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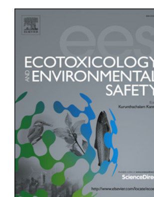

Agrochemicals: Effect on genetic resistance in yeasts colonizing winter wheat kernels

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ABSTRACT

Crop protection agents are widely used in modern agriculture and exert direct effects on non-target microorganisms such as yeasts. Yeasts abundantly colonize wheat grain and affect its chemical composition. They can also limit pathogen growth. This study evaluated the sensitivity of yeast communities colonizing winter wheat kernels to benzimidazole, strobilurin, triazole and morpholine fungicides, trinexapac-ethyl, a commercial mixture of *o*-nitrophenol + *p*-nitrophenol + 5-nitroguaiacol, and chitosan applied during the growing season of winter wheat and in vitro in a diffusion test. A molecular identification analysis of yeasts isolated from winter wheat kernels was performed, and nucleotide polymorphisms in the *CYTb* gene (G143A) conferring resistance to strobilurin fungicides in yeast cells were identified. The size of yeast communities increased during grain storage, and the total counts of endophytic yeasts were significantly (85%) reduced following intensive fungicide treatment (fenpropimorph, a commercial mixture of pyraclostrobin, epoxiconazole and thiophanate-methyl). This study demonstrated that agrochemical residues in wheat grain can drive selection of yeast communities for reduced sensitivity to xenobiotics. A mutation in the *CYTb* gene (G143A) was observed in all analyzed isolates of the following azoxystrobin-resistant species: *Aureobasidium pullulans*, *Debaryomyces hansenii*, *Candida albicans* and *C. sake*. Agrochemicals tested in vitro were divided into four classes of toxicity to yeasts: (1) tebuconazole and a commercial mixture of flusilazole and carbendazim - most toxic to yeasts; (2) fenpropimorph and a commercial mixture of pyraclostrobin and epoxyconazole; (3) propiconazole, chitosan, thiophanate-methyl and a commercial mixture of *o*-nitrophenol, *p*-nitrophenol and 5-nitroguaiacol; (4) trinexapac-ethyl and azoxystrobin – least toxic to yeasts. It was found that agrochemicals can have an adverse effect on yeast abundance and the composition of yeast communities, mostly due to differences in fungicide resistance between yeast species, including the clinically significant *C. albicans*.

1. Introduction

Winter wheat is the major cereal crop grown for consumption. Its yield is determined by various factors, including microbial colonies that colonize wheat plants (Salvador et al., 2006). Wheat is susceptible to infections caused by numerous pathogens, mostly *Zymoseptoria tritici*, *Fusarium* spp., *Blumeria graminis* and *Oculimacula* spp. These pathogens can be controlled with benzimidazole, strobilurin, triazole and morpholine fungicides with various mechanisms of action targeting fungi. The most popular triazole and morpholine fungicides belong to the group of sterol biosynthesis inhibitors (SBIs). Benzimidazoles target a specific site in the β-tubulin subunit of fungal cells (Hahn, 2014). Strobilurins inhibit mitochondrial respiration in fungal cells by binding with the coenzyme ubiquinol in cytochrome b and c1 (enzyme complex III) (Bartlett et al., 2002; Grasso et al., 2006a). Apart from their yield-forming benefits, agrochemicals also exert adverse environmental impacts (Bartlewicz et al., 2016). Broad-spectrum fungicides can affect non-target organisms including humans (Guyton et al., 2015; Bartlewicz et al., 2016). The limited effectiveness of fungicides resulting from the fungicides arising from the emergence of resistant pathogen strains is another important consideration. Strobilurins were applied for the first time in 1996, and within a few years resistant forms of *Zymoseptoria tritici*, *Blumeria graminis* and other plant pathogens were reported across Europe (Grasso et al., 2006b; Ishii and Holloman, 2015). The majority of strobilurin-resistant pathogens had point mutations in the cytochrome b (*CYTb*) gene (Heick et al., 2017).

The mechanism of action responsible for the effect of these crop protection compounds on microorganisms colonizing wheat grain has to be understood to create the best growing conditions for plants and to produce crops of the highest quality. Yeasts, which are investigated less

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Table 1
Fungicide treatments on winter wheat used in this study.

Treatments		BBCH 31	BBCH 55	BBCH 71
ECON1	Economical 1	Alert 375 SC 1 (fusilazole – 125 g/l, carbendazim – 250 g/l)	No treatment	Tarcza Łan 250 EW [2] (tebuconazole – 250 g/l)
INT1	Intensive 1	Corbel 750 EC [3] (fenpropimorph – 750 g/l)	Opera Max 147,5 SE [4] (pyraclostrobin – 85 g/l, epoxyconazole 62,5 g/l)	Tarcza Łan 250 EW (tebuconazole – 250 g/l)
INT2	Intensive 2	Corbel 750 EC (fenpropimorph – 750 g/l)	Opera Max 147,5 SE (pyraclostrobin – 85 g/l, epoxyconazole 62,5 g/l)	Topsin M 500 SC [5] (thiophanate-methyl – 500 g/l)
REG1	With growth regulator 1	Moddus 250 EC [6] (trinexapac-ethyl – 250 g/l)	Amistar 250 SC [7] (azoxystrobin – 250 g/l)	Topsin M 500 SC (thiophanate-methyl – 500 g/l)
REG2	With growth regulator 2	Moddus 250 EC (trinexapac-ethyl – 250 g/l)	Amistar 250 SC (azoxystrobin – 250 g/l)	Tarcza Łan 250 EW (tebuconazole – 250 g/l)
BIOT	Biotechnical	Asahi SL[8] (*o*-nitrophenol – 2%, *p*-nitrophenol – 3%, 5-nitroguaiacol – 2%)	Biochicol 020 PC [9] (chitosan – 2%)	Biochicol 020 PC (chitosan – 2%)
ECON2	Economical 2	no treatment	Bumper 250 EC[10] (propiconazole – 25.1%)	Topsin M 500 SC (thiophanate-methyl – 500 g/l)

