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Original Research

Response of Epiphytes and Endophytes Isolated from Winter Wheat Grain to Biotechnological and Fungicidal Treatments

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Abstract

A greenhouse experiment was carried out to determine the effect of biotechnological and fungicide treatments on the colonization of winter wheat kernels by epiphytic and endophytic *Pseudomonas* and *Azotobacter* bacteria, filamentous, and yeast-like fungi. Microbiological analyses were performed using two techniques that involved placing kernels on PDA medium, and washing kernels to remove the microbes and plating the wash on selective media.

The predominant microbial groups washed from the surface of winter wheat kernels were the pseudomonads and yeast-like fungi. Bacteria of the genus *Azotobacter* were present primarily inside kernels, and their counts increased after six months of grain storage. The application of Moddus 250 EC and Amistar 250 SC increased the abundance of most microorganisms, including *F. poae*. The fungicides Corbel 750 EC and Opera Max 147.5 SE, the growth promoter Asahi SL, and the plant resistance inducer Biochicol 020 PC inhibited the growth of endophytic yeast-like and filamentous fungi. Yeast-like fungi formed multicellular clusters on the surface of wheat kernels.

Keywords: winter wheat, kernels, fungi, bacteria, fungicides, growth regulators, growth promoters, resistance inducers

Introduction

Current trends in crop protection include a limited use of ecotoxic chemicals, and the development and introduction of integrated pest management strategies that combine agri-technological, biotechnological, and fungicidal methods [1-3]. Plant resistance inducers are applied to improve the health status of crops [4, 5]. Chitosan, an elicitor of defense responses in plants, can effectively reduce the infection of winter wheat spikes by *F. culmorum* as well as grain contamination with the mycotoxin deoxynivalenol (DON) [5, 6].

Stem-shortening substances whose ecological toxicity effects remain unknown have been applied recently to winter wheat. One such compound is trinexapac-ethyl (TE), an inhibitor of gibberellin (GA) biosynthesis. Gibberellins are a group of plant growth regulators influencing a range of developmental processes in plants, including stem elongation, flowering, leaf growth, and synthesis of various plant enzymes such as sucrose syntethase, amylases, and proteases [4]. Asahi SL, which stimulates plant development, may have a significant effect on biochemical and physio-

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logical processes in plants [7]. When applied to asters during flowering, it increased seed vigor, the electrical conductivity of plant secretions, and the activity levels of ACC oxidase and dehydrogenase.

The effects of fungicides and biotech products on non-target microbial populations remains poorly investigated. Such microorganisms often provide natural protection against pathogens [5], and promote crop growth and development [8-10]. Undesirable changes in the structure of their communities may lead to deterioration in the microbiological quality of winter wheat grain and the sowing value of seeds, as well as the accumulation of toxin-producing fungi of the genus *Fusarium*, including *F. culmorum*, *F. graminearum*, *F. sporotrichioides*, *F. poae*, and *F. avenaceum* [2, 11-13]. Due to their toxic effects, the above species pose a serious threat to food safety [14-16]. Commission Regulation (EC) No. 856/2005 of 6 June 2005 sets the maximum levels of Fusarium toxins in cereals and foodstuffs.

The objective of this study was to determine the effect of fungicides, a plant growth regulator, a plant growth promoter, and a plant resistance inducer on changes in the population size of epiphytic and endophytic *Pseudomonas* and *Azotobacter* bacteria, filamentous and yeast-like fungi colonizing winter wheat kernels.

Materials and Methods

Chemicals Used

The fungicides used in the experiment, Corbel 750 EC (fenpropimorph), Opera Max 147.5 SE (pyraclostrobin, epoxiconazole), and Amistar 250 SC (azoxystrobin), contain active ingredients from the morpholine, strobilurin, and azole groups. The other products used were the plant growth regulator Moddus 250EC (trinexapac-ethyl), the plant resistance inducer Biochicol 020 PC (chitosan), and the plant growth promoter Asahi SL (ortho-nitrophenol, para-nitrophenol, 5-nitroguaiacol).

Greenhouse Experiment

Grain samples for microbiological analyses were collected from the spikes of winter wheat cv. Bogatka grown in a greenhouse. A pot experiment was performed in three replications. Wheat seeds, surface disinfected in a 1% sodium hypochlorite solution, were sown in 30 cm diameter pots filled with soil. During the growing season, two treatments were carried out: at the stem elongation stage (BBCH 31) and at the heading stage (BBCH 53). The following treatment combinations were applied: Corbel 750 EC (fenpropimorph) and Opera Max 147.5 SE (pyraclostrobin, epoxiconazole), Moddus 250EC (trinexapac-ethyl) and Amistar 250 SC (azoxystrobin), and Asahi SL (ortho-nitrophenol, para-nitrophenol, 5-nitroguaiacol) and Biochikol 020 PC (chitosan). All products were applied at the doses recommended by their respective manufacturers.

