast isolates which decreased the ellipticity index of pathogenic colonies to 0.70 or below were regarded as highly active.

Field Experiment

Field-plot experiment 1 with biological, fungicidal and integrated crop protection was carried out in Bałcyny (53°36'N, 19°51'E) (Table 3) on bread wheat (cv. Skagen, resistant to infections caused by *Fusarium* spp.) and durum wheat (cv. Komnata, highly susceptible to infections caused by *Fusarium* spp.) [42, 43]. The experiment had a randomized block design with three replications. Plot area was 15 m^2. In biological control treatments (Biol 1), the following yeast suspensions were sprayed on wheat plants during three growth stages: *C. sake* Cs58 – first node detectable at least 1 cm above the tillering node (BBCH 31) [24], *Rh. glutinis* Rg64 – middle of heading (BBCH 55), and *D. hansenii* Dh53 – full flowering (BBCH 65) (Table 3). In fungicide treatments (Fung1), the following fungicides were applied during three growth stages: a commercial mixture of fenpropimorph, metrafenone and epoxiconazole (1.5 dm^3 per ha, concentration of fungicide 0.25%) in stage BBCH 31, a commercial mixture of prothioconazole and fluoxastrobin (1 dm^3 per ha) in stage BBCH 55, and tebuconazole (1 dm^3 per ha, concentration of fungicide 0.17%) in the watery ripe stage (BBCH 71). Integrated crop protection (Integ1) involved three treatments: laminarin resistance inducer (1 dm^3 per ha, the concentration of product 0.17%) in stage BBCH 31, a commercial mixture of prothioconazole and fluoxastrobin (1 dm^3 per ha, concentration of fungicide 0.17%) in stage BBCH 55, and a suspension of *D. hansenii* Dh50 cells in stage BBCH 65 (Table 3). The experiment was conducted in three replications. Unprotected plants were the control.

Field-plot experiment 2 involving integrated (Integ 2) and chemical protection (Fung 2, Fung 3) treatments was carried out in Bałdy in north-eastern Poland (53°36'N, 20°36'E) – Table 3. Crop protection treatments composed of azole (Fung 2, Fung 3, Integ 2), morpholine (Integ 2), benzimidazole (Fung 2) and strobilurin (Fung 2, Fung 3). Fungicides were applied twice (Fung 3, Integ 2) or three times (Fung 2) to winter wheat (*T. aestivum* L., cv. Bogatka) sown in plots with an area of 20 m^2 each in growth stages BBCH 31, BBCH 55 and BBCH 71 (Table 3). The experiment had a randomized block design. The biological treatment involving a suspension of *D. hansenii* Dh53 cells (Integ 2) was performed at full flowering (BBCH 65). The experiment was conducted in four replications. Unprotected plants were the control.

Field-plot experiment 3 with biological crop protection was performed in Bałdy (N 53° 36', E 20° 36'). Winter wheat (*T. aestivum* L., cv. Tonacja) was sown in plots with an area of 1 m^2 each. The experiment had a randomized block design with three replications. Suspensions of *A. pullulans* Ap24 or *D. hansenii* Dh53 cells were applied three times in stages BBCH 31, BBCH 55 and BBCH 65 (Table 3). Unprotected plants were the control.

Preparation of Yeast Isolates for Crop Protection Treatments

Five yeast isolates, *A. pullulans* Ap24 (NCBI GenBank accession number KX444670), *D. hansenii* Dh53 (KX444669), *D. hansenii* Dh50 (KX444668), *C. sake* Cs58 (KX444660) and *Rh. glutinis* Rg64 (KX424386) were used in biological protection treatments. Yeast isolates were incubated (En 120 incubator, Poland) on PDA (Merck, Poland) at 24ºC. Yeasts were rinsed off with 5 cm^3 of sterile water (per plate) with the use of an inoculation loop into 1 dm^3 flasks. Yeast suspensions were brought to a concentration of 10^6-10^7 cells in 1 cm^3 of water (Thoma cell counting chamber, Fein-Optik, Germany). Backpack sprayers with 12 dm^3 tank capacity (Marolex Titan 12, Poland) were filled with 1 dm^3 of the yeast suspension each, diluted with 9 dm^3 of water, and they were used to spray plots with an area of 15-20 m^2. Plots with an area of 1 m^2 were sprayed with a manual sprayer (1.5 dm^3, Marolex Master, Poland) containing 250 cm^3 of the yeast suspension and 750 cm^3 of water. Biological treatments were performed on windless days in the afternoon.

Table 3. Treatments applied to winter wheat.