[1] – Du Pont International Operations Sarl - Switzerland, [2] – Zakłady Chemiczne "Organika-Sarzyna" SA - Poland, [3,4] – BASF SE - Germany, [5] – Nippon Soda Co. Ltd., Shin-Ohtemachi Building, Japan, [6] – Syngenta - Switzerland, [7] – Syngenta, United Kingdom of Great Britain and Northern Ireland, [8] – Asahi Chem. MFG Co. Ltd., Japan, [9] – Gumitex Poli – Farm Sp. z o.o., Poland, [10] – Makhteshim Chemical Works Ltd - Israel.

frequently than other saprotrophic fungi such as *Cladosporium* spp. and *Epicoccum* spp., play a particularly important role in grain production (Bertelsen et al., 2001). Epiphytic and endophytic yeasts are the predominant fungi colonizing the above-ground parts of cereals (Karlsson et al., 2014). Yeasts are found in both Ascomycota and Basidiomycota, with three subdivisions of Ascomycota containing the bulk of species: (1) Schizosaccharomyces in Taphrinomycotina, (2) Saccharomycotina and (3) Pezizomycotina filamentous fungi (Kurtzman and Robnett, 2013; Fitzpatrick et al., 2006; Kurtzman, 2014). Many yeast species belong to the division Basidiomycota, which additionally complicates their classification (Kurtzman, 2014). Some yeasts are found only in the asexual phase, including the genera *Rhodotorula* and *Cryptococcus* (Yurkov et al., 2015). Confounding their discrete classification is the fact that phylogenetic analyses have frequently revealed the polyphyletic nature of yeast genera (Kurtzman et al., 2011). The first studies documenting the ability of yeasts to inhibit the growth of phytopathogens in monocotyledons (Fokkema et al., 1979) were continued and developed by several research teams (Dik et al., 1991; Khan et al., 2004; Schisler et al., 2002; Karlsson et al., 2014), which led to the selection of isolates useful for the protection of cereals (De Curtis et al., 2012). In yeasts that are used for biological protection against plant pathogens, the key mechanisms of action are competition and antibiosis (Wachowska and Borowska, 2014), but they can also influence physiological processes in plants (El-Tarabily and Sivasithamparam, 2006). Yeast abundance increases naturally in stored grain (Druvefors et al., 2002), and it modifies flour strength (Salvador et al., 2006). The application of yeasts during the growing season induces changes also in the content of lipophilic bioactive compounds (including sterols and carotenoids) in wheat grain (Wachowska et al., 2016).

Fungicides eliminate fungal pathogens, but they can also limit the proliferation of saprotrophic fungi, including yeasts. For this reason, the influence of fungicides on non-target fungi has to be well understood (Newton et al., 2010). In some cases the beneficial fungi are less susceptible to fungicides than pathogens (Dawidziuk et al., 2016). A study into species of the genus *Fusarium*, the causative agents of Fusarium head blight in cereals, demonstrated that the use of fungicides to protect crops against specific pathogens can exacerbate infections caused by other pathogens (Birzele et al., 2002). Such complications can be attributed to the inhibitory effect of fungicides on saprotrophic fungi which compete with pathogens for resources (Henriksen and Elen, 2005). Phyllospheric saprotrophs also accelerate leaf aging, therefore, fungicides can contribute to productivity despite the absence of pathogenic infections (Bertelsen et al., 2001). The effect of fungicides on yeasts colonizing grain has to be thoroughly analyzed to optimize fungicide application. A better understanding of the interactions between fungicides and yeasts is required to facilitate the combination biological control using yeast suspensions with fungicide treatments. The objective of this study was to evaluate the sensitivity of yeasts to three different classes of antifungals that are commonly used in agriculture. These treatments were applied both during the growing season of winter wheat and under in vitro conditions. Isolates of the following yeast species: *Aureobasidium pullulans* (De Bary) G. Arnaud, *Candida albicans* (Robin) Berkhout, *Candida sake* (Saito & Oda) van Uden & H.R. Buckley, *Debaryomyces hansenii* (Zopf) Lodder & Kreger-van Rij (anamorph: *Candida famata* (Harrison) S.A Meyer & Yarrow var. *famata), Metchnikowia pulcherrima* Pitt & M.W. Miller (anamorph: *Candida pulcherrima* (Lindner) Windisch) and *Rhodotorula glutinis* (Fresenius) F.C. Harrison, obtained from wheat grain, and the reference isolate of *Saccharomyces cerevisiae* Meyen ex E.C. Hansen were analyzed in vitro to evaluate their sensitivity to agrochemicals. The isolates obtained from wheat grain were identified based on ITS 1, 5.8S and ITS 2 rDNA sequences along with the morphology of colonies, cells and pseudofilaments. The sequences of selected isolates were deposited in GenBank, and were used to estimate the relatedness between the analyzed species. Selected azoxystrobin-resistant isolates were analyzed to detect point mutations in the *CYTb* gene (G143A) responsible for resistance to QoIs. The A143 mutation has been found in the strobilurin-producing basidiomycete *Mycena galopoda* and in plant pathogens, whereas its presence in yeasts has not been previously documented.