Inoculation of Wheat Spikes with the Pathogen

At the end of flowering (BBCH 69), control spikes (KF) and spikes treated with biotech products and fungicides were inoculated with a cell suspension culture of *Fusarium culmorum*. The pathogen came from our own collection. It was isolated from the grain of winter wheat cv. Bogatka grown in a field experiment established in northeastern Poland. The isolate, denoted by the symbol Fc 38, was characterized by high pathogenicity against winter wheat seedlings. Wheat spikes were inoculated with a spore suspension at a concentration of 10^5 CFU/ml of sterile water.

Microbiological Analyses of Wheat Grain

The microbiological analyses of winter wheat grain were performed at harvest and after six months' storage at 11ºC. In order to wash epiphytes from wheat kernels, random grain samples of 10 g each were placed in 250 ml flasks containing 90 ml sterile water and shaken for 30 min (180 rpm) in a laboratory shaker, type 358 S. Endophytes were obtained by grinding surface-disinfected kernels in sterile water. 10 g homogenate samples were placed in 250 ml flasks containing 90 ml of sterile water, and were shaken for 30 min (180 rpm). 0.1 ml dilute microbial suspensions were transferred to Petri dishes (9 cm diameter), and selective media cooled to 42ºC were poured on top. The pseudomonads, *Azotobacter* bacteria and fungi were enumerated on King's B medium [17], nitrogen-free agar medium [18] and Martin's medium [19], respectively. The experiment was performed in four replications. The number of colonies in dishes was counted. Immediately after harvest, the colonies of filamentous fungi were transferred to agar slants and identified to the species level based on sporulation characteristics [20-22]. The species composition of filamentous fungi colonizing wheat grain was determined at harvest by placing kernels on solid PDA (potato dextrose agar) medium. The colonies of filamentous fungi were transferred on agar slants, and were identified based on sporulation characteristics using the relevant keys [20-22].

Scanning Microscopy (SEM)

Wheat kernels were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer with pH 7.4 for 12 h at 4ºC, and were rinsed in phosphate buffer for 1 h. The solution was changed three times. The resultant preparations were dehydrated in a graded ethanol series (30, 50, 70, and 96%, 10 min each), and twice in absolute alcohol (30 min each time). The dehydrated samples were dried by the critical point method (CDP 030, BALTEC), and were sputter coated with gold (JFC-1200 Fine Coater, JEOL). The specimens were viewed under a scanning microscope (JSM 5310L, JEOL) at 15 kV. Black-and-white photographs were taken using ILFORD FP4 Plus 125 roll film.

Table 1. Abundance of epiphytic microorganisms colonizing winter wheat grain.

Microorganisms	Date of analysis	KO	KF	Cor+OpM	As+Bioch	Mod+Ami	Mean
		Log(CFU + 1) per 1 g grain					
Azotobacter spp.	after harvest	1.84 bc	0.00 a	1.00 abc	0.81 abc	0.52 ab	0.83 X
	after 6 months' storage	2.22 c	2.19 c	1.85 bc	1.73 bc	1.92 bc	1.98 Y
	mean	2.03 B	1.09 AB	1.42 AB	1.27 AB	1.22 AB	
Pseudomonas bacteria	after harvest	2.47 abc	0.95 a	3.18 bc	3.37 bc	3.32 bc	2.66 X
	after 6 months' storage	1.56 ab	3.10 bc	3.18 bc	2.75 bc	3.47 c	2.81 X
	mean	2.02 c	2.03 A	3.18 AB	3.06 AB	3.40 B	
Yeast-like fungi	after harvest	3.48 ab	3.70 cd	3.48 ab	1.44 a	3.79 cd	3.18 X
	after 6 months' storage	1.47 a	3.60 c	2.90 c	2.19 b	4.17 d	2.87 X
	mean	2.47 A	3.65 C	3.19 B	1.82 A	3.98 D	
Filamentous fungi	after harvest	1.32 ab	2.35 cd	1.83 bc	1.98 bc	2.49 cd	2.00 X
	after 6 months' storage	1.04 ab	1.55 bc	0.52 a	1.47 b	2.76 d	1.47 X
	mean	1.18 A	1.96 B	1.18 A	1.73 B	2.63 C	