Treatment	BBCH 31	BBCH 55	BBCH 65	BBCH 71
	(First node at least 1 cm above tillering node)	(Middle of heading)	(Full flowering)	(Second fruit fall)
	Experiment 1 (Bałcyny)			
Fung 1	Fenpropimorph, metrafenone, epoxyconazole[1] (0.25%, 1.5 dm^3 per ha)	Prothioconazole, fluoxastrobin[2] (0.17%, 1 dm^3 per ha)	No treatment	Tebuconazole[3]
Integ 1	Laminarin[4] (0.17%, 1 dm^3 per ha)	Prothioconazole, fluoxastrobin [2] (0.17%, 1 dm^3 per ha)	*Debaryomyces hansenii* Dh50 KX444668	No treatment
Biol 1	*Candida sake* Cs58 KX444660	*Rhodotorula glutinis* Rg 64 KX424386	*Debaryomyces hansenii* Dh53 KX444669	No treatment
	Experiment 2 (Bałdy)			
Integ 2	Fenpropimorph, epoxiconazole[5] (0.17%, 1 dm^3 per ha)	Propiconazole[6] (1 dm^3 per ha)	*Debaryomyces hansenii* Dh53 KX444669	No treatment
Fung 2	Chlorothalonile[7]	Epoxiconazole, piraclostrobin[8] (0.25%, 1.5 dm^3 per ha)	No treatment	Tebuconazole[3]
Fung 3	No treatment	Epoxiconazole, piraclostrobin[8] (0.25%, 1.5 dm^3 per ha)	No treatment	Tebuconazole[3]
	Experiment 3 (Bałdy)			
Biol 2	*Aureobasidium pullulans* Ap24 KX444670	*Aureobasidium pullulans* Ap24 KX444670	*Aureobasidium pullulans* Ap24 KX444670	No treatment
Biol 3	*Debaryomyces hansenii* Dh53 KX444669	*Debaryomyces hansenii* Dh53 KX444669	*Debaryomyces hansenii* Dh53 KX444669	No treatment

[1] – Capallo 337.5 SE (fenpropimorph - 19.49% , metrafenone - 7.31%, epoxyconazole - 6.09%, BASF SE, Germany), [2] – Fandango 200 EC (prothioconazole 10.0%, fluoxastrobin - 10.0%, BASF SE, Germany), [3] – Tarcza Łan 250 EW (tebuconazole - 250 g/ dm^3, Sharda Polska Sp. z o.o., Poland), [4] – Vaxiplant SL (laminarin - 5.0%, Laboratoires GOËMAR SAS [1], France), 5 – Duett Star 334 SE (fenpropimorph - 24.56%, epoxiconazole - 8.25%, BASF SE, Germany), [6] – Bumper 250 SC (propiconazole - 25.1%, Makhteshim Chemical Works Ltd, Israel), 7 – Gwarant 500 SC (chlorothalonile - 40.16%, France), 8 – Opera Max 147,5 SE (epoxiconazole - 6.01 %, piraclostrobin - 8.18 %, Germany).

Inoculation of Wheat Spikes with *Fusarium culmorum*

All and entire field-plot experiments were conducted in duplicate. Flowering spikes were inoculated with an aqueous suspension of *F. culmorum* Fc32 spores in stage BBCH 65. The concentration of fungal cells in the suspension was 10^4 cells in 1 cm^3 of water. Fungal spores were rinsed off with 5 cm^3 of sterile water from fungal colonies cultured for 14 days on PDA (Merck, Poland) at 28ºC. Spikes were inoculated at full flowering (BBCH 65) with a backpack sprayer (Marolex Titan 12, Marolex, Poland).

Evaluation of Wheat Spike Health and Grain Yield

The severity of FHB symptoms was evaluated in the hard dough stage (BBCH 87) in 100 wheat plants randomly selected from each treatment based on the scale proposed by the European and Mediterranean Plant Protection Organization [45]. The results were expressed as the average percentage of spike surface area affected by the disease. In weakly infected spikes, a single spikelet exhibited FHB symptoms, whereas in severely infected spikes, FBH symptoms were observed on several spikelets. Grain was harvested with a plot harvester in the fully ripe stage (BBCH 92). Grain yield was expressed in grams per 1 m^2 of plot area.

Grain Colonization By Yeasts and Fungi of the Genus *Fusarium*

The abundance of epiphytic fungi of the genus *Fusarium* and yeasts colonizing wheat kernels was determined immediately after harvest according Wachowska et al. [37]. Additionally non-disinfected (to obtain epiphytic colonies) and surface-disinfected (to obtain endophytic colonies) kernels were placed on PDA (Merch, Poland) in Petri plates [37]. Colonies of *Fusarium* spp. were identified [46] after 7 days of incubation at 24ºC (En 120 incubator, Poland).

The counts of yeasts and *Fusarium* fungi (N) rinsed off from 1 g of grain were converted according to the following formula: $N = n/10^{-r} \cdot v$, where v is the number of colonies on the plate, 10^{-r} is the dilution coefficient, and v is the volume of the plated suspension.

The colony counts of *Fusarium* growing on wheat kernels were expressed as a percentage of total kernels within epiphytes and endophytes.