2. Materials and methods

2.1. Field experiment

The effects of agrochemicals on yeast communities colonizing winter wheat kernels were evaluated under field conditions. A field plot experiment with a randomized block design with four replications was performed in 2010–2012 in Tomaszkowo, Poland (53.71 N, 20.41 E). Winter wheat (*Triticum aestivum* L., cv. Bogatka) was sown in plots of 25 m^2 at 14 g of grain per m^2. Seven different plant protection regimes were devised for the plots: four intensive protection plans where plants were treated a three stages throughout the growing season, in combination with (REG1, REG2) or without (INT1, INT2) growth regulators; two economical plans where only two treatments were applied (ECON1, ECON2) and a biotechnical treatment plan (BIOT) (Table 1). Wheat plants were severely infected with *Zymoseptoria tritici*, *Oculimacula* spp. and *Fusarium* spp., and agrochemicals were applied in the stem elongation stage (BBCH 31), heading stage (BBCH 55) or watery ripe stage

(BBCH 71) (Meier, 2003). Crop protection products were sprayed onto plants with the use of a backpack sprayer (Marolex, Poland), in the afternoon, on windless and cloudy days, at 15–25 °C. In plots subjected to intensive protection (INT1, INT2) and treated with growth regulators (REG1, REG2), the fungicide Corbel 750 EC (fenpropimorph) or Moddus 250 EC (trinexapac-ethyl) was applied on the first application date, and the fungicide Opera Max 147.5 SE (pyraclostrobin, epoxiconazole) or Amistar 250 SC (azoxystrobin) was applied on the second date for foliar protection, spikes were protected with the fungicide Tarcza Łan 250 EW (tebuconazole) or Topsin M 500 SC (thiophanate-methyl). In treatment ECON1, Alert 375 SC (carbendazim, flusilazole) was applied in the stem elongation stage (BBCH 31), and the fungicide Tarcza Łan 250 EW (tebuconazole) was sprayed in the watery ripe stage (BBCH 71). In treatment ECON2, winter wheat plants were treated with the fungicide Bumper 250 EC (propiconazole) in the heading stage (BBCH 55) and Topsin M 500 SC in the watery ripe stage (BBCH 71). All agrochemicals were applied in doses recommended by the manufacturers. Alert 375 SC, Corbel 750 EC, Amistar 250 SC and Tarcza Łan 250 EW were applied at 1 L/ha, Bumper 250 EC – at 0.5 L/ha, Topsin M 500 SC – at 1.4 /ha and Opera Max 147,5 SE – at 2 L/ha. In the biotechnical treatment (BIOT), plants were sprayed with the Asahi SL growth stimulator (ortho-nitrophenol, para-nitrophenol, 5-nitrogauiacol) at 0.6 L/ha and were twice sprayed with the Biochicol 020 PC resistance inducer (chitosan) at 2.5 L/ha. Unprotected plots constituted the control.

Winter wheat was fertilized with nitrogen (N) at 120 kg/ha, potassium (K) at 82 kg/ha, and phosphorus (P) at 26 kg/ha. Grain was harvested in the over-ripe stage (BBCH 92) with a plot harvester. Grain with 14% relative moisture content was transported to the laboratory. The size of yeast and microbial populations colonizing wheat kernels was analyzed upon harvest and after six months of storage in paper bags at low humidity and temperature of 11 °C.

2.2. Isolation of yeasts from grain

The abundance of yeasts was evaluated using 10 g samples of non-disinfected whole kernels (epiphytes) or kernels disinfected with 1% sodium hypochlorite (endophytes). Samples of non-disinfected and disinfected grain, mechanically ground (Predom mill, Poland) into 2–3 mm fragments weighing 10 g were placed in 250 ml flasks filled with 90 ml sterile water. Microorganisms were rinsed from the samples by shaking the flasks for 60 min at 180 rpm on a shaker table (358S, Elpin Plus, Poland). Microbial suspensions (0.1 cm^3 each) were transferred by pipette to sterile Petri plates with a diameter of 9 cm (Equimed, Poland). Selective Martin's medium (Martin, 1950) cooled to 42 °C was poured into the plates. The experiment was performed with four replicates. Yeast cells were incubated at 24 °C in darkness for seven days. The number of yeast colonies was counted and then the colonies were transferred to Petri plates containing potato dextrose agar (PDA, Merck, Poland). Pure cultures were obtained by repeated subculturing. Yeasts were initially identified microscopically (Nicon Eclipse E200, Japan) and with the use of API ID 32 C test strips (bioMérieux, Poland) (Kurtzman et al., 2011). Precise yeast identification was performed by sequencing of the ITS1-5.8S-ITS2 fragment. The predominant species was *A. pullulans* which accounted for 38.62% of all isolated and identified yeasts, mainly in the biotechnical treatment (BIOT) and the control treatment (Table 2).