Values that do not differ signifcantly in Duncan's test are denoted by identical letters (p=0.05)
KO – control, KF – unprotected plants inoculated with *F. culmorum*, Cor+OpM – Corbel 750 EC (fenpropimorph) and Opera Max 147.5 SE (pyraclostrobin, epoxiconazole), As+Bioch – Asahi SL (oortho-nitrophenol, para-nitrophenol, 5-nitroguaiacol) and Biochikol 020 PC (chitosan), Mod+Ami – Moddus 250EC (trinexapac-ethyl) and Amistar 250 SC (azoxystrobin), Bakt – bacteria of the genus *Pseudomonas* applied during the growing season.

Statistical Analysis

The data regarding the abundance of microbial communities were log transformed (CFU+1). ANOVA was performed using Statistica 9.0 software, and the significance of differences was determined by Duncan's test (p=0.05). The community structure of filamentous fungi was expressed as a percentage based on all colonies that developed on Martin's medium. The community biodiversity of filamentous fungi was estimated as follows: Margalef index = number of species – 1/ln (abundance of fungal communities).

Results

The average abundance of epiphytic yeast-like fungi and *Pseudomonas* bacteria was higher than their counts determined inside wheat kernels (Tables 1 and 2). The communities of *Azotobacter* bacteria and filamentous fungi colonized mostly the inside of wheat kernels (Tables 1 and 2). The counts of all analyzed eukaryotic endophytes (Table 2) and epiphytic and endophytic bacteria of the genus *Azotobacter* (Tables 1 and 2) increased significantly over a six-month storage period.

All protective treatments modified the population size of microbial communities (Tables 1, 2). Epiphytic bacteria of the genus *Azotobacter* were not isolated from the grain of wheat plants treated with a cell suspension culture of *F. culmorum* (Table 1). Biotechnological and fungicidal treatments inhibited the growth of epiphytic bacteria of the genus *Azotobacter* on wheat kernels, yet the differences noted in comparison with the control treatment were statistically non-significant.

Epiphytic pseudomonads were found in significantly greater abundance on the grain of wheat plants treated with the growth promoter Moddus 250 EC and the fungicide Amistar 250 SC, stored for six months, compared to control treatment. Both products also had a significant (relative to the control) stimulating effect on epiphytic yeast-like and filamentous fungi, at harvest and during a six-month storage period. All fungicidal and biotechnological treatments and the application of a spore suspension of *F. culmorum* to wheat plants contributed to a significant increase in the abundance of epiphytic yeast-like fungi after six months' storage. Following intensive budding, yeast-like fungi formed multicellular clusters on the surface of wheat kernels (Fig. 1).

The counts of endophytic diazotrophs increased significantly after six months' storage (Table 2). The application of Moddus 250 EC (trinexapac-ethyl) and Amistar 250 SC (azoxystrobin) had a significant stimulating effect on the abundance of the above microorganisms during a six-month storage period. *Azotobacter* bacteria were not isolated from the grain of wheat plants sprayed with a suspension of *F. culmorum*. The counts of endophytic pseudomonads generally remained at a stable level throughout the experiment. Significantly more abundant communities of *Pseudomonas* bacteria were isolated at harvest only from wheat plants treated with Moddus 250 EC (trinexapac-ethyl) and Amistar 250 SC (azoxystrobin), and the fungicides Corbel 750 EC and Opera Max 147.5 SE, compared to the control treatment.

Table 2. Abundance of endophytic microorganisms colonizing winter wheat grain.

Microorganisms	Date of analysis	KO	KF	Cor+OpM	As+Bioch	Mod+Ami	Mean
		Log(CFU + 1) per 1 g grain					
Azotobacter spp.	after harvest	1.78 cd	0.00 a	0.66 ab	2.58 b	2.47 d	1.50 X
	after 6 months' storage	2.08 b	1.82 bc	2.49 b	2.06 b	2.46 cd	2.18 Y
	mean	1.93 B	0.91 A	1.58 AB	2.32 B	2.46 B	
Pseudomonas bacteria		2.75 ab	1.00 a	3.65 c	2.84 ab	3.63 c	2.78 X
	after 6 months' storage	2.77 ab	3,21 ab	2.84 ab	3.12 ab	2.84 ab	2.96 X
	mean	2.76 A	2,1 A	3.25 B	2.98 A	3.23 B	
Yeast-like fungi	after harvest	2.44 abc	1.45 a	2.78 bc	1.77 ab	1.18 a	1.92 X
	after 6 months' storage	4.74 d	3.11 cde	2.67 abc	2.16 abc	3.26 c	3.19 Y
	mean	3.59 C	2.28 AB	2.73 B	1.96 A	2.22 AB	
Filamentous fungi	after harvest	3.04 c	2.03 ab	3.38 c	2.36 abc	1.76 a	2.52 X
	after 6 months' storage	4.74 d	3.11 cde	2.67 bc	2.16 ab	3.26 c	3.19 Y
	mean	3.89 C	2.57 AB	3.03 B	2.26 AB	2.51 A	

