Food Control 138 (2022) 109039

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

The application of antagonistic yeasts and bacteria: An assessment of *in vivo* and under field conditions pattern of *Fusarium* mycotoxins in winter wheat grain

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ARTICLE INFO

Keywords:
Mycotoxins
Fusarium
bread wheat
Antagonistic microorganisms

ABSTRACT

Bacteria of the genus *Sphingomonas, Aureobasidium pullulans* yeast-like fungus and yeast *Debaryomyces hansenii* naturally colonize wheat grain. Selected isolates of these microorganisms, antagonistic against *Fusarium* spp., can be applied to wheat spikes to complement chemical control methods. The aim of this study was to determine the content of *Fusarium* mycotoxins in winter wheat grain *in vivo* and under field conditions, and to analyze the interactions between antagonistic microorganisms and *F. culmorum* and *F. avenaceum* pathogens in dual cultures. Seventeen toxic metabolites produced by *Fusarium* fungi were identified in grain: deoxynivalenol, deoxynivalenol-3-glucoside, culmorin, 15-hydroxyculmorin, 5-hydroxyculmorin, HT-2 toxin, nivalenol, nivalenol-3-glucoside, aurofusarin, enniatins, zearalenone, moniliformin, equisetin, and apicidin. Integrated fungicide and biological treatments decreased deoxynivalenol levels in grain in the field conditions from 141.36 to 72.76 μg/kg. Deoxynivalenol was not detected in grain treated with *D. hansenii* suspension, and culmorin content was 38 times lower than in unprotected grain. Fungicides and integrated treatments involving *D. hansenii* also decreased enniatin A1 content by 76.61 and 48.17%. In vitro yeasts considerably reduced moniliformin and enniatin concentrations by 7.67–92.87%. Antagonistic microorganisms, which inhibit the growth of *Fusarium* fungi through antibiosis and competition for nutrients, can effectively reduce the production of selected *Fusarium* toxins when combined with fungicides in the integrated approach.

1. Introduction

Bread wheat (*Triticum aestivum* L.) is grown mainly for consumption, and global wheat production reached 763.93 tons in 2019 (2019FAOSTAT). Wheat is infected by numerous pathogens, and several dozen *Fusarium* species that cause Fusarium head blight (FHB) are considered to be the most dangerous wheat pathogens. In Europe, bread wheat is colonized mainly by *F. culmorum*, *F. avenaceum*, *F. graminearum* and *F. poae*, depending on location and weather conditions (Becher et al., 2013; Duba et al., 2019; Góral et al., 2018; Karlsson et al., 2021). These pathogens infect wheat during the entire growing season, and they also cause Fusarium root rot (FRR) and Fusarium crown rot (FCR) (Beccari et al., 2011). Fungal pathogens produce toxic secondary metabolites that compromise the quality of wheat grain (Lindblad et al., 2013). Deoxynivalenol (DON), nivalenol (NIV) and estrogenic zearalenone (ZEA) produced by *F. culmorum* and *F. graminearum* are the most ubiquitous and toxicologically significant fungal metabolites. Nivalenol is also produced by *F. poae* (Karlsson et al., 2021; Lindblad et al., 2013; Venkatesh, Keller 2019). These mycotoxins can exert acute and chronic toxic effects, which is why the maximum levels of DON and ZEA in foodstuffs and feedstuffs are legally regulated (Antonissen et al., 2014; European Commission, 2006). Modern screening techniques support simultaneous qualitative and quantitative analyses of multiple mycotoxins (Karlsson et al., 2021). However, little is known about the combined effects of

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https://doi.org/10.1016/j.foodcont.2022.109039
Received 29 October 2021; Received in revised form 1 April 2022; Accepted 13 April 2022
Available online 18 April 2022
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multiple mycotoxins on humans. Research conducted on human gastric epithelial (GES-1) cells demonstrated that multiple deoxynivalenol-family mycotoxins (DON, NIV and their acetyl derivatives of 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), deoxynivalenol-3-glucoside (D3G), and fusarenon-X (FX)) were more or less toxic than individual mycotoxins (Yang et al., 2017). In a study by Gao et al. (2016, 2020), the cytotoxicity of aflatoxin M1 (AFM1) for human cell lines increased dramatically in the presence of ochratoxin A, ZEA, and/or α-zearalenol. The cited authors postulated that the maximum residue limit (MRL) for infant foods containing milk and cereals should be revised due to possible cross-contamination with multiple mycotoxins. Limited information is available about the toxicity and concentrations of *F. avenaceum* mycotoxins, including enniatins (ENNs) and moniliformin (MON), in food and feed (Jestoi et al., 2004, 2008; Karlsson et al., 2021; Kosiak et al., 2003; Uhlig et al., 2004; Uhlig, Torp, & Heier, 2006).

Each year, food and feed contamination with the mycotoxins produced by *Fusarium* fungi is evaluated around the world to protect consumers and animals against the toxic effects of these compounds. The maximum residue limits of many mycotoxins in foodstuffs are also legally regulated (Ji et al., 2019). However, the results of studies investigating mycotoxin prevention and detoxication strategies in food and feed are rarely presented (Wachowska, Waśkiewicz, & Jędryczka, 2017). Recent years have witnessed an upsurge of interest in bacteria and fungi that occur naturally in plants and can help control diseases through the production of antibiotics, mycoparasitism, competition for space and nutrients, and induction of plant immunity (Karlsson et al., 2021; Pieterse et al., 2014; Wachowska, Waśkiewicz, & Jędryczka, 2017). *Bacillus subtilis*, *B. velezensis* and *Pseudomonas piscium* bacteria secrete compounds that inhibit the development and virulence of *F. graminearum* (Chen et al., 2018; Palazzini et al., 2016; Zhao et al., 2014). A new biocontrol strain of *P. piscium* identified by Chen et al. (2018) produced phenazine-1-carboxamide and directly affected the activity of FgGcn5*F* protein in *F. graminearum*. As a result, the pathogen's growth, virulence and ability to biosynthesize mycotoxins were inhibited, which decreased the symptoms of FHB on wheat spikes. *Bacillus subtilis* SG6 produced chitinase, fengycins and surfactins which inhibited mycelial growth, sporulation and DON production by *F. graminearum* (Zhao et al., 2014). In a study by Palazzini et al. (2016), *B. velezensis* decreased FHB symptoms and deoxynivalenol accumulation by producing the lantibiotic ericin. Iron-chelating siderophores produced by *Pseudomonas* spp., *Bacillus* spp. and *Aureobasidium pullulans* yeast isolates enable antagonists to compete for nutrients with *F. graminearum* and inhibit the pathogen's development (Chi et al., 2012; Pal et al., 2001). The Earth's crust is abundant in iron, but its bioavailability is limited due to low solubility in water. Fungi and bacteria produce siderophores which are low-molecular-weight organic compounds with strong iron-chelating properties (Chi et al., 2012; Oide et al., 2014). Chi et al. (2012) demonstrated that fusigen produced by the marine-derived *A. pullulans* HN6.2 plays a unique role in iron chelation and uptake. *Fusarium graminearum* can also produce malonichrome, ferricrocin and TAFC siderophores which influence the pathogen's virulence and ability to produce sexual spores (Oide et al., 2014). Research has also revealed that selected *Fusarium* mycotoxins are biodegraded by bacteria and yeasts (Ito et al., 2013; Vanhoutte et al., 2016). The *Sphingomonas* sp. strain KSM1 isolated from the aquatic environment by Ito et al. (2013) was able to assimilate both DON and NIV under aerobic conditions. Three enzymes produced by that strain catabolized DON to produce 16-HDON. *Devosia mutans* bacterium isolated from soil by He (2015) transformed DON into less toxic compounds: 3-epi-DON (main product) and 3-keto-DON (by-product). Böswald et al. (1995) reported that ZEA was reduced stereoselectively to both α-ZOL and β-ZOL by several strains of *Saccharomyces* yeasts. McCormick et al. (2012) found that yeasts belonging to the *Trichomonascus* clade detoxified T-2 toxin via 3-OH conjugations: 3-acetylation and 3-glucosylation. Our previous study of the profile of *Fusarium* mycotoxins demonstrated that fungicides, yeasts and bacteria reduced the content of ENNs and MON in wheat grain to the levels lower than those noted in unprotected grain (Wachowska, Waśkiewicz, & Jędryczka, 2017). Therefore, the aim of this study was to analyze: (1) the profile of *Fusarium* mycotoxins in winter wheat grain after the application of fungicides, *Debaryomyces hansenii* and *Aureobasidium pullulans* yeast isolates and *Sphingomonas* spp. bacterial isolates under field conditions; (2) the *in vivo* content of DON, MON and ENNs in winter wheat grain after the application of antagonistic microorganisms, and toxin-producing *F. culmorum* and *F. avenaceum* pathogens; (3) the interactions between antagonistic bacteria, yeasts and *F. culmorum* and *F. avenaceum* pathogens in dual cultures.