2.3. Molecular identification of yeasts

The cells of single yeast isolates were diluted 10× in Czapek-Dox liquid medium (Merck, Poland) and subcultured on PDA medium to obtain single colonies. The colonies were then grown for 2 days on 20 cm^3 of Czapek-Dox medium in conical flasks on an orbital shaker (SHKE4000-1CE, LaboPlus, Poland) at 125 rpm at 20 °C. A 1.5 cm^3 cell suspension was then transferred to sterile Eppendorf tubes and centrifuged at 5000 rpm for 5 min (Eppendorf Centrifuge 5418, Poland). DNA was isolated with the use of the DNA Genomic Mini AX kit (A&A Biotechnology, Poland). Fragments containing ITS 1, 5.8 S and ITS 2 rDNA regions were amplified with species-specific primers ITS5 (F) GTATCGGACGGAGATCCAGC and ITS4 (R) TTGCTCAGTGCATTGT CGG (White et al., 1990) with the use of the FailSafe PCR system (Epicentre, Poland). The reaction was performed on 20 ng DNA in a Mastercycler Ep Gradient thermocycler (Eppendorf, USA). The reaction had the following thermal profile: 95 °C for 3 min, followed by 34 cycles of: 95 °C for 1 min, 58 °C for 1 min, 74 °C for 3 min and 74 °C for 10 min. Electrophoresis of amplicons with the size of 750 bp was carried out in 1.2% agarose gel (Prona, Poland) in TBE buffer (Sigma, Poland). Amplicons were sequenced at the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw (www.ibb.waw.pl). Purified labeled DNA fragments were sequenced by an external service and the obtained sequences were compared with those in the NCBI database: BLAST (Basic Local Alignment Search Tool) (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Toxic effects of agrochemicals on selected yeast isolates in a diffusion test

The sensitivity of 189 yeast isolates of *A. pullulans*, *C. albicans*, *C. sake, D. hansenii*, *M. pulcherrima* and *Rh. glutinis* (Table 2) to agrochemicals was analyzed in a diffusion test. All products were applied at three concentrations equivalent to: maximum residue limits (MRL), 10× MRL and field concentrations (FCon) (Table 3). These limits were set for each crop protection product in accordance with Regulation EC no 396/2005 of the European Parliament and of the Council and with product safety sheets (Table 3). In the diffusion test, pure yeast cultures were transferred to PDA (Merck) in Petri plates with a diameter of 9 cm. The reference isolate of *Saccharomyces cerevisiae* was obtained from a gene bank (CBS 2451, Utrecht GeneBank). Yeast suspensions with a standard 2 ° cell density according to McFarland scale were introduced in the amount of 0.1 cm^3 per plate and spread evenly on the surface of PDA with a glass spreader. Six antibiogram discs (per Petri dish) measuring 5 mm in diameter (BTL, Poland) were placed on the medium saturated with the tested products in three concentrations. During incubation, the analyzed products were diffused from paper discs into the medium, creating zones of inhibition. Petri plates were photographed with a Sony A330 digital camera, and images were processed using the ImageJ 1.48 application (http://imagej.nih.gov/ij/, Rasband, 1997–2016). The size of inhibition zones (in cm^2) was measured in 8-bit images (256 grayscale).

2.5. Detection of point mutations conferring resistance to azoxystrobin

Nineteen isolates of the following azoxystrobin-resistance species: *A. pullulans*, *D. hansenii*, *C. albicans* and *C. sake* were analyzed. Spontaneous point mutations in the *CYTb* gene, responsible for resistance to strobilurin, were detected using the mismatch amplification mutation assay developed by Siah et al. (2010). In resistant isolates, 302 bp fragments were amplified using StrobSNPrcF7 (5`-CAATAAGT TAGTTATAACTGTTGCGG-3`) and StrobSNPrcR1 (5`-CTATGCATTATA ACCCTAGCGT-3`) primers. The reaction was carried out on 50 µL samples with the use of 50 ng DNA of selected isolates. The reaction had the following thermal profile: 94 °C for 10 min, followed by 40 cycles of: 94 °C for 1 min, 55 °C for 30 s, 60 °C for 30 s – for StroSNP2fwd and StrobSNP1rws primers, and 72 °C for 1 min – for StrobSNPrcF7 and StrobSNPrcR1 primers, ending with 72 °C for 10 min. The reaction's products were visualized by electrophoresis in 1.5% agarose gels (Prona, Poland) in TBE buffer (Sigma, Poland).

2.6. Statistical analysis

Results were processed using the Statistica 13.0 (StatSoft, Inc, 2016)

Table 2
Number of yeast isolates obtained from different treatments during the growing season.

Species	Control	ECON1[†]	INT1	INT2	REG1	REG2	BIOT	ECON2	Total
Aureobasidium pullulans	20	7	5	4	5	3	20	9	73
Candida albicans	3	7	0	1	0	2	0	0	13
Candida sake	3	4	1	1	3	9	1	2	24
Debaryomyces hansenii	3	1	5	6	6	4	0	1	26
Metschnikowia pulcherrima	1	9	2	8	7	3	1	2	33
Rhodotorula glutinis	2	4	2	2	0	5	3	2	20
Total	32	32	15	22	21	26	25	16	189

[†] Names of treatments as in Table 1.

Table 3
Concentrations of agrochemicals used in diffusion tests.

Fungicide	1× MRL mg/kg	10× MRL mg/kg	FCon (%)
Azoxystrobin	0.3	3.0	0.25
Chitosan	2	20	2.5
Fenpropimorph	0.5	5.0	0.25
Fusilazole + carbendazim	0.1	1.0	0.25
o-nitrophenol + *p*-nitrophenol + 5-nitroguaiacol	2	20	0.15
Propiconazole	0.05	0.5	0.13
Pyraclostrobin + epoxyconazole	0.2	2	0.5
Tebuconazole	0.2	2.0	0.18
Thiophanate-methyl	0.05	0.5	0.35
Trinexapac-ethyl	0.5	5.0	0.1

MRL - maximum residue limits, FCon - field concentration.

Table 4
The mean number (Log (CFU + 1) per 1 g grain) of yeast communities colonizing the grain of winter wheat under experimental conditions in 2010–2012. The (min - max) values are given in brackets.