Chemical Analysis of Ergosterol

Ergosterol was determined by high-performance liquid chromatography (HPLC). Samples containing 100 mg of ground grain were placed in 17 ml culture tubes, suspended in 2 ml of methanol, treated with 0.5 ml of 2M aqueous sodium hydroxide, and tightly sealed. The tubes were placed inside 250 ml plastic bottles, tightly sealed and microwaved (AVM 401/1WH microwave oven, Whirlpool, Sweden) at 2450 MHz with 900 W maximum output. Grain samples were irradiated (370 W) for 20 s and, approximately 5 min later, for another 20 s. After 15 minutes, the contents of culture tubes were neutralized with 1M aqueous hydrochloric acid, 2 ml of MeOH was added, and the contents were extracted with pentane (3 x 4 ml). Pentane extracts were pooled and evaporated to dryness in a nitrogen stream. Before analysis, the samples were dissolved in 4 ml of MeOH, filtered through 13 mm syringe filters with 0.5 µm pore diameter (Fluoropore Membrane Filters, Millipore, Ireland), and evaporated to dryness in a nitrogen stream. The sample extract was dissolved in 1 ml of MeOH, and it was analyzed in the Acquity H class UPLC system equipped with a Waters Acquity PDA detector (Waters, USA). Chromatographic separation was performed on the Acquity UPLC® BEH C18 column (100 mm×2.1 mm, particle size 1.7 µm) (Waters, Ireland). Ergosterol was detected with a Waters Acquity PDA detector (Waters, USA) at 282 nm. The presence of ERG was confirmed by comparing retention times and co-injecting every tenth sample with an ergosterol standard.

Trichothecene Analysis

Sub-samples (10 g) were extracted with acetonitrile/water (82:18) and purified on a charcoal column (Celite 545/charcoal Draco G/60/activated alumina neutral 4:3:4; w/w/w). Group A trichothecenes (H-2 toxin, T-2 toxin, T-2 tetraol) were analyzed as TFAA derivatives (Table 1). The dried sample was combined with 100 µl of trifluoroacetic acid anhydride. After 20 minutes, the reagent was evaporated to dryness under nitrogen. The residue was dissolved in 500 µl of isooctane, and 1 µl was injected onto a gas chromatograph-mass spectrometer. Group B trichothecenes (DON, NIV, 3-AcDON, 15-AcDON) were analyzed as trimethylsilyl ether (TMS) derivatives. The dried extract was combined with 100 µl of the trimethylsilyl imidazole/trimethylchlorosilane (TMSI/TMCS; 100/1 v/v) mixture. After 10 minutes, 500 µl of isooctane were added, and the reaction was quenched with 1 ml of water. The isooctane layer was used for analysis, and 1 µl of the sample was injected onto the GC/MS system. The analyses were run on a gas chromatograph (Hewlett Packard GC 6890) connected to a mass spectrometer (Hewlett Packard 5972 A, Waldbronn, Germany) with an HP-5MS 0.25 mm x 30 m capillary column. Injection port temperature was 280ºC and transfer line temperature was 280ºC. The analyses were conducted at programmed temperatures, separately for group A and group B trichothecenes. The temperature program for group A trichothecenes was as follows: initial temperature of 80ºC for 1 min, followed by a temperature increase of 80ºC-280ºC at 10ºC/min, with the final temperature maintained for 4 min. The temperature program for group B trichothecenes was as follows: initial temperature of 80ºC for 1 min, followed by a temperature increase of 80ºC-200ºC at 15ºC/min for 6 minutes and 200-280ºC at 10ºC/min, with the final temperature maintained for 3 min. The helium flow rate was held constant at 0.7 ml/min. A quantitative analysis was performed in the single ion monitored mode (SIM) with the following detection ions: STO – 456 and 555; T-2 tetraol – 455 and 568; T-2 triol – 455, 569 and 374; HT-2 – 455 and 327; T-2 – 327 and 401; DON – 103 and 512; 3-AcDON – 117 and 482; 15-AcDON – 193 and 482; NIV – 191 and 600. A qualitative analysis was performed in the SCAN mode (100-700 amu). The recovery rates for the analyzed toxins were as follows: STO – 82±5.3%; T-2 triol – 79±5.1%; T-2 – 86±3.8%; T-2 tetraol – 88±4.0%; HT-2 – 91±3.3%; DON – 84±3.8%; 3AcDON – 78±4.8%; 15 AcDON –74±2.2%; and NIV – 81±3.8%. The limit of detection was 0.01 mg/kg.

Statistical Analysis

The analysis of variance was performed in the Statistica 12 program. The data regarding the abundance of yeasts and *Fusarium* spp. communities were log transformed (CFU+1). The significance of differences between means was estimated by analysis of variance and the significance of differences between the average was estimated by the Student-Newman-Keuls (SNK) test ($p<0.01$).

Results

Identification of Yeast Species

Yeasts isolates belonged to three orders within the divisions Basidiomycota and Ascomycota. *A. pullulans* belonged to Ascomycota, order Dothideales, family Dothideaceae. In the order Saccharomycetales, *D. hansenii and C. albicans* belonged to the family Debaryomycetaceae, whereas *M. pulcherrima* was a member of the family Metschnikowiaceae. *Rhodotorula glutinis* belonged to the division Basidiomycota, subdivision Pucciniomycotina, class Microbotryomycetes, order Sporidiobolales, family Sporidiobolaceae.