2. Materials and methods

2.1. Isolation of biocontrol strains and culture conditions

The *Sphingomonas* sp. Sph isolate (GenBank accession number JX444564) was obtained from the rhizosphere of winter wheat (King et al., 1954); the *Debaryomyces hansenii* (Zopf) Lodder & Kreger Dh isolate (GenBank accession number KX444668) was obtained from the surface of apples, and the *Aureobasidium pullulans* (de Bary) Arnaud Ap isolate (GenBank accession number KX444670) was obtained from wheat grain. The biomass of the bacterial isolate was obtained according to a previously described method (Wachowska 2017). The resulting yeast suspension was brought to a concentration of 5×10^6 cells per 1 cm^3 of water, and the bacterial suspension – to a concentration of 7×10^8 cells per 1 cm^3 of water.

2.2. Production of pathogenic F. culmorum inoculum

Two strains of *Fusarium culmorum* (W.G. Smith) Sacc (15-ADON chemotype, Wiwart et al., 2016). and *Fusarium avenaceum* (Fr.) Sacc (GenBank accession number KX375416). were isolated from bread wheat grain with symptoms of FHB, grown in north-eastern Poland. The toxigenic profiles of *F. culmorum* were determined in a previous study (Wiwart et al., 2016). *Fusarium culmorum* and *F. avenaceum* were incubated for 7–10 days on PDA (Merck, Poland) in Petri plates with a diameter of 9 cm (Medical, Italy) at a temperature of 25 °C. After incubation, fungal spores were rinsed off with sterile demineralized water (5 mL per plate) using an inoculation loop. Spore suspensions were centrifuged (3500 rpm; 10 min), re-suspended in sterile distilled water containing Tween® 40 (0.05%, Merck, Germany), and filtered through five layers of sterile gauze to obtain conidial suspensions. The density of conidial suspensions was determined at 7×10^5 conidia per cm^3 with the use of a hemocytometer (Marienfeld, Germany). A suspension of the *F. culmorum* inoculum (1 L) was poured into a backpack sprayer (Marolex Titan 12, Poland) and combined with 9 L of sterile water. Spikes were inoculated in the evening, on a cloudy day.

2.3. Co-inoculation of antagonistic microorganisms and pathogenic Fusarium strains to evaluate the production of DON, MON and ENNs

Samples of 100 g of healthy-looking wheat kernels were sterilized twice (121 °C, 2 MPa, 2 h) in 250 mL flasks. Two inoculation variants were applied. In the first variant, 5 mL of the antagonistic yeast suspension (5×10^6 cells/cm^3, Ap0, Dh0) and 5 mL of the bacterial suspension (7×10^8 cells/cm^3, Sph0) were applied to wheat grain, and grain was simultaneously inoculated with 2 cm^3 of *F. avenaceum* or *F. culmorum* spore suspension (7×10^5 spores/cm^3). In the second variant, (Ap 48, Dh 48 and Sph 48), the same concentrations of antagonist and pathogen suspensions were applied, but the spore suspension was applied 48 h after the biological treatment. Grain that was not treated with the antagonistic microorganisms and was inoculated only with *F. avenaceum* and *F. culmorum* isolates was the control. The experiment was conducted in triplicate and repeated twice. After 21 days of incubation at a temperature of 25 °C, grain was ground and used

in mycotoxin analyses.

2.4. Inoculation of DON-contaminated grain with antagonistic microorganisms

Sterile grain samples (sterilized twice for 2 h at 121 °C and 2 MPa) of 100 g each were contaminated with 1250 or 750 μg of DON per one kg (Sigma Aldrich, Poland) in flasks. At the same time, 5 cm^3 of the antagonistic yeast suspension (5 × 10^6 cells/cm^3, Ap0, Dh0) and 5 cm^3 of the bacterial suspension (7 × 10^8 cells/cm^3, Sph0) were applied to grain. The experiment was conducted in triplicate. Grain samples were incubated at a temperature of 25 °C for 21 days. Incubated grain was ground and used in mycotoxin analyses.

2.5. Dual culture bioassays

A 5-mm agar discs with actively growing *F. avenaceum* or *F. culmorum* mycelia were placed on PDA (Merck, Poland) in a Petri plates with a diameter of 9 cm (Medical, Italy). Using an inoculation loop, antagonistic microbial cultures (bacterial or yeast suspensions) were applied at a distance of 2 cm from the agar disc, in two parallel lines on both sides of the disc. All plates were incubated for 5 days at a temperature of 27 °C (bacteria) or 24 °C (fungi). The experiment was conducted in triplicate. The growth inhibition of *F. avenaceum* and *F. culmorum* was determined based on the surface area and ellipticity coefficient of colonies. The ellipticity coefficient (E) was calculated with the use of simple formula $E = d/D$, where d is the small diameter, and D is the large diameter of the pathogen colony. Colony surface area was measured with the use of the ImageJ® program (Rasband, 2016) based on digital images acquired using the CCD scanner (Epson Perfection V370 Photo, Epson, Shinjuku, Tokyo, Japan) and saved as 24-bit color BMP files with 200 dpi resolution. Dual cultures of pathogenic fungi and antagonistic microorganisms were established on PDA, with and without the addition of 0.5 μM of $FeCl_3$. The culture protocol was previously described by Vero et al. (2009). Using an inoculation loop, bacteria and yeasts were applied in a line at a distance of 4 cm from the agar disc that was positioned 2.5 cm from the edge of the Petri plate. The experiment was conducted in triplicate.