Values that do not differ signifcantly in Duncan's Test are denoted by identical letters (p=0.05)
KO – control, KF – unprotected plants inoculated with *F. culmorum*, Cor+OpM – Corbel 750 EC (fenpropimorph), and Opera Max 147.5 SE (pyraclostrobin, epoxiconazole), As+Bioch – Asahi SL (ortho-nitrophenol, para-nitrophenol, 5-nitroguaiacol), and Biochikol 020 PC (chitosan), Mod+Ami – Moddus 250EC (trinexapac-ethyl), and Amistar 250 SC (azoxystrobin)

The grain of wheat plants treated with fungicides or biotech products, stored for six months, were colonized by significantly less abundant communities of endophytic yeast-like fungi, in comparison with the control treatment (Table 2). All chemical treatments reduced the counts of endophytic filamentous fungi after a six-month storage period.

At harvest, a total of 298 fungal colonies identified to eight genera and species (Table 3) were washed from the surface of wheat kernels. The community of ephiphytes was characterized by low diversity. The values of the Margalef diversity index ranged from 0.43 in the community isolated from the grain of wheat plants inoculated with *F. culmorum* to 0.75 in the community obtained from the grain of plants treated with Moddus 250 EC (trinexapac-ethyl) and Amistar 250 SC (azoxystrobin). The predominant epiphytic species was *Fusarium poae*, which accounted for 31.2% of all colonies. The fungus was most common on the grain of wheat plants sprayed with Moddus 250 EC and Amistar 250 SC during the growing season. Saprotrophs (*Alternaria alternata* and *Cladosporium herbarium*), which accounted for 35.1% of all isolates of filamentous fungi, were obtained mostly from the grain of unprotected plants and plants inoculated with *F. culmorum*. Genera of the order *Mucorales* were isolated from wheat kernels in all treatments. The genus *Mucor* dominated on the grain of wheat plants treated with Asahi SL and Biochicol 020 PC.

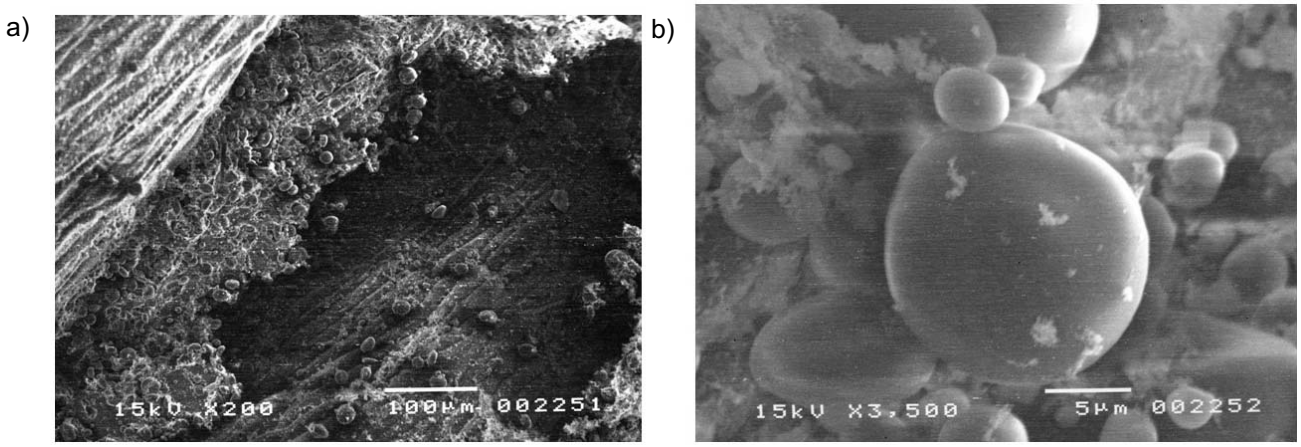

Fig. 1. Kernels of winter wheat cv. Bogatka colonized by multicellular aggregates of yeast-like fungi (a), a budding cell of a yeast-like fungus (b).