Treatment	Epiphytes	Endophytes
Control	3.46 (2.69–4.65)	1.66[a] (0.59–3.76)
ECON1[†]	3.34 (2.58–4.33)	1.97[a] (1.12–3.14)
INT1	3.22 (2.41–4.46)	1.78[a] (0.76–2.45)
INT2	3.39 (2.76–4.62)	0.83[b] (0.00–2.91)
REG1	3.39 (2.60–4.72)	1.87[a] (0.00–0.35)
REG2	3.41 (2.32–4.71)	2.18[a] (0.59–3.38)
BIOT	3.19 (1.96–4.72)	1.59[a] (0.59–2.98)
ECON2	3.01 (1.47–3.86)	1.24[a] (0.00–2.88)
H	2.93[x]	1.09[x]
S	3.68[y]	2.19[y]

Values followed by the same letter do not differ significantly within columns according to the SNK test at $p < 0.01$.
[†] Names of treatments as in Table 1. H - post harvest, S - post storage of six months.

software package. Yeast colony counts were log transformed (CFU + 1) and these results were presented as log(CFU + 1) per 1 g of kernels, where log is a base-ten logarithm. The data were subjected to analysis of variance (ANOVA), and the significance of differences between mean values was determined by the Student-Newman-Keuls (SNK) test. The size of inhibition zones in the diffusion test was expressed in cm^2. The significance of differences between mean areas of inhibition zones, depending on the origin of isolates, yeast species, chemical treatments applied during the growing season and the products used in the diffusion test, was tested by the Student-Newman-Keuls (SNK) test. The percentage of isolates of the species *A. pullulans*, *C. albicans*, *C. sake, D. hansenii*, *M. pulcherrima* and *Rh. glutinis,* characterized by different sensitivity to the tested agrochemicals (0 – not sensitive, 0–2 cm^2 – weakly sensitive, 2–5 cm^2 – moderately sensitive, > 5 cm^2 – highly sensitive), was presented as heat maps for each product (Wilkinson, Friendly, 2009).

3. Results

3.1. The abundance of yeasts isolated from winter wheat grain after chemical treatment

The abundance of epiphytic yeasts on wheat kernels (3.31 log (CFU + 1) on average) was 47-fold higher than the abundance of endophytic yeasts (1.64 log (CFU + 1) on average) (Table 4). During six months of storage, yeast abundance increased by 5.6-fold in epiphytes and 12.6-fold in endophytes. Protective treatments did not exert a significant effect on the average abundance of yeasts colonizing the surface of wheat kernels, and the size of endophytic communities was reduced significantly by 85% relative to the control only after the application of fungicides (INT2): fenpropimorph, a commercial mixture of pyraclostrobin, epoxyconazol and thiophanate-methyl.

3.2. Yeast identification

Six yeast species and genera were isolated from wheat kernels: *Aureobasidium pullulans* (De Bary) G. Arnaud, *Candida albicans* (Robin) Berkhout, *Candida sake* (Saito & Oda) van Uden & H.R. Buckley, *Debaryomyces hansenii* (Zopf) Lodder & Kreger-van Rij (anamorph: *Candida famata* (Harrison) S.A Meyer & Yarrow var. *famata*), *Metchnikowia pulcherrima* Pitt & M.W. Miller (anamorph: *Candida pulcherrima* (Lindner) Windisch) and *Rhodotorula glutinis* (Fresenius) F.C. Harrison (Table 2). The identification was based on the sequences of ITS1, 5.8S and ITS2 rDNA fragments, which in most cases exceeded 99% homology with the corresponding regions in the type strains of the species (NCBI, BLAST). The majority of identified species, including *C. albicans*, *C. sake, D. hansenii*, *M. pulcherrima* belonged to the division Ascomycota, subdivision Saccharomycotina. The species *A. pullulans* belongs to the division Ascomycota, subdivision Pezizomycotina. The division Basidiomycota was represented by *Rh. glutinis.* The ITS1, 5.8S and ITS2 rDNA sequences of 21 isolates were deposited in GenBank under the following accession numbers: *A. pullulans* - KX444656-KX44459, KX444670, KX424381-KX424384, *C. sake* - KX444660, *C. albicans* - KX444661, *D. hansenii* - KX444669, KX444668, *M. pulcherrima* - KX424389, *Rh. glutinis* - KX424385-KX424388, KX424653-KX424655.

3.3. The effect of agrochemicals on yeast isolates in a diffusion test

On average, epiphytes were significantly less sensitive to the analyzed agrochemicals than endophytes (Table 5). The standard isolate of *S. cerevisiae* was generally most sensitive to the tested concentrations of agrochemicals. Yeast colonies were classified into three groups: (1) most sensitive: *A. pullulans* and *Rh. glutinis*, (2) sensitive: *M. pulcherrima,*

Table 5
The sensitivity of yeast species to different concentrations of fungicides, selective effects of crop protection products applied during the growing season and fungicide ecotoxicity classes determined in diffusion tests.