2.6. Field experiments

Two field experiments were conducted in 2018–2019 in the Agricultural Experiment Station in Bałdy, in north-eastern Poland (N 53°36′, E 20°36′). In the first experiment, winter wheat (cv. Bogatka, susceptible to infections caused by *Fusarium* spp.) was sown in plots with an area of 20 m^2 each. The experiment had a randomized block design. Azole, morpholine and benzimidazole fungicides were applied two or three times during the growing season in the following growth stages: first node visible at least 1 cm above the tillering node (BBCH 31), middle of heading (BBCH 55) and watery ripe (BBCH 71) (Meier, 2003) (Table 1). The third treatment involved 1 dm^3 of the yeast suspension (5 × 10^6 cells per 1 cm^3 of water) or 1 dm^3 of the bacterial suspension (7 × 10^8 cells per 1 cm^3 of water) combined with 9 dm^3 of sterile demineralized water with the addition of Tween® 40 (0.05%, Merck, Germany). The suspensions were left to stabilize for 30 min before application. They were applied to wheat spikes with the use of a backpack sprayer (Marolex Titan 12, Poland). Unprotected plants were the control.

The second experiment involved biological control was conducted in the same location in a randomized block design. Winter wheat (cv. Tonacja, susceptible to infections caused by *Fusarium* spp.) was sown in microplots with an area of 1 m^2 each. Yeast or bacterial suspensions were applied to wheat plants three times during the growing season in the following growth stages: first node visible at least 1 cm above the tillering node (BBCH 31), middle of heading (BBCH 55) and full flowering (BBCH 65) (Table 1). The suspensions (250 cm^3 of initial bacterial or yeast suspension and 750 cm^3 of demineralized water) were applied with the use of a hand sprayer (Marolex Master, Poland). Unprotected plants were the control.

2.7. Grain colonization by fungi

The counts of *Fusarium* fungi causing FHB and yeasts colonizing wheat kernels were determined on Martin's medium (Martin, 1950) immediately after harvest, according to a previously described procedure (Duba et al., 2019). Non-disinfected and surface-disinfected kernels (for obtaining epiphytic and endophytic colonies, respectively) were cultured on PDA (Merck, Poland) in Petri plates. Kernels were disinfected in 1% NaOCl aqueous solution for 1 min, rinsed three times in sterile demineralized water, and dried. Yeast and *Fusarium* spp. colonies were identified under a light microscope (Nikon E200, Japan) (Kurtzman et al., 2011; Lesslie et al., 2006). All colonies were counted after 7 days of incubation at a temperature of 24 °C. The number of fungal colony forming units (CFU) in 1 cm^3 (N), rinsed off kernels, was calculated with the use of the following formula [1]:

$$N = \frac{n}{10^{-r}} \times v \quad [1]$$

where:

N – colony forming units,

Table 1
Winter wheat treatments.

Treatment	Growing stage			
	BBCH 31 (First node at least 1 cm above tillering node)	BBCH 55 (Middle of heading)	BBCH 65 (Full flowering)	BBCH 73 (Early milk)
Experiment 1 (cv. Bogatka)				
Int Sph	fenpropimorph [a]	propiconazole [b]	*Sphingomonas* spp.	no treatment
Int Dh	fenpropimorph, epoxiconazole [c]	propiconazole [b]	*Debaryomyces hansenii*	no treatment
Fung 1	chlorothalonil [d]	epoxiconazole, pyraclostrobin [e]	no treatment	tebuconazole [f]
Fung 2	no treatment	epoxiconazole, pyraclostrobin [e]	no treatment	tebuconazole [f]
Experiment 2 (cv. Tonacja)				
Biol Ap	*Aureobasidium pullulans*	*Aureobasidium pullulans*	*Aureobasidium pullulans*	no treatment
Biol Dh	*Debaryomyces hansenii*	*Debaryomyces hansenii*	*Debaryomyces hansenii*	no treatment
Biol Sph	*Sphingomonas* sp.	*Sphingomonas* sp.	*Sphingomonas* sp.	no treatment

[a] Corbel 750 EC, 1 dm^3 per ha (fenpropimorph - 79.87%, BASF SE, Germany).
[b] Bumper 250 SC, 1 dm^3 per ha (propiconazole - 25.1%, Makhteshim Chemical Works Ltd., Israel).
[c] Duett Star 334 SE, 1 dm^3 per ha (fenpropimorph - 250 g/dm^3, epoxiconazole - 84.0 g/dm^3, BASF SE, Germany).
[d] Gwarant 500 SC, 1 dm^3 per ha (chlorothalonil - 40.16%, France).
[e] Opera Max 147,5 SE, 1.5 dm^3 per ha (epoxiconazole - 6.01%, pyraclostrobin - 8.18%, Germany).
[f] Tarcza Łan 250 EW, 1 dm^3 per ha (tebuconazole - 250 g/dm^3, Sharda Ltd., Poland).

n – number of colonies on the plate,
10^{-r} – dilution factor,
v – volume of plated suspension.

The number of colonies growing on wheat kernels was expressed as a percentage of total kernels within epiphytes and endophytes.

2.8. Mycotoxin profile analysis by LC-MS/MS

The content of mycotoxins in grain harvested during field experiments was determined with the use of a method described by Sulyok et al. (2020). In brief, 5.00 g of ground wheat was extracted with 20 mL of extraction solvent composed of acetonitrile (AcN): water (W): acetic acid (HAC) = 79:20:1 (v:v:v) on a rotary shaker (GFL 3017, GFL; Burgwedel, Germany) for 90 min at room temperature in a horizontal position. After extraction, 500 μL of the extract was diluted with 500 μL of dilution solvent composed of AcN:W:HAC = 20:79:1 in vials. Finally, 5 μL was injected into an LC-MS/MS system composed of a QTrap 5500 MS/MS (Sciex, Foster City, CA, USA) coupled with an Agilent 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). The separation of analytes was performer on a Gemini C18 column (150 × 4.6 mm i.d., 5 μm particle size) with a 4 × 3 mm precolumn with the same characteristics (Phenomenex, Torrance, CA, USA). The eluents used were composed of methanol (MeOH): W: HAC = 10:89:1 (v:v:v) as eluent A, and MeOH:W:HAC = 97:2:1 (v:v:v) as eluent B. The analysis was performed on the fully validated method described in detail by Sulyok et al. (2020) for measurement of 500+ mycotoxins and other secondary metabolites.

2.9. Statistical analysis

All results were processed statistically. Toxin concentrations were analyzed by multivariate clustering, and the results were presented in heat maps and a dendrogram. Data for multivariate analyses were ranked because in some cases, metabolite concentrations differed by three orders of magnitude. Data on the surface area of fungal mycelia, grain colonization by fungi, spike density and one thousand kernel weight were processed by ANOVA, and the significance of differences between means was determined by the SNK multiple comparison test at $p < 0.01$. All statistical analyses were performed in the Statistica 13 program (TIBCO, 2017).