Table 4. Grain yield, FHB severity and colonization of winter wheat grain by *Fusarium* spp. and yeast.

Cultivar/ inoculation	Treatment	Yield g/m^2	FHB severity	ERG (mg/kg)	*Fusarium* spp.			Yeast
					CFU x 10^2	Percentage of [b]		CFU x 10^2
					1 g^{-1} grain [a]	Epiphytes	Endophytes	1 g^{-1} grain [a]
Experiment 1 (Bałcyny)								
Komnata	Control	669.44 [b]	0.55	1.27	0.35 [a]	5.56 [abc]	12.50 [bc]	3.99 [a]
	Fung 1	638.89 [b]	3.33	2.07	0.07 [de]	13.89 [abc]	5.56 [c]	3.96 [ab]
	Integ 1	638.61 [b]	1.03	6.88	0.14 [d]	4.17 [bc]	2.78 [c]	3.65 [bcd]
	Biol 1	660.83 [b]	2.01	9.48	0.27 [abc]	5.56 [abc]	2.78 [c]	4.36 [a]
Komnata/ inoculation with *F. culmorum*	Control	608.33 [b]	5.41	20.50	0.29 [abc]	13.89 [abc]	8. 33 [bc]	3.79 [bc]
	Fung 1	646.29 [b]	7.70	4.58	0 [e]	12.50 [abc]	4.17 [bc]	3.69 [cde]
	Integ 1	611.11 [b]	5.43	8.66	0.26 [c]	11.11 [abc]	2.78 [bc]	4.38 [a]
	Biol 1	644.44 [b]	2.38	11.42	0.27 [abc]	12.50 [abc]	5.56 [bc]	4.42 [a]
Skagen	Control	1118.89 [a]	0.01	0.95	0.28 [abc]	4.17 [bc]	4.17 [bc]	3.72 [cde]
	Fung 1	1057.33 [a]	0.01	1.12	0.27 [c]	1.39 [c]	4.17 [bc]	2.93 [f]
	Integ 1	1123.89 [a]	0.01	2.63	0.25 [c]	8.33 [abc]	5.56 [c]	3.26 [def]
	Biol 1	1114.17 [a]	0.04	4.27	0.14 [d]	6.94 [abc]	9.72 [bc]	3.38 [cde]
Skagen/ inoculation with *F. culmorum*	Control	1106.67 [a]	0.75	9.35	0.29 [abc]	20.83 [a]	9.72 [bc]	2.46 [g]
	Fung 1	1180.56 [a]	1.73	3.44	0.30 [abc]	15.28 [abc]	9.72 [bc]	3.05 [ef]
	Integ 1	1068.33 [a]	0.82	2.95	0.27 [c]	19.44 [ab]	19.44 [ab]	3.90 [b]
	Biol 1	1135.28 [a]	0.95	5.48	0 [e]	8.33 [abc]	23.61 [a]	0 [h]
Experiment 2 (Bałdy)								
Bogatka	Control	479.67 [ab]	0.08	2.06	0.31 [a]	15.28 [a]	12.50	3.55 [a]
	Integ 2	547.67 [ab]	0	10.55	0.27 [ab]	11.11 [ab]	8.33	2.72 [ab]
	Fung 2	537.00 [ab]	0.25	3.27	0.28 [ab]	9.72 [ab]	15.28	2.67 [ab]
	Fung 3	580.33 [a]	0.42	3.81	0.28 [ab]	2.78 [ab]	12.50	3.03 [ab]
Bogatka/ inoculation with *F. culmorum*	Control	384.33 [ab]	1.00	19.41	0.16 [b]	15.29 [a]	12.50	3.09 [ab]
	Integ 2	315.67 [b]	0	5.98	0.25 [ab]	11.11 [ab]	15.28	2.93 [ab]
	Fung 2	338.00 [ab]	0	2.68	0.27 [ab]	4.17 [ab]	11.11	3.41 [a]
	Fung 3	364.33 [ab]	0	3.49	0.28 [ab]	9.72 [ab]	6.94	3.16 [ab]
Experiment 3 (Bałdy)								
Tonacja	Control	303.30	1.36	1.11	0.29 [a]	1.39 [b]	8.33	3.78 [ab]
	Biol 2	296.13	0.70	5.29	0.07 [b]	2.78 [ab]	8.33	3.13 [ab]
	Biol 3	299.97	0.33	8.49	0.30 [a]	2.77 [ab]	12.50	3.43 [ab]
Tonacja/ inoculation with *F. culmorum*	Control	258.51	0.78	12.36	0.32 [a]	9.72 [ab]	0.10	3.42 [ab]
	Biol 2	188.44	0	8.29	0.31 [a]	12.50 [a]	20.83	4.28 [a]
	Biol 3	181.32	2.89	6.22	0.29 [a]	8.33 [ab]	16.67	3.79 [ab]

[a] – epiphytes obtained from kernel surfaces in experiment 1 (Bałcyny): *F. culmorum* - 28.28%, *F. poae* - 35.69%, *F. graminearum* - 3.36%, *F. sporotrichioides* - 32.65%), in experiment 2 (Bałdy): (*F. culmorum* - 26.26%, *F. poae* - 69.19%, *F. graminearum* - 1.51%) and in experiment 3 (Bałdy): (*F. culmorum* - 15.68%, *F. poae* - 75.98%, *F. graminearum* - 4.90%). Values that did not differ significantly in experiments in the SNK test ($p<0.001$) are marked with the same letters in columns.