Treatments	MRL†	10xMRL	FCon††
	Size of inhibition zones in cm^2		
A. Ecological niche			
Epiphytes	0.54	1.17^{b}	1.99^{b}
Endophytes	0.69	1.57^{a}	2.48^{a}
B. Species of yeasts			
Saccharomyces cerevisiae	4.81^{a}	5.88^{a}	5.12^{a}
Aureobasidium pullulans	1.11^{b}	2.24^{bc}	3.66^{ab}
Candida albicans	0.32^{b}	0.91^{c}	1.18^{cd}
Candida sake	0.33^{b}	1.06^{c}	1.58^{cd}
Debaryomyces hansenii	0.22^{b}	0.57^{c}	1.49^{cd}
Metschnikowia pulcherrima	0.98^{b}	2.63^{b}	3.12^{bc}
Rhodotorula glutinis	0.95^{b}	2.10^{bc}	4.09^{ab}
C. Crop protection products in a diffusion test			
Azoxystrobin	0.26^{c}	0.57^{d}	0.85^{b}
Chitosan	0.59^{bc}	0.63^{cd}	0.33^{b}
Fenpropimorph	0.76^{bc}	1.44^{bc}	2.67^{a}
Fusilazole, carbendazim	1.29^{a}	1.93^{b}	2.92^{a}
o-nitrophenol + *p*-nitrophenol + 5-nitroguaiacol	0.51^{bc}	0.72^{cd}	0.94^{b}
Propiconazole	0.39^{c}	0.98^{cd}	2.87^{a}
Pyraclostrobin, epoxyconazole	0.59^{bc}	1.47^{bc}	3.16^{a}
Tebuconazole	0.98^{ab}	3.20^{a}	3.21^{a}
Thiophanate-methyl	0.25^{c}	0.73^{cd}	1.69^{ab}
Trinexapac-ethyl	0.19^{c}	0.45^{d}	0.91^{b}
D. Treatment during the growing season			
Control	0.58^{b}	1.40^{b}	2.58^{b}
ECON1‡	0.52^{b}	1.31^{b}	2.04^{bc}
INT1	0.46^{b}	1.18^{b}	2.05^{bc}
INT2	0.54^{b}	1.19^{b}	1.89^{bc}
REG1	0.48^{b}	0.94^{b}	1.73^{bc}
REG2	0.41^{b}	0.79^{b}	1.21^{c}
BIOT	1.17^{a}	2.41^{a}	3.82^{a}
ECON2	0.42^{b}	0.75^{b}	1.32^{c}

Values followed by the same letter do not differ significantly within columns and treatments according to the SNK test at $p < 0.01$.
† MRL - maximum residue limits.
†† FC - field concentrations.
‡ Names of treatments as in Table 1.

and (3) least sensitive: *D. hansenii*, *C. albicans* and *C. sake* (Table 5).

Crop protection products were divided into four toxicity groups based on their effect on yeasts in the diffusion test (Table 5). The first group comprised tebuconazole (TBC) and a commercial mixture of flusilazole and carbendazim (FC). TBC was characterized by the strongest average inhibitory effect on 189 yeast isolates at a concentration of 10xMRL and FCon (68% *C. sake* isolates and 31% *Rh. glutinis* isolates were sensitive to FCon) (Fig. 1). In the diffusion test, a commercial mixture of flusilazole and carbendazim exerted the strongest toxic effect at a concentration of 10xMLR, mostly on *Rh. glutinis* isolates (82%) (Table 5, Fig. 1). Fenpropimorph (F) and a commercial mixture of pyraclostrobin and epoxyconazole (PE) belonged to the second toxicity group, due to their strong inhibitory effect on yeast isolates, observed at FCon (Table 5). Fenpropimorph (F) was toxic to 87% of *Rh. glutinis* isolates (Fig. 1), whereas 60% of *A. pullulans* and *C. albicans* isolates were sensitive to a commercial mixture of pyraclostrobin and epoxyconazole (Fig. 1). The fungicide propiconazole (PPC) belonged to the third toxicity group, due to its highly toxic effect on yeast isolates, noted at FCon. All *C. albicans* isolates responded very strongly (> 5 cm^2 inhibition zones) to the presence of this fungicide at the highest tested concentration (Fig. 1). The third toxicity group comprised also the fungicide thiophanate-methyl (TM), which exerted a relatively strong toxic effect on yeast isolates when applied at FCon (64% of *Rh. glutinis* isolates) (Table 5, Fig. 1), as well as chitosan and a commercial mixture of *o*-nitrophenol, *p*-nitrophenol and 5-nitroguaiacol (NNN) (Table 5). Only 26% of *A. pullulans* isolates, 17% of *D. hansenii* isolates and 28% *Rh. glutinis* isolates were weakly sensitive to chitosan (CH) applied at FCon (Fig. 1). All analyzed species responded to the presence of a commercial mixture of *o*-nitrophenol, *p*-nitrophenol and 5-nitroguaiacol (NNN), and up to 50% of all isolates responded to the tested product applied at FCon. The fourth toxicity groups consisted of the fungicide azoxystrobin (A) and trinexapac-ethyl (TE); 86–100% of isolates of the tested yeast species were not sensitive to these agrochemicals (Fig. 1).

The isolates obtained from the kernels of wheat plants protected with fungicides azoxystrobin and TBC (REG2), and with PPC and TM (ECON2) were significantly less sensitive to crop protection products (FCon) in the diffusion test than the isolates from control grain (Table 5). Isolates from the grain of fungicide-protected wheat plants (all treatments) were generally less sensitive to commercial mixtures of pyraclostrobin, epoxyconazole and flusilazole, carbendazim and tiophanate-methyl in the diffusion test (Table 6). The sensitivity of yeast isolates to azoxystrobin, propiconazole and fenpropimorph was also noticeably reduced when the above fungicides were applied during the growing season.