3. Results and discussion

3.1. In vivo reduction in the content of DON, MON and ENNs in wheat grain by bacteria and yeasts

In the first *in vivo* experiment, the content of MON in grain inoculated with *F. avenaceum* was determined at 19.05 μg/kg, and it was reduced to 1.36 μg/kg when grain was inoculated with *F. avenaceum* and treated with *A. pullulans* Ap (Fig. 1). The content of MON was reduced to 1.06 μg/kg when *Sphingomonas* sp. Sph was applied to grain 48 h before inoculation with *F. avenacum*. In control grain, the total content of ENNs was determined at 4327 μg/kg, and yeasts reduced ENN levels (in particular A1, B1, B2 and B3) in all samples (Fig. 1, Suppl. 1). Enniatin levels were not reduced only in treatments combining fungal inoculation with the application of *Sphingomonas* sp. Sph bacteria. Deoxynivalenol was not detected in grain inoculated *in vivo* with *F. culmorum*.

In the current field study, all grain samples contained small amounts of MON in the range of 0.73–90.24 μg/kg (Table 4). Moniliformin occurs naturally as water-soluble sodium or potassium salt, and it exerts cardiotoxic and hepatotoxic effects on humans and animals, but the risk of MON toxicity for humans is very low (EFSA, Panel Contam 2018b). Enniatins A, A1, B and B1 were identified in all grain samples at surprisingly high concentrations (0.51–826 μg/kg), in particular in wheat cv. Tonacja. These toxins are produced not only by *Fusarium* fungi, but also by fungi of the genera *Alternaria*, *Halosarpheia* and *Verticillium* (Bertero et al., 2020; Pallarés et al., 2020). Enniatins are widely identified in wheat grain and feed at varied concentrations. The maximum concentration of ENNs in feed was determined at 89.5 μg/kg by Tolosa et al. (2019). However, Uhlig et al. (2007) analyzed the content of ENNs in Finnish and Norwegian wheat grain and found that ENN B content reached 18300 and 5800 μg/kg, respectively. In a review article, Bertero et al. (2020) pointed to the cytostatic effects of ENNs on intestinal cells, which was observed by Dornetshuber et al. (2007). According to Roig et al. (2014), ENN A1 and ENN B1 are characterized by bactericidal activity against lactic acid bacteria, and they can disrupt the homeostasis of the gut microbiome. In the present study, ENN levels in grain were reduced *in vivo* by all tested microorganisms, but only by *D. hansenii* in field experiments. In our previous study, *Sphingomonas* sp. and *A. pullulans* isolates reduced the content of ENNs under field conditions (Wachowska, Waśkiewicz, & Jędryczka, 2017).

In the second *in vivo* experiment, all antagonistic microorganisms

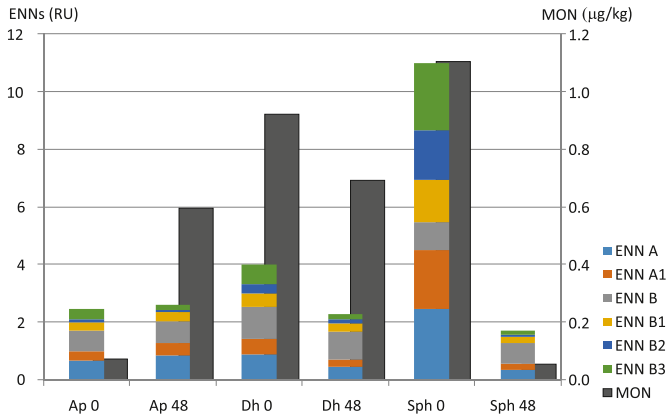

Fig. 1. Moniliformin (MON, right axis) and enniatin (ENNs) (A, A1, B, B1, B2, B3, left axis) levels in grain inoculated with *Fusarium avenaceum* directly after and 48 h after the application of *Aureobasidium pullulans* (Ap 0 and Ap 48), *Debaryomyces hansenii* (Dh 0 and Dh 48) and *Sphingomonas* (Sph 0 and Sph 48) in the first *in vivo* experiment. For ENNs, relative units (RU) correspond to concentration relative to control.

Table 2
Growth of pathogen colonies in dual cultures with antagonistic microorganisms.

Pathogen	Antagonistic microorganism	Surface area of pathogen colonies in dual culture 1	Ellipticity coefficient of pathogen colonies in dual culture 1	Surface area of pathogen colonies in dual culture 2	
				0 $FeCl_3$	0.5 mM $FeCl_3$
F. culmorum	Control	45.02 $^{a-c}$	0.98	61.97 ^{a}	22.14 ^{cde}
	Sphingomonas sp.	50.02 ^{ab}	0.82	45.39 ^{b}	29.28 ^{c}
	A. pullulans	18.87 ^{cd}	0.79	24.81 ^{cd}	28.57 ^{c}
	D. hansenii	40.01 $^{a-d}$	0.74	57.66 ^{a}	30.69 ^{c}
F. avenaceum	Control	13.54 ^{cd}	0.99	11.14 ^{e}	10.16 ^{e}
	Sphingomonas sp.	15.58 ^{cd}	0.76	10.16 ^{e}	9.26 ^{e}
	A. pullulans	12.06 ^{d}	0.84	18.45^{cde}	11.27 ^{e}
	D. hansenii	21.02 ^{cd}	0.71	17.95^{cde}	13.68 ^{de}

^{a} … ^{e} – values followed by the same letter differ not significantly within a column at $p < 0.01$.

Table 3
Content of trichothecenes (μg/kg) and limits of detection (LOD, in brackets) in winter wheat grain.

Treatment	DON (1.2)	D3G (0.8)	NIV (1.1)	N3G (0.8)	CULM (8)	15-hydroxy-culmorin (7)	5-hydroxy-culmorin (10)	HT-2 toxin (1.9)	Σ > LOD
Experiment 1 (cv. Bogatka)									
Control	141.36	4.89	41.54	0.81	243.12	57.94	< LOD	< LOD	489.7
Int Sph	75.19	4.06	49.59	1.02	52.54	< LOD	< LOD	< LOD	182.4
Int Dh	72.76	1.56	42.20	0.97	101.44	< LOD	< LOD	< LOD	218.9
Fung 1	60.06	1.22	48.26	1.01	26.17	< LOD	< LOD	< LOD	136.7
Fung 2	171.12	6.09	56.61	1.21	195.76	192.72	< LOD	< LOD	623.5
Experiment 2 (cv. Tonacja)									
Control	344.56	13.14	51.69	2.55	544.72	446.16	144.48	< LOD	1547.3
Biol Ap	433.2	3.74	73.35	5.14	347.20	877.60	220.96	< LOD	1961.2
Biol Dh	< LOD	0.17	75.30	7.50	14.02	< LOD	< LOD	< LOD	96.99
Biol Sph	419.04	17.92	58.68	4.39	421.20	451.52	309.84	8.99	1691.6

DON, D3G, NIV, N3G, CULM – deoxynivalenol, deoxynivalenol-3-glucoside, nivalenol, nivalenol-3-glucoside, and culmorin, respectively.