Dual Culture Assay to Determine the Antagonistic Effects of Epiphytes and Endophytes Against *Fusarium culmorum in vitro*

A total of 77 yeast isolates, identified by sequencing, were selected for *in vitro* screening by dual culture with *F. culmorum* (Table 2). Ten out of the 38 epiphytic isolates obtained from the surface of wheat kernels, including four isolates of *D. hansenii*, two isolates of *A. pullulans* and *M. pulcherrima* each, and one isolate of *Rh. glutinis* and *C. sake* each, induced substantial changes in the shape of pathogenic colonies. Fungal colonies cultured in the presence of the above isolates had the shape of elongated ellipsoids with an ellipticity index of less than 0.70. Eight yeast isolates (six isolates of *A. pullulans* and two isolates of *M. pulcherrima*), including five isolates from kernel tissues, reduced the area of pathogenic colonies by more than 90%.

Fusarium Head Blight and Wheat Grain Yield

In experiment 1 the yield of control bread wheat cv. Skagen was 1.7-fold higher (statistically significant) on average in comparison with durum wheat cv. Komnata (Table 3). Fungicides (Fung 1) increased (non-significant) the grain yield of wheat cv. Komnata by 6.24% (inoculated with *F. culmorum*) relative to unprotected plants, whereas the biological treatment (Biol 1) increased grain yield by 5.93%. The grain yield of wheat cv. Skagen increased (non-significant) by 6.68% in response to fungicide protection (Fung 1) and by 2.58% in response to the biological treatment (Biol 1). The increase in the severity of FHB symptoms did not exceed 8% and was higher in wheat cv. Komnata than in wheat cv. Skagen. The severity of FHB in wheat cv. Komnata (inoculated with *F. culmorum*) was non-significant reduced by 56.01% only by the biological treatment (Biol 1) relative to unprotected plants.

In experiment 2 spike inoculation with *F. culmorum* decreased yield by 19.82%, and none of the applied treatments minimized that drop. In non-inoculated treatments subjected to fungicide or integrated protection, grain yield non-significant increased by 11.95% (Fung 2), 14.17% (Integ 2) and 20.98% (Fung 3) relative to non-inoculated control. The severity of FHB symptoms was non-significant reduced by 100% relative to inoculated control.

In experiment 3 the yield of control plants inoculated with *F. culmorum* non-significant decreased by 14.77%. None of the analyzed treatments increased yields. The severity of FHB symptoms was non-significant reduced by 51.47% (Biol 2, non-inoculated plants) and 100% (Biol 2, inoculated plants) relative to control.

Content of ERG and Grain Colonization by Yeast and Fungi of the Genus *Fusarium*

Spike inoculation with suspensions of *F. culmorum* spores or yeast cells containing ERG increased ERG concentration in the inoculated grain of all wheat cultivars in control treatments and in non-inoculated grain in biological treatments (Tables 4). Fungicide (Fung 1) and integrated (Integ 1) treatments significantly decreased the abundance of pathogenic fungi in the non-inoculated grain of wheat cv. Komnata. Fungal colonies of the genus *Fusarium* were not isolated from the surface of inoculated kernels protected with Fung 1. In the grain of wheat cv. Skagen, fungal abundance was most effectively reduced by Biol 1. In experiment 3 the biological treatment involving *A. pullulans* Ap24 (Biol 2) also significantly decreased the abundance of *Fusarium* spp. in the grain of wheat cv. Tonacja (Table 4).

Yeasts were at least 10-fold more abundant than *Fusarium* fungi on the surface of grain in all wheat cultivars (Table 4). Grain was colonized predominantly by *F. culmorum* and *F. poae* which accounted for 60.63% of all fungal colonies of the genus *Fusarium*. *F. graminearum* was most prevalent (11.94%) on the disinfected kernels of wheat cv. Tonacja and *F. sporotrichioides* was most abundant on the grain of wheat cvs. Komnata and Skagen.

Trichothecene Content of Grain

All grain samples were contaminated with DON and NIV. Safe levels of DON were exceeded in the inoculated and non-protected grain of all wheat cultivars (Tables 5), in the non-inoculated and biologically protected grain of wheat cv. Skagen and in the inoculated grain of wheat cv. Bogatka subjected to integrated protection (Integ 2). The content of type A trichothecenes differed across wheat cultivars. T-2 tetraol (0.005-0.256 mg kg^{-1}) was detected in most grain samples of wheat cvs. Komnata, Skagen and Bogatka, and its content was highest in the non-inoculated control grain of wheat cv. Komnata. Scirpentriol was detected in most grain samples of wheat cvs. Bogatka and Tonacja at 0.007-0.039 mg kg^{-1}.