3.4. The resistance of yeast isolates to strobilurin fungicides

Selected yeast isolates, which were resistant to azoxystrobin in the diffusion test, were analyzed to detect a point mutation in the *CYTb* gene (G143A). Resistant isolates, which contained the A143 allele conferring resistance to azoxsystrobin, produced a single amplicon of 302 bp in length (Supplementary data). All analyzed isolates of *A. pullulans*, *C. albicans*, *C. sake* and *D. hansenii* carried mutations in the *CYTB* gene responsible for fungicide resistance. None of the isolates produced an amplicon of 639 bp, which would point to their sensitivity to the tested fungicide.

4. Discussion

Yeast communities isolated from winter wheat grain during the three-year field experiment were very abundant, which confirms earlier observations that this group of microorganisms plays a dominant role in this particular ecological niche (Laca et al., 2006; Druvefors et al., 2002). The yeast species identified in the present study were typical of this environment. The predominance of *A. pullulans* in yeast communities colonizing wheat grain, observed in our experiment, had been previously reported by Barkat et al. (2016). In the cited study, *A. pullulans* abundantly colonized wheat grain particularly during storage. The percentage of *C. albicans* isolates obtained from wheat kernels was low in both our experiment and the study conducted by Larran et al. (2007) where this species was isolated sporadically (occurrence frequency of 0.4%). The presence of *D. hansenii* and *Rh. glutinis* in cereal grain was reported by Druvefors and Schnürer (2005). In the current study, significantly more epiphytic than endophytic yeasts were isolated from wheat kernels. Laca et al. (2006) also demonstrated that the number of microbial communities is significantly reduced when external layers of grain are removed. In our study, the size of yeast colonies increased during storage, which is consistent with the findings of Druvefors et al. (2002) who reported an increase in the abundance of *Pichia anomala* from 10^2 to 10^7 colony-forming units (CFU) after nine months of storage.

In the present study, fungicides significantly decreased total yeast counts on wheat kernels relative to the control in one case only, after the application of fungicides with different modes of action (fenpropimorph, a commercial mixture of pyraclostrobin, epoxyconazol and thiophanate-methyl). These results corroborate the findings of Buck and Burpee (2002) who reported an inhibitory effect of intensive fungicide treatments (azoxystrobin, chlorothalonil, fluotanil and propiconazole) on yeasts colonizing grasses. Fungicides can also influence the composition of yeast communities in the phyllosphere. A recent study of two Swedish regions demonstrated moderate but significant effects of

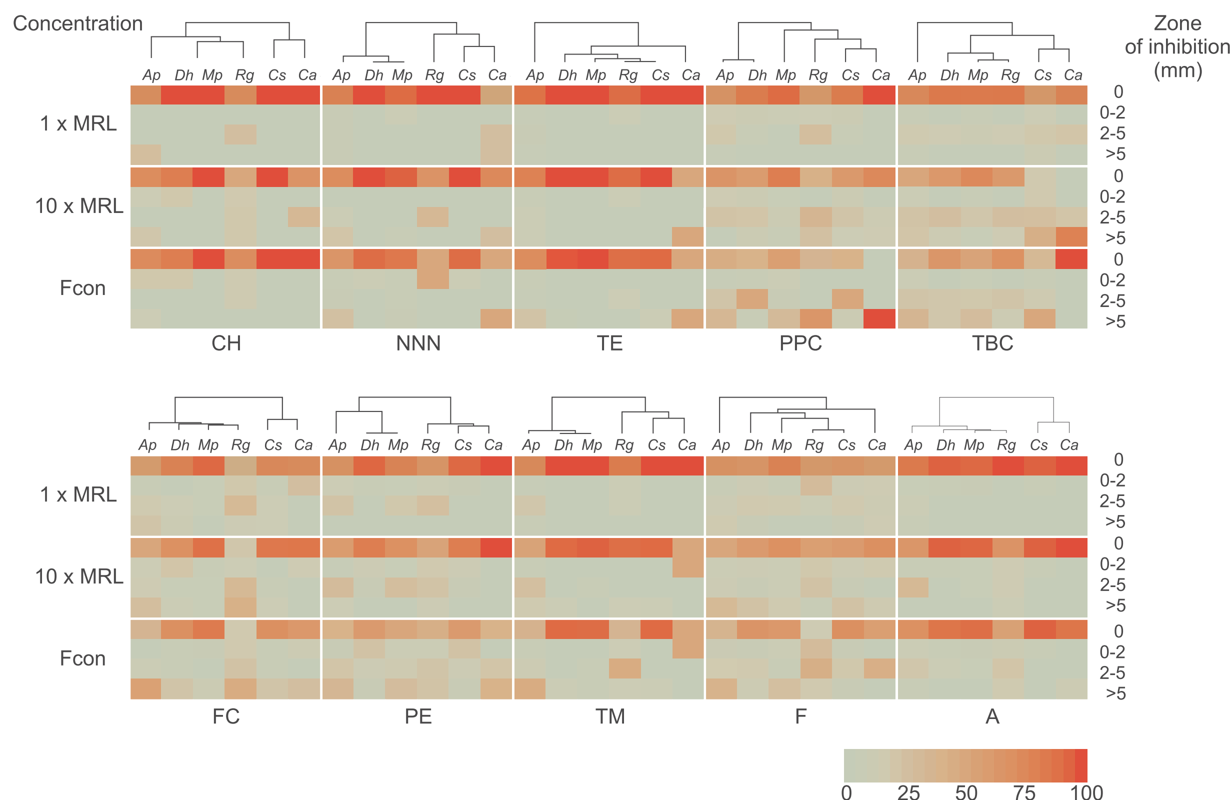

Fig. 1. The sensitivity of yeast isolates to different concentrations of agrochemicals: maximum residue limits (MRL), 10 × MRL and field concentrations (FCon). CH - chitosan, NNN - O-nitrophenol + p-nitrophenol + 5-nitroguaiacol, TE - trinexapac-ethyl, PPC – propiconazole, TBC – tebuconazole, FC - fusilazole, carbendazim, PE - pyraclostrobin, epoxyconazole, TM - thiophanate-methyl, F – fenpropimorph, A – azoxystrobin.