Table 4
Content of other identified metabolites (μg/kg) and limits of detection (LOD, in brackets) in winter wheat grain.

Treatment	ZEA (0.2)	ENNA (0.01)	ENNA1 (0.06)	ENN B (0.01)	ENN B1 (0.04)	MON (1.5)	Aurofusarin (4.6)	Equisetin (0.7)	Apicidin (0.65)	Siccanol (8)
Experiment 1 (cv. Bogatka)										
Control	<LOD	2.18	13.98	10.46	28.79	<LOD	37.88	3.51	4.65	<LOD
Int Sph	<LOD	2.14	16.85	46.68	56.97	7.63	42.19	9.12	8.33	<LOD
Int Dh	<LOD	1.13	6.95	5.54	11.04	<LOD	36.88	13.53	22.94	<LOD
Fung 1	<LOD	20.82	190.56	276.88	556.56	23.03	72.01	4.63	16.66	<LOD
Fung 2	0.58	0.51	4.68	20.41	22.08	8.09	28.03	<LOD	5.46	<LOD
Experiment 2 (cv. Tonacja)										
Control	<LOD	2.32	8.91	90.56	57.14	20.47	<LOD	<LOD	4.59	3979
Biol Ap	<LOD	3.01	29.55	147.44	104.40	18.32	28.00	<LOD	4.28	<LOD
Biol Dh	<LOD	3.69	48.26	379.04	307.36	60.09	104.56	<LOD	0.72	4338
Biol Sph	<LOD	16.78	176.56	789.52	826.40	90.24	18.69	<LOD	1.47	6591

ZEA, ENNA, ENNA1, ENN B, ENN B1, MON – zearalenone, enniatin A, A1, B, and B1 and moniliformin, respectively.

reduced DON levels in sterilized wheat grain (inoculated with 1250 or 750 μg DON/kg), but the noted reduction did not exceed 14% for *Sphingomonas* spp. and *A. pullulans* (Fig. 2).

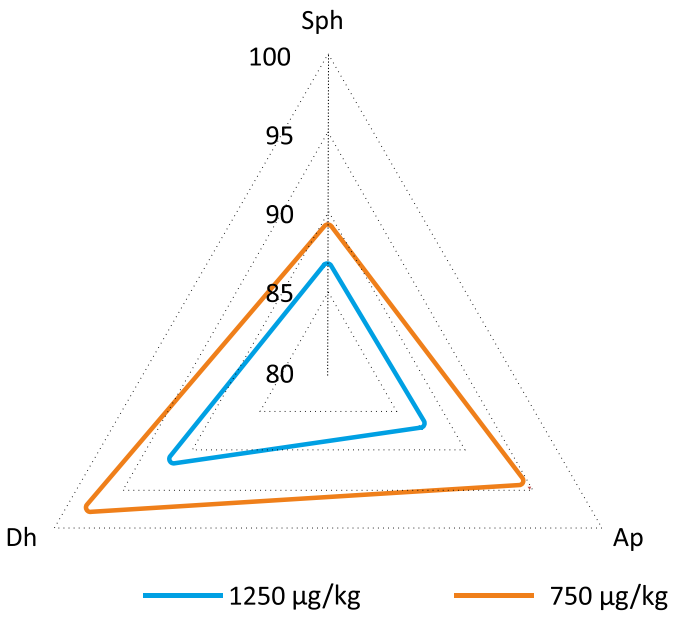

Fig. 2. Percentage reduction in deoxynivalenol (DON) content in grain contaminated with 1250 or 750 μg DON/kg, directly after the application of *Aureobasidium pullulans* (Ap), *Debaryomyces hansenii* (Dh) and *Sphingomonas* (Sph) and after 21-day incubation at a temperature of 21 °C in the second *in vivo* experiment.

3.2. Interactions between bacteria and yeasts vs. F. culmorum and F. avenaceum in dual cultures

The development of *F. culmorum* and *F. avenaceum* colonies in dual cultures 1 and 2 was most effectively inhibited by *A. pullulans*. It is worth noting that the inhibitory effect of *A. pullulans* was lost when a small amount of $FeCl_3$ was added to dual culture 2, which can be probably attributed to siderophore-mediated competition for nutrients with pathogens (Table 2). *Debaryomyces hansenii* did not inhibit the colony growth of the pathogens, but it strongly decreased the ellipticity coefficients of their colonies. *Sphingomonas* sp. and *A. pullulans* decreased the surface area of *F. culmorum* colonies in dual culture 2, but its inhibitory effect was lost after the addition of a small amount of $FeCl_3$, most likely due to the siderophores. Epiphytic and endophytic bacteria and yeasts abundantly colonize wheat leaves and spikes, and they can naturally inhibit the development of pathogens, including fungi of the genus *Fusarium*. Antagonistic strains isolated from wheat quickly adapt to difficult conditions on the surface of crop plants. This protection strategy has been evaluated in numerous research studies (Khan et al., 2001; Palazzini et al., 2007; Pan et al., 2015; Rojas-Solis et al., 2020; Schisler et al., 2015).

3.3. Profile of Fusarium mycotoxins in field-grown wheat grain

Fig. 3 illustrates the weather conditions in two years of the experiment. In the second year of the experiment in June, i.e. in the period of wheat flowering, there was higher precipitation which, in connection with higher temperatures, was more favorable for infection than in the first year of the study. Seventeen *Fusarium* toxins were identified in field-grown wheat grain: DON, D3G, CULM, 15-hydroxyculmorin, 5-hydroxyculmorin, HT-2 toxin, NIV, NIV3G, aurofusarin, ENNs (A, A1, B, B1), ZEA, MON, equisetin and apicidin. Deoxynivalenol, NIV and their glucosides were detected in nearly all grain samples of winter wheat cv.

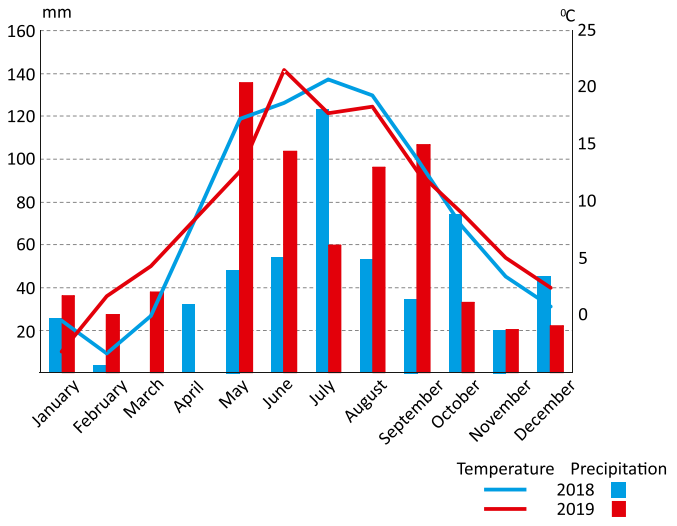

Fig. 3. Sums of precipitation and average temperatures in the growing seasons under experimental conditions.