Table 3. Epiphytic filamentous fungi washed from the surface of winter wheat kernels at harvest.

Species of filamentous fungi	KO	KF	Cor+OpM	As+Bioch	Mod+Ami	Total
	Percentage of isolates					
Alternaria alternata (Fr.) Keissl.		17.45				17.45
Cladosporium herbarum Link ex Fr.		17.78				17.78
Fusarium culmorum (W. G. Sm.) Sacc.				0.34	0.34	0.68
Fusarium poae (Peck) Wollenweber	6.71	1.67	5.36		52.00	65.74
Fusarium sp.					0.34	0.34
Mortierella sp.	1.01		5.03			6.04
Mucor spp.	5.70		0.47	19.46		25.83
Rhizopus nigricans Ehrenberg					0.67	0.67
Number of colonies per treatment	40	110	33	59	56	298
Number of species per treatment	3	3	3	2	4	9
Biodiversity (Margalef index)	0.54	0.43	0.57	0.25	0.75	1.40

KO – control, KF – unprotected plants inoculated with *F. culmorum*, Cor+OpM – Corbel 750 EC (fenpropimorph), and Opera Max 147.5 SE (pyraclostrobin, epoxiconazole), As+Bioch – Asahi SL (ortho-nitrophenol, para-nitrophenol, 5-nitroguaiacol), and Biochikol 020 PC (chitosan), Mod+Ami – Moddus 250EC (trinexapac-ethyl), and Amistar 250 SC (azoxystrobin)

Table 4. Epiphytic filamentous fungi isolated from winter wheat grain by placing kernels on PDA medium.

Species	KO	KF	Cor+OpM	As+Bioch	Mod+Ami	Total
	Percentage of isolates					
Alternaria alternata (Fr.) Keissl.		4.05	4.05		2.70	10.80
Arthrinium phaeospermum (Corda) M.B.Ellis					5.41	5.41
Cladosporium herbarum Link ex Fr.		13.51				13.51
Drechslera sp.				2.70		2.70
Fusarium culmorum (W. G. Sm.) Sacc.				2.70		2.70
Fusarium poae (Peck) Wollenweber		2.70	18.91		18.97	40.58
Hansfordia sp.			2.70			2.70
Mucor spp.		2.70		5.41		8.11
Penicillium spp.			6.75			6.75
Rhizopus nigricans Ehrenberg	4.05			1.35		5.40
Trichothecium roseum Link			1.35			1.35
Non-sporulating colonies				5.41		5.41
Number of colonies per treatment	3	17	25	9	20	74
Number of species per treatment	1	4	6	4	3	13
Biodiversity (Margalef index)	0.00	1.06	1.55	1.37	0.67	2.79

KO – control, KF – unprotected plants inoculated with *F. culmorum*, Cor+OpM – Corbel 750 EC (fenpropimorph), and Opera Max 147.5 SE (pyraclostrobin, epoxiconazole), As+Bioch – Asahi SL (ortho-nitrophenol, para-nitrophenol, 5-nitroguaiacol), and Biochikol 020 PC (chitosan), Mod+Ami – Moddus 250EC (trinexapac-ethyl), and Amistar 250 SC (azoxystrobin)

The community of epiphytic filamentous fungi cultured by placing wheat kernels in Petri dishes was small, but its biodiversity (Margalef index) was higher than that of the community of filamentous fungi obtained by washing off microbes (Tables 3 and 4). A total of 74 isolates were identified to 11 genera and species. The community was dominated by members of the genus Fusarium, which made up 43.2% of all isolates, including *F. poae,* which accounted for 40.58% of the *Fusarium* population (Table 4). *F. poae* was isolated primarily from the grain of wheat plants treated

Table 5. Endophytic filamentous fungi washed from ground winter wheat grain at harvest.