The content of DON was determined at 0.473 mg kg^{-1} in the unprotected grain of durum wheat cv. Komnata, and it increased to 11.704 mg kg^{-1} after inoculation with *F. culmorum*. In experiment 1, all protective treatments decreased DON concentration in grain inoculated with *F. culmorum* (Table 5). The greatest reduction was noted in the grain of wheat cv. Komnata (Fung 1, 11.41-fold decrease relative to control) where the DON content of control grain exceeded safe levels 6.7-fold. In the non-inoculated and protected grain of both wheat cultivars, the content of T-2 tetraol

Table 5. Content of other *Fusarium* metabolites in winter grain (mg/kg sample).

Cultivar/ inoculation	Treat-ment	DON	3Ac-DON	15Ac-DON	FUS-X	NIV	Sum TOX	STO	T-2 Tetraol	DAS	HT-2	Sum TOX
Experiment 1(Bałcyny)												
Komnata	Control	0.473	0.061	0.036	0.031	0.024	0.626	<LOD	0.256	<LOD	<LOD	0.256
	Fung 1	0.294	0.040	0.020	0.031	0.043	0.429	<LOD	0.005	<LOD	<LOD	0.005
	Integ 1	0.666	0.001	0.032	0.032	0.057	0.789	<LOD	0.009	<LOD	<LOD	0.009
	Biol 1	0.346	0.054	0.026	<LOD	0.735	1.161	0.003	0.008	<LOD	<LOD	0.011
Komnata/ in-oculation with *F. culmorum*	Control	11.704	0.185	0.029	0.027	0.073	12.018	<LOD	0.005	<LOD	<LOD	0.005
	Fung 1	1.026	0.072	0.034	0.025	0.115	1.273	<LOD	0.012	<LOD	<LOD	0.012
	Integ 1	1.166	0.049	0.010	<LOD	0.035	1.260	<LOD	0.009	<LOD	<LOD	0.010
	Biol 1	1.119	0.072	0.022	<LOD	0.114	1.327	<LOD	0.015	<LOD	<LOD	0.015
Skagen	Control	0.315	0.046	0.024	<LOD	0.231	0.616	<LOD	0.019	<LOD	<LOD	0.020
	Fung 1	0.039	<LOD	0.009	<LOD	0.029	0.078	0.006	<LOD	<LOD	<LOD	0.006
	Integ 1	0.04	0.001	0.009	<LOD	0.032	0.082	<LOD	0.013	<LOD	<LOD	0.013
	Biol 1	1.647	0.018	0.008	<LOD	0.008	1.681	0.004	0.005	<LOD	<LOD	0.009
Skagen/ in-oculation with *F. culmorum*	Control	7.383	0.116	0.017	0.036	0.072	7.623	0.004	0.014	<LOD	<LOD	0.018
	Fung 1	0.105	0.140	0.015	<LOD	0.051	0.311	<LOD	0.005	<LOD	<LOD	0.005
	Integ 1	0.322	0.061	0.034	0.026	0.122	0.566	<LOD	0.012	<LOD	<LOD	0.012
	Biol 1	1.776	0.041	0.025	0.025	0.027	1.895	<LOD	0.043	<LOD	<LOD	0.043
Experiment 2 (Bałdy)												
Bogatka	Control	0.106	0.003	0.001	0.001	0.025	0.136	<LOD	0.006	<LOD	<LOD	0.006
	Integ 2	0.053	0.001	0.001	0.001	0.028	0.084	0.007	0.005	<LOD	<LOD	0.012
	Fung 2	0.133	0.014	0.006	0.023	0.041	0.217	0.012	0.005	<LOD	<LOD	0.017
	Fung 3	0.145	0.029	0.065	0.188	0.127	0.554	0.006	0.016	<LOD	0.004	0.026
Bogatka/ inoculation with *F. culmorum*	Control	6.128	0.314	0.042	0.026	0.064	6.575	0.017	0.006	<LOD	<LOD	0.023
	Integ 2	1.759	0.026	0.009	0.125	0.191	2.109	0.010	0.006	<LOD	<LOD	0.016
	Fung 2	0.659	0.015	0.005	0.069	0.098	0.846	0.008	0.020	<LOD	0.003	0.031
	Fung 3	0.878	0.019	0.008	0.088	0.090	1.084	0.025	0.004	<LOD	0.002	0.031
Experiment 3 (Bałdy)												
Tonacja	Control	1.099	0.044	0.013	0.012	0.453	1.621	0.007	0.009	<LOD	0.004	0.020
	Biol 2	0.939	0.032	0.011	0.012	0.236	1.228	0.006	<LOD	0.001	0.011	0.018
	Biol 3	0.567	0.019	0.006	0.009	0.025	0.626	0.003	<LOD	<LOD	<LOD	0.003
Tonacja/ inoculation with *F. culmorum*	Control	7.808	0.143	0.048	0.027	0.167	8.193	0.010	<LOD	<LOD	<LOD	0.010
	Biol 2	0.849	0.035	0.005	0.005	0.071	0.965	<LOD	<LOD	<LOD	<LOD	<LOD
	Biol 3	0.689	0.024	0.007	0.012	0.023	0.757	0.039	<LOD	0.002	<LOD	0.041