Bogatka (Table 3). Deoxynivalenol, NIV (41.54–75.30 μg/kg), their respective glucoside conjugates (D3G and NIV3G), CULM (14.02–544.72 μg/kg) and its hydroxyl derivatives (15-hydroxyculmorin and 5-hydroxyculmorin) were detected in nearly all samples of both bread wheat cultivars (Table 3). Deoxynivalenol levels in the analyzed wheat cultivars (60.06–419.04 μg/kg) were lower than in durum wheat (Gorczyca et al., 2017) and common wheat (Chełkowski et al., 2012) grown in various Polish regions. In selected years, similar concentrations of DON were reported in common wheat grown in Switzerland (Pallez-Barthel et al., 2021), Poland (Perkowski et al., 2004), Italy (Bertuzzi et al., 2020) and other European regions (Lindblad et al., 2013; Nathanail et al., 2015). Based on the results of a 12-year study, Pallez-Barthel et al. (2021) observed that DON concentrations in wheat exceeded 1250 μg/kg every two years on average, and that the risk of contamination was influenced by various factors, including weather conditions. Chełkowski et al. (2012) demonstrated that *F. graminearum* and *F. culmorum* are the main producers of DON in Poland, and that their prevalence is affected by the origin of grain and weather conditions. According to Pallez-Barthel et al. (2021), *F. graminearum*, *F. avenaceum*, *F. poae* and *F. culmorum* are the main causative agents of FHB in Europe, where *F. graminearum* thrives in periods of wet weather and *F. culmorum* – during dry spells. In the cited study, the percentage of Swiss grain samples where DON levels exceeded the maximum legal limit varied considerably from 0% (in 2010, 2011, 2013–2015, 2017) to 36% in 2012. They also argued that research results are more difficult to publish if the analyzed materials are characterized by low mycotoxin levels. Therefore, there is a risk that data collected in years with high levels of DON contamination are published more frequently. It should be noted that mycotoxins can have adverse health effects, even if consumed in small quantities. The acute reference dose (ARfD) for the sum of DON, 3-ADON, 15-ADON and D3G has been set at 8 μg/kg BW per eating occasion (EFSA, CONTAM, 2018a). In addition, other toxins and secondary metabolites of *Fusarium* fungi have not been sufficiently investigated. In the present study, the CULM content of grain ranged from 14.02 to 544.72 μg/kg, and it was most frequently correlated with DON levels. Such correlations were also reported by other authors (Khaneghah et al., 2019; Spanic et al., 2020; Uhlig et al., 2013). There are no published reports describing the toxic effects of this metabolite for animals, but Wipfler et al. (2019) demonstrated that CULM was not toxic for wheat seedlings when applied alone, but it exerted strong phytotoxic effects when applied in combination with DON. Woelflingseder et al. (2019) reported that CULM inhibited uridine diphosphate glucosyltransferase (UGT), an enzyme that catalyzes the glycosylation of DON into the less toxic DON-3-glucuronide.

Only the grain of winter wheat cv. Tonacja protected with a suspension of *D. hansenii* yeast (Biol Dh) was practically free of DON. The content of DON in the grain of cv. Bogatka protected with fungicides as well as *D. hansenii* (Int Dh) and *Sphingomonas* spp. (Int Sph), and protected only with fungicides (Fung 1) was 48.53, 46.81 and 57.52% lower, respectively, than in unprotected grain. All protective treatments reduced the content of CULM, and the biological treatment involving *D. hansenii* was most effective (Biol Dh; CULM content was reduced from 544.72 to 14.02 μg/kg). Pan et al. (2015) demonstrated that *Bacillus megaterium* (BM1) and *B. subtilis* (BS43, BSM0 and BSM2) significantly reduced fungal growth, germination of *F. graminearum* spores, and decreased DON production by 89.3% in the field. In the work of Palazzini et al. (2007), DON levels in the grain of greenhouse-grown wheat were reduced by 60–100%. According to the cited authors, two bacterial strains of the genera *Brevibacillus* and *Streptomyces* were most potent inhibitors of *F. graminearum.* Their antagonistic effects were attributed to antibiosis and competition. The *Cryptococcus flavescens* OH 182.9 strain isolated from the anthers of flowering wheat heads decreased FHB symptoms in the field by up to 42% (Khan et al., 2001), and its ability to reduce DON levels in grain in only one experiment was relatively high (41%), but not statistically significant (Schisler et al., 2015). In the present study, unlike in the above experiments, *D. hansenii* isolated from the surface of apples was most effective in reducing DON levels in wheat grain. Interestingly, this isolate also significantly suppressed the growth of yeasts that naturally colonize grain. It appears that the predominant mode of action of *D. hansenii* was the secretion of proteins, which has been widely described in the literature (Banjara et al., 2016).

The results of this study indicate that fungicides (Fung 1) effectively targeted a broad spectrum of mycotoxins, i.e. DON, D3G, CULM, 15-hydroxyculmorin and 5-hydroxyculmorin. Interestingly, the above toxins were not eliminated when the first fungicide treatment was omitted (Fung 2). It should be noted that DON is highly mobile in plants, and it is transferred to grain when the stem base is infected with *F. culmorum* (Covarelli et al., 2012). The integrated treatment combining fungicides and the *D. hansenii* Dh isolate targeted the broadest spectrum of mycotoxins. Integrated chemical and biological treatments were also recommended by Schisler et al. (2015) despite the fact that in their study, the combined application of *Cryptococcus flavescens* OH 182.9 and prothioconazole was not significantly more effective in reducing FHB symptoms and DON levels in wheat grain than the fungicide alone. However, the cited authors argued that yeasts colonize wheat spikes, thus providing long-term protection against successive *F. graminearum* infections. Biological treatments can also minimize the risk of fungicide resistance in pathogen populations.

Enniatins A, A1, B and B1 were detected in all grain samples, and their content ranged from 1.13 to 826.40 μg/kg (Table 4). The content of all ENNs was lower in the grain of wheat cv. Bogatka protected with fungicides and *D. hansenii* (Int Dh) than in the control grain, and the content of ENN B1 was reduced by as much as 61.66% in protected grain.

The results of multivariate clustering analysis of the concentrations of *Fusarium* toxins in the grain of wheat cv. Bogatka are presented in Fig. 4. Trichothecene concentrations were highest in control grain and in grain protected with fungicides in the Fung 2 treatment. The content of these metabolites was lowest in grain protected with fungicides in the Fung 1 treatment, and in the integrated treatment (Int Sph) where the *Sphingomonas* sp. isolate was applied in the full flowering stage (BBCH 65). Interestingly, enniatin concentrations were high in grain subjected to both of the above treatments. Based on these results, three major clusters were formed. The first cluster comprised control, Int Dh and Fung2 treatments. The second cluster contained Int Sph and Fung1 treatments and third control Biol Ap and Biol Sph.