Species of filamentous fungi	KO	KF	Cor+OpM	As+Bioch	Mod+Ami	Total
	Percentage of isolates					
Alternaria alternata (Fr.) Keissl.					8.69	8.69
Aspergillus sp.			2.17			2.17
Cladosporium herbarum Link ex Fr.			2.17			2.17
Fusarium culmorum (W. G. Sm.) Sacc.	2.17					2.17
Fusarium poae (Peck) Wollenweber	6.52	23.90	4.34	10.86	23.91	69.54
Non-sporulating colonies	15.21					15.21
Number of colonies per treatment	11	11	4	5	15	46
Number of species per treatment	2	1	3	1	2	6
Biodiversity (Margalef index)	0.42	0	1.44	0	0.37	1.31

KO – control, KF – unprotected plants inoculated with *F. culmorum*, Cor+OpM – Corbel 750 EC (fenpropimorph), and Opera Max 147.5 SE (pyraclostrobin, epoxiconazole), As+Bioch – Asahi SL (ortho-nitrophenol, para-nitrophenol, 5-nitroguaiacol), and Biochikol 020 PC (chitosan), Mod+Ami – Moddus 250EC (trinexapac-ethyl), and Amistar 250 SC (azoxystrobin)

with Corbel 750 EC (fenpropimorph) and Opera Max 147.5 SE (pyraclostrobin, epoxiconazole), and Moddus 250 EC (trinexapac-ethyl) and Amistar 250 SC (azoxystrobin).

A total of 46 colonies of filamentous fungi representing five genera and species were obtained from ground- and surface-disinfected wheat kernels (Table 5). The predominant species was *F. poae,* which made up over 50% of all isolates. *F. culmorum* colonies were not isolated from the grain of chemically protected plants. The kernels collected from wheat plants treated with the growth regulator Moddus 250EC were colonized exclusively by toxin-producing species *F. poae* and *A. alternata*.

Discussion

Winter wheat kernels provide a dynamically changing environment for the growth of bacteria, filamentous, and yeast-like fungi [23]. The structure of microbial communities is affected, among other factors, by the time of grain storage [24] and protective treatment during the growing season [25]. In our study, the counts of *Azotobacter* bacteria increased after six months' storage, which most probably improved the sowing value of wheat seeds. The results of greenhouse and field experiments show that *Azotobacter* bacteria have a beneficial influence on the growth and yield of winter wheat [8-10].

Fungal communities obtained by washing microbes from the surface or tissue of wheat kernels were considerably more abundant than those obtained by placing wheat kernels on filter paper or agar. It should be noted that the populations of bacteria [23] and yeast-like fungi [26, 27] are reported to predominate on wheat kernels when the washing off technique is used, whereas they are practically absent when other techniques are employed [24]. The above probably results from the fact that bacteria and yeasts usually form biofilms – superficial colonies or multicellular aggregates immersed in a matrix [28]. In our study, aggregates of yeast-like fungi, made up of several dozen cells, were found on the surface of wheat kernels.

In the present experiment, the plant growth promoter Asahi SL had no significant effect on the counts of microorganisms colonizing winter wheat grain, although in previous studies the product had been found to affect biochemical and physiological processes in plants. When applied to asters during flowering, it increased seed vigor, the electrical conductivity of plant secretions, and the activity levels of ACC oxidase and dehydrogenase [7].

Chitosan, an elicitor of defense responses in plants, did not inhibit the growth of epiphytic *Fusarium* species. According to other authors [5, 6] chitosan can effectively reduce the infection of winter wheat spikes by *F. culmorum* as well as DON contamination of wheat grain.

In our experiment, the toxin-producing species *F. poae* tended to accumulate on the surface of wheat kernels, in particular those collected from plants sprayed with Moddus 250 EC (trinexapac-ethyl) and Amistar 250 SC (azoxystrobin), which could pose serious health risks. It seems that the above products had a strong effect on plant physiology. In a study by Liu et al. [4], trinexapac-ethyl, in addition to shortening stem internodes, exerted a significant effect on the protein content of sorghum grain, but it did not affect starch structure. Azoxystrobin has been found to considerably impact the physiological activity of winter wheat plants [29], thus increasing the availability of nutrients to plant-dwelling microbes.

Conclusions

The predominant microbial groups washed from the surface of winter wheat kernels were bacteria of the genus *Pseudomonas* and yeast-like fungi. The abundance of bacteria of the genus *Azotobacter* was relatively low at harvest,

but their counts increased after six months of grain storage. The application of Moddus 250 EC and Amistar 250SC increased the abundance of most microorganisms, and exerted a stronger effect on epiphytes than on endophytes. Spike inoculation with *F. culmorum* contributed to a decrease in the average abundance of all analyzed endophytes. The fungicides Corbel 750 EC and Opera Max 147.5 SE, the growth promoter Asahi SL, and the plant resistance inducer Biochicol 020 PC inhibited the growth of endophytic yeast-like and filamentous fungi. Yeast-like fungi formed multicellular clusters on the surface of wheat kernels.

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