LOD - limit of detection, LOD for all mycotoxins is 0.001 (mg/kg)

was lower than in unprotected grain. In experiment location 2, the concentration of DON in the inoculated and protected grain of wheat cv. Bogatka was at least 3-fold lower than in control grain. In non-inoculated grain, DON levels were reduced only by integrated protection. In experiment 3 all biological treatments decreased the content of DON and NIV both with and without inoculation with *F. culmorum* (Table 5). Isolate *D. hansenii* Dh53 (Biol 3) was most effective, and it reduced DON concentration in inoculated grain

11.33-fold and NIV concentration in non-inoculated grain by 18.12-fold relative to control.

Discussion

The results of this study indicate that yeasts obtained from winter wheat grain are promising biological control agents (BCAs) that inhibit the development of *F. culmorum* and decrease trichothecene concentrations in the grain of bread wheat and durum wheat. In this study, three foliar BCA treatments applied to wheat plants inoculated with *F. culmorum* decreased the DON content of grain below 1.250 mg kg^{-1} in most cases. Skagen was the only wheat cultivar where the concentration of DON remained high after biological treatment at 1.647 mg kg^{-1} in non-inoculated grain and 1.776 mg kg^{-1} in inoculated grain. Similar results were reported by Matarese et al. [47], where only one of the ten analyzed isolates of the genus *Trichoderma* reduced DON concentration in *F. culmorum*-inoculated grain *in vivo*, but only to 1.75 mg kg^{-1}. In a field study conducted by Schisler et al. [26], isolate *Cryptococcus flavescens* OR 182.9 decreased trichothecene concentrations to up to 2.8 ppm in naturally infected grain. In a greenhouse experiment evaluating seven FHB antagonists, only *Bacillu*s strains 43.3 and 43.4 and the *Cryptococcus* OH182.9 strain reduced disease severity by 48-95% and decreased DON concentration in grain by 83-98% [25]. However, the same antagonistic strains delivered variable results under field conditions. *Bacillus* strains had no effect on either FHB severity or DON concentration in grain, whereas strain OH 182.9 reduced FHB severity and DON content by 50% [25, 27]. According to Khan and Doohan [48], *Pseudomonas flourescens* strains induced a significant 74% decrease in DON levels. Kim and Vujanovic [49] found that fungal isolate *Sphaerodes mycoparasitica* SMCD 2220-01 was a host-specific mycoparasitic against plant-pathogenic *Fusarium* species. The tested isolate also reduced DON content by 89%. The above authors also demonstrated that *S. mycoparasitica* SMCD 2220-01 was capable of transforming DON to the less toxic deoxynivalenol sulfate [49].

In our study, 19% of *D. hansenii, Rh. glutinis, A. pullulans, M. pulcherrimia* and *C. sake* isolates grown in dual culture with *F. culmorum* significantly inhibited the pathogen's growth. A screening study conducted by Comby et al. [50] revealed that isolates of yeast species *Rh. kratochvilovae, Rh. lysiniphila* and *Sporobolomyces roseus* inhibited the development of *F. culmorum* and *F. graminearum* colonies by 3-14.5%. In the above study, *Aureobasidium protae* reduced the development of pathogenic colonies by 25-37.5%. The potential ability of *A. pullulans* to inhibit the growth of *F. culmorum* on wheat grain was also reported by Wachowska et al. [37]. *A. pullulans* is used in commercial preparations for post-harvest control of pathogens (BoniProtect) and fireblight (BlossomProtect) [34, 51].

There is extensive evidence to indicate that *A. pullulans* isolates produce aureobasidin, a compound with fungicidal properties [52], or fusigen, a siderophore which chelates iron and enables the antagonist to compete more effectively for iron with the pathogen [53]. Castoria et al. [54] studied the effectiveness of *A. pullulans* against the *B. cinerea* pathogen in stored apples and observed high levels of activity of β-1,3-glucanase, an enzyme produced by this antagonist. In the work of Ippolito et al. [55], *A. pullulans* produced extracellular enzymes exochitinase and β-1,3-glucanase in wounded apples and *in vitro*. These enzymes degrade the walls of pathogenic cells and induce resistance in plants [55]. In the current study, *A. pullulans* most probably exerted protective effects by competing with *Fusarium* fungi for space and nutrients. This hypothesis is supported by the following observations: (1) *A. pullulans* was most effective in decreasing the area of fungal colonies in dual cultures; (2) the discussed yeast was most abundant on the grain of bread wheat inoculated with *F. culmorum;* (3) *A. pullulans* effectively minimized the symptoms of FHB; (4) the analyzed yeast decreased grain colonization by fungi of the genus *Fusarium;* (5) *A. pullulans* was less effective than other yeasts only in its ability to reduce DON levels in grain (by 14.64% in non-inoculated grain). In a field study by Schisler et al. [26], yeast isolate *Cryptococcus flavescens* OH 71.4 was not highly effective in reducing DON concentration (up to 20%) in the grain of two bread wheat cultivars.