Total mycotoxin concentrations in wheat grain in field experiments 1 and 2 are presented in Fig. 5. The biological treatment involving

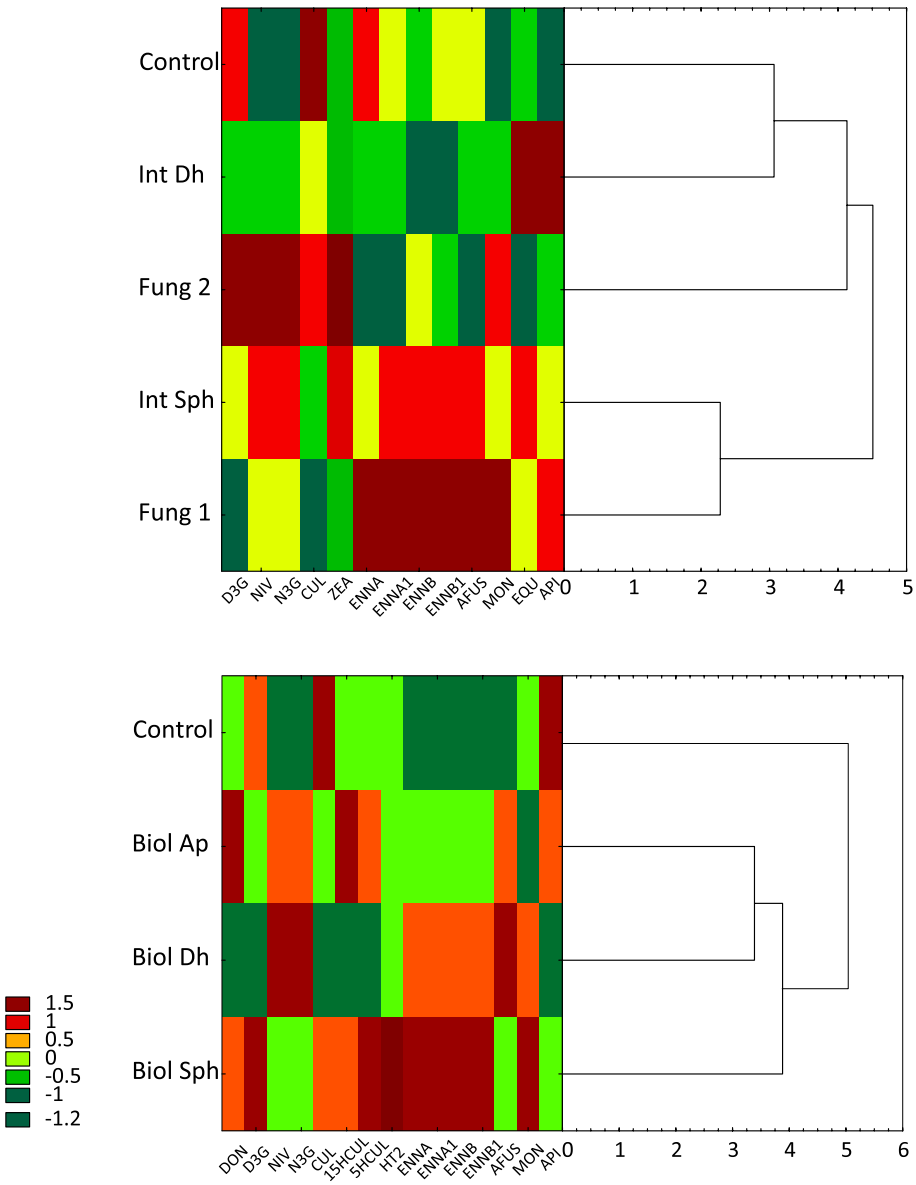

Fig. 4. The result of multivariate cluster analysis for mycotoxin concentration in the grain of wheat cv. Bogatka (at the top) and cv. Tonacja (bottom). DON - deoxynivalenol, D3G - deoxynivalenol-3-glucoside, NIV - nivalenol, N3G - nivalenol-3-glucoside, CUL - culmorin, ZEA - zearalenone, 15HCUL - 15-hydroxyculmorin, 5HCUL - 5-hydroxyculmorin, AFUS - aurofusarin, MON - moniliformin, EQUI - equisetin, API - apicidin, HT-2 - HT-2 toxin, ENNA, ENNA1, ENNB, ENNB1 - enniatin A, A1, B, and B1, respectively.

D. hansenii (Biol Dh) and the integrated treatment involving *D. hansenii* and fungicides (Int Dh) reduced the total mycotoxin content of grain by 42.2% and 48.1%, respectively, relative to the control treatment.

Interestingly, siccanol concentrations were high in both unprotected (3979 μg/kg) and biologically protected (Biol Dh – 4338, Biol Sph – 6591 μg/kg) wheat cv. Tonacja. Siccanol is produced by *Drechslera siccans*, a pathogenic fungus and a bicyclic sesterterpene (Lim et al., 1996). A siccanol dose of 100 mg/L completely inhibited root growth in *Lolium multiflorum* seedlings (Vurro, 2007). Chan and Jamison (2004) concluded that siccanol is not 11-*epi*-terpestacin, but (−)-terpestacin itself. Nihashi et al. (2002) demonstrated that siccanol is produced by the fungal strain *Bipolaris sorokiniana* NSDR-011, previously referred to as *Drechslera siccans*. *Bipolaris sorokiniana* causes foliar spot blotch, root rot and black point in grain. This pathogen is ubiquitous around the world, and it also produces other mycotoxins, including helminthosporol and sorokinianin (Kumar et al., 2002). Other microorganisms capable of producing siccanol have not been identified to date, which suggests that biological treatments involving Biol Dh and Biol Sph were not effective in suppressing this pathogen, and wheat cv. Tonacja was particularly susceptible to infections caused by *B. sorokiniana*.

3.4. *Fusarium spp. and yeasts in field-grown wheat grain*

Integrated and biological treatments decreased the abundance *Fusarium* fungi in 7 out of the 27 analyzed grain samples (Table 5). The number of *Fusarium* colonies was 30.88% lower in the grain of wheat plants protected with *D. hansenii* Dh (Biol Dh) than in control grain. Surprisingly, the number of yeast colonies on the kernels of wheat cv. Tonacja protected with *D. hansenii* (Biol Dh) was nearly 12 times lower than in control grain. In the remaining samples, yeast abundance was higher in protected grain than in control grain. The most efficient biocontrol agents simultaneously employ a combination of different interactions for controlling pathogens (Pal, McSpadden Gardener 2006). Antagonistic microorganisms can protect crops against direct, mixed-path and indirect interactions between pathogens (Vujanovic, Goh 2011). This study demonstrated that *A. pullulans* and *Sphingomonas* spp. compete for space and nutrients with pathogens, probably by producing siderophores, whereas *D. hansenii* secretes antimicrobial substances. This hypothesis is confirmed by previous reports indicating that *A. pullulans* produces siderophores (Chi et al., 2012) and *D. hansenii* secretes killer toxins (Banjara et al., 2016).

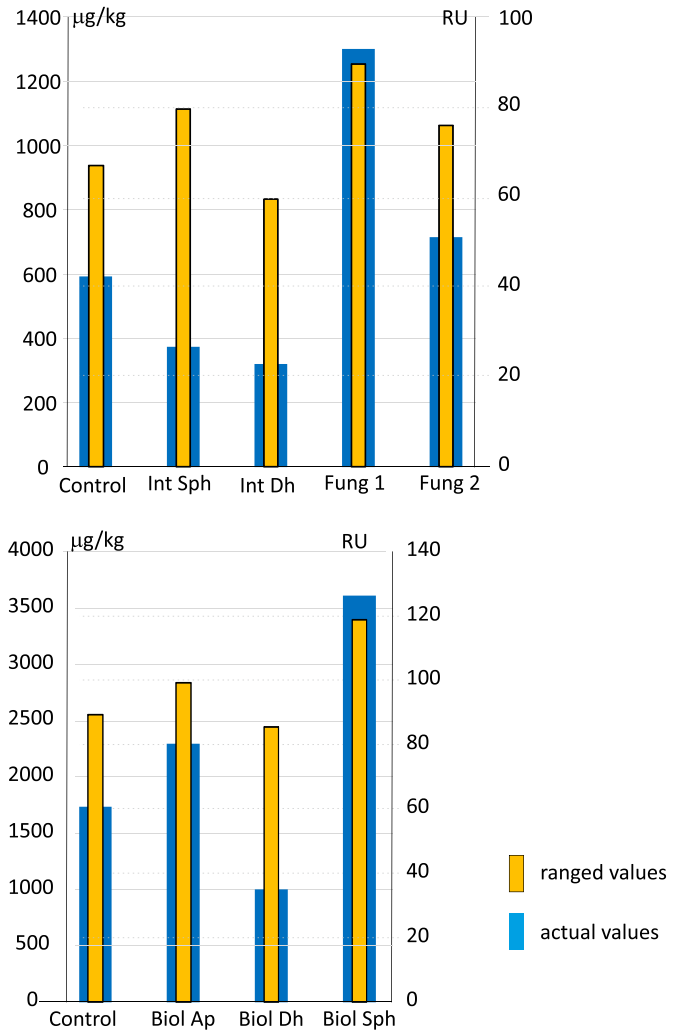

Fig. 5. Total mycotoxin content of wheat grain in field experiments 1 (at the top, 1 – Control, 2 – Int Sph, 3 – Int Dh, 4 – Fung 1, 5 – Fung 2) and 2 (bottom, 1 – Control, 2 – Biol Ap, 3 – Biol Dh, 4 – Biol Sph). RU – relative units. The sum of ranks is marked in yellow; total mycotoxin concentrations are marked in blue. All other abbreviations see Table 1.

4. Conclusions

Biological treatments performed during flowering, after the application of fungicides in the stem elongation and heading stages, effectively inhibit the growth of *Fusarium* fungi on wheat spikes. In addition, antagonistic yeasts have a complex mechanism of action which prevents the accumulation of mycotoxins in wheat grain in the long term. The tested yeast isolates simultaneously reduced the content of several mycotoxins: deoxynivalenol, culmorin, moniliformin and enniatins. *Debaryomyces hansenii* (GenBank accession number KX444668) was the most promising isolate which will be analyzed extensively under field conditions in the future.

CRediT authorship contribution statement

Urszula Wachowska: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, preparation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Michael Sulyok:** Methodology, Validation, Investigation, Data curation. **Marian Wiwart:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Elżbieta Suchowilska:** Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing. **Wolfgang Kandler:** Methodology, Validation, Investigation, Data curation. **Rudolf Krska:** Methodology, Validation, Investigation, Data curation.

Table 5
Colonization of winter wheat grain by *Fusarium* spp. and yeasts, spike density and one thousand kernel weight (TKW).

Treatment	*Fusarium* spp. [a]			Yeast	Spike density [d]	TKW
	CFU $\cdot 10^2$/ 1 g of grain [b]	Percentage of: [c]		CFU x 10^2/1 g of grain [b]		
		Epiphytes	Endophytes			
Experiment 1 (cv. Bogatka)						
Control	1.00 ^{ab}	11.46 ^{ab}	10.42 ^{ab}	16.00 ^{b}	21.86 ^{a}	50.74 ^{ab}
Int Sph	2.00 ^{ab}	12.50 ^{ab}	9.89 ^{abc}	27.57 ^{ab}	21.89 ^{a}	49.94 ^{bc}
Int Dh	2.13 ^{ab}	10.93 ^{ab}	10.42 ^{ab}	51.43 ^{ab}	22.29 ^{a}	51.55 ^{a}
Fung 1	4.00 ^{a}	11.46 ^{ab}	11.99 ^{a}	34.00 ^{ab}	22.28 ^{a}	49.87 ^{bc}
Fung 2	3.63 ^{a}	14.58 ^{a}	9.37 ^{abc}	54.57 ^{ab}	21.55 ^{a}	50.75 ^{ab}
Experiment 2 (cv. Tonacja)						
Control	3.00 ^{ab}	9.38 ^{ab}	6.77 ^{ab}	57.86 ^{ab}	19.07 ^{b}	49.22 ^{c}
Biol Sph	3.13 ^{ab}	8.85 ^{ab}	7.29 ^{abc}	36.00 ^{ab}	18.86 ^{b}	49.43 ^{c}
Biol Dh	0.13 ^{b}	10.94 ^{ab}	4.68 ^{a}	4.86 ^{b}	18.36 ^{b}	48.70 ^{c}
Biol Ap	3.75 ^{a}	6.77 ^{b}	6.77 ^{ab}	90.57 ^{a}	18.25 ^{b}	48.67 ^{c}

[a] *F. culmorum, F. poae, F. graminearum, F. sporotrichiodes, F. avenaceum.*
[b] Rinsed off kernels.
[c] Colonies growing on 100 kernels on PDA.
[d] Number of spikelets per 10 cm of spike. Values followed by the same letter inside the table differ not significantly within a column in the SNK test ($p < 0.01$).

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

This research is the result of a long–term study carried out at the University of Warmia and Mazury in Olsztyn, Poland, Faculty of Agriculture and Forestry, Department of Entomology, Phytopathology and Molecular Diagnostics (topic number 30.610.011-110) and Department of Genetics, Plant Breeding and Bioresource Engineering (topic number 30.610.007-110). This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

The authors express their gratitude to A. Poprawska for language editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.109039.

Abbreviations

15-ADON 15-acetyl-deoxynivalenol
3-ADON 3-acetyl-deoxynivalenol
ANOVA Analysis of variance
ARfD Acute Reference Dose

BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
BW	Body weight
CFU	Colony-forming unit
CULM	Culmorin
D3G	Deoxynivalenol-3-glucoside
DON	Deoxynivalenol
ENNs	Enniatins
FCR	Fusarium Crown Rot
FHB	Fusarium Head Blight
FRR	Fusarium Root Rot
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
MON	Moniliformin
NIV	Nivalenol
NIV3G	Nivalenol-3-glucoside
PDA	Potato Dextrose Agar
SNA	Small Nutrient Agar
SNK	Multiple Student-Newman-Keuls test
UGT	Uridine 5′-diphospho-glucuronosyltransferase
ZEA	Zearalenone

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