In the present study, the following observations suggest that the isolate of *D. hansenii* (applied three times) produced biocidal compounds: (1) the shape of *F. culmorum* colonies grown with *D. hansenii* in dual cultures changed significantly; (2) *D. hansenii* was not highly effective in reducing the severity of FHB symptoms or decreasing grain colonization by *Fusarium* fungi; (3) *D. hansenii* significantly decreased DON concentration in grain. *Debaryomyces hansenii* (anamorph of *Candida famata*) is generally a non-pathogenic species which is resistant to salinity and low temperature. The species easily adapts to various ecosystems, and it is used in the biological protection of crops against *Penicillium* spp. [56] and *Botrytis cinerea* [57]. *Debaryomyces hansenii* also exerts antagonistic effects on pathogens colonizing foods, such as dairy products [58], dry-cured meat products [59] and dry-fermented sausage [60]. Interestingly, *D. hansenii* isolated from cheeses also exerted fungicidal effects on *Candida albicans* and *C. neoformans* which are dangerous for humans [61, 62]. The most widely described biocidal mechanism of *D. hansenii* against fungi relies on killer toxins [63] with a molecular mass of 22-23 kDA [64, 65, 66]. According to Żarowska [63], *D. hansenii* killer toxins probably bind to β-(1,6)-glucan in the cell walls of sensitive fungi and to an unidentified receptor in the cytoplasmic membrane, and they eliminate fungal cells by inhibiting their division. The European Food Safety Authority [66] has

qualified *D. hansenii* has as an isolate with qualified presumption of safety (QPS) status for industrial and commercial applications [67].

Three biological treatments involving the cell suspensions of yeast species *C. sake*, *Rh. glutinis* and *D. hansenii* were least effective against FHB in wheat. In the biologically protected grain of wheat cv. Skagen, DON concentration was determined at 1.647 mg kg^{-1}, and it exceeded the safe limit of 1250 µg kg^{-1}. The isolate of *Rh. glutinis* was characterized by a low growth rate and moderate activity *in vitro*. Lima et al. [68] found that *Rh. glutinis* exerted antagonistic effects on pathogens only at low temperatures. In the present study, *C. sake* effectively inhibited the development of *F. culmorum* in vitro, but it was not a reliable antagonist under field conditions. Laitila et al. [34] also demonstrated that *C. sake* exerted inhibitory effects against *F. cerealia* and *F. equiseti*. *Candida sake* is an ingredient of Candifruit, a commercial product for post-harvest control of pathogens. However, Candifruit is registered as a plant strengthening agent in Spain [51].

In this study, DON concentration was at least 1.5-fold higher in durum wheat grain inoculated with *F. culmorum* than in bread wheat grain. In a study conducted in southern Poland, Gorczyca et al. [7] demonstrated that wheat cv. Komnata had a high propensity for accumulating DON. The concentration of DON was 3.5-fold higher in the grain of wheat cv. Komnata than in the Austrian cultivar Auradur. In the work of Langevin et al. [69], durum wheat spikes were rapidly colonized by both trichothecene-producing and non-producing *F. graminearum* strains. The above authors suggested that durum wheat harboring genomes A and B does not have type II resistance against fungal pathogens. Bread wheat also harbors genomes A and B, but in a field study conducted by Langevin et al. [69], this species was significantly less infected by *F. graminearum* strains non-producing trichothecenes. For this reason, the cited authors probably concluded that genome D is more likely to harbor genes that encode type II resistance.

It should also be noted that the effectiveness of BCAs is largely determined by the severity of FHB, cultivar, environmental conditions and the mycotoxin content of grain. In our study, DON (40-1647 µg kg^{-1} grain) and NIV (23-735 µg kg^{-1} grain) were detected in all samples of non-inoculated grain harvested in 2017. The concentrations of these mycotoxins were generally higher than those reported by Bryła et al. [8]. In north-eastern Poland, bread wheat and durum wheat were colonized mainly by *F. culmorum* and rarely by *F. poae*. In contrast, *F. avenaceum* [7] and *F. graminearum* [5] were the predominant pathogens of durum wheat in southern Poland. *Fusarium graminearum* is the predominant pathogen of wheat in Europe, including in Hungary, Serbia-Montenegro and Austria [70, 71], Finland and Russia (European part) [72], Germany [73] and France [74].

Conclusions

The results of this study indicate that yeasts colonizing wheat grain can be applied as biological treatments to reduce the severity of FHB and decrease trichothecene concentrations in grain during heading, flowering and ripening. Yeast isolates should be thoroughly identified and tested before application. Yeasts have a complex mechanism of action and are sensitive to environmental factors; therefore, further research is required to select yeast isolates characterized by high levels of activity, high survivability on the protected plants, and low sensitivity to adverse environmental conditions in the field. Yeast biocontrol mechanisms represent unexplored field of research and plentiful opportunities for the development of commercial, yeast-based applications for plant protection.

Conflict of Interest

The authors declare no conflict of interest.

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