Table 6

Selective effects of protective treatments applied during the growing season of winter wheat on the sensitivity of yeasts to crop protection agrochemicals (interactions between treatments and yeast sensitivity in diffusion tests).

Crop protection products in a diffusion test	Crop protection regimes, used in a field experiment							
	Control	ECO 1[†]	INT1	INT2	REG1	REG2	BIOT	ECO2
	The size of inhibition zones in cm^2							
Azoxystrobin	0.48^{d-h}	0.78^{c-h}	0.83^{b-h}	0.11^{h}	0.19^{gh}	0.26^{fgh}	0.86^{b-h}	0.51^{d-h}
Chitosan	0.08^{h}	0.06^{h}	0.05^{h}	0.46^{d-h}	0.04^{h}	1.19^{b-h}	2.30^{a-f}	0.36^{fgh}
Fenpropimorph	1.73^{a-h}	1.28^{b-h}	0.98^{b-h}	2.03^{a-h}	2.16^{a-h}	1.19^{b-h}	2.54^{a-d}	1.09^{b-h}
Fusilazole + carbendazim	2.53^{a-d}	2.04^{a-h}	2.32^{a-f}	1.57^{b-h}	1.82^{a-h}	0.76^{c-h}	3.53^{a}	0.93^{b-h}
o-nitrophenol + *p*-nitrophenol + 5-nitroguaiacol	0.40^{e-h}	0.49^{d-h}	0.29^{fgh}	1.12^{b-h}	0.66^{c-h}	0.32^{fgh}	2.45^{a-e}	0.27^{fgh}
Propiconazole	1.72^{a-h}	1.60^{b-h}	1.48^{b-h}	1.27^{b-h}	0.98^{b-h}	0.83^{b-h}	2.23^{a-g}	0.56^{c-h}
Pyraclostrobin, epoxyconazole	2.14^{a-h}	1.32^{b-h}	1.56^{b-h}	1.60^{b-h}	1.15^{b-h}	0.84^{b-h}	3.52^{a}	1.29^{b-h}
Tebuconazole	2.86^{ab}	2.60^{a-c}	2.36^{e-f}	1.98^{a-h}	2.05^{a-h}	1.71^{a-h}	3.48^{a}	1.62^{b-h}
Thiophanate-methyl	1.13^{b-h}	0.75^{c-h}	0.95^{b-h}	0.77^{c-h}	0.40^{e-h}	0.08^{h}	2.02^{a-h}	0.93^{b-h}
Trinexapac-ethyl	0.34^{fgh}	0.51^{d-h}	0.33^{fgh}	0.38^{e-h}	0.31^{fgh}	0.85^{b-h}	0.92^{b-h}	0.18^{gh}

Values followed by the same letter do not differ significantly according to the SNK test at $p < 0.01$. [†]Names of treatments as in Table 1.

fungicides on fungal communities colonizing wheat leaves (Karlsson et al., 2014).

The present study revealed that *C. albicans,* responsible for clinical infections, is also present in the agricultural environment where it is subjected to selection pressure due to the widespread use of triazole fungicides. Propiconazole strongly inhibited the growth of all *C. albicans* isolates, and tebuconazole was ineffective in limiting their development. Our findings confirm previous suggestions that the emergence of resistance to triazoles among yeast strains responsible for clinical infections is the result of selection pressure exerted by these fungicides (Warrilow et al., 2013). A point mutation in the *CYP51*A gene, conferring resistance to azole fungicides, was detected in *A. fumigatus,* and it was hypothesized that azole resistance might develop through azole exposure in the environment (Snelders et al., 2009). There are four known mechanisms that confer azole resistance in *C. albicans* (Parker et al., 2014). These include mutations that alter the amino acid sequence of CYP51, overexpression of efflux transporters (CaCDR1, CaCDR2 and CaMDR1), overexpression of CYP51 and alterations in the ergosterol biosynthetic pathway such as mutations in *ERG3* (Parker et al., 2014).

Only a few studies have demonstrated a positive influence of yeast-based biocontrol agents on the quality of wheat grain (Wachowska et al., 2016). Many types of bread are still traditionally fermented with the addition of non-commercial strains of baker's yeast (Fleet, 2007). Yeasts significantly influence the rheological properties of dough by loosening its consistency (Salvador et al., 2006). Despite the addition of baker's yeast, grain is colonized predominantly by native yeast strains that can form symbiotic relationships with lactic acid bacteria to give

the final product its distinctive flavor. A field experiment conducted by Ruske et al. (2004) demonstrated that strobilurin and triazole fungicides influence the quality of bread made of winter wheat grain. In some cases, higher fungicide doses lowered protein and sulfur content and decreased the falling number of grain. A few studies have investigated the interactions between the use of agrochemicals, yeast abundance and grain quality. Therefore, it would be difficult to associate the abundance of yeasts that naturally colonize grain with the quality of the final product. Previous research shows, however, that fungicide residues affect the quality of yeasts and consequently, the quality of wine. Comitini and Ciani (2008), who investigated the fungicide sensitivity of yeasts colonizing grapes, demonstrated the prevalence of *Hanseniaspora uvarum* which accounted for 70% of the yeast population on unprotected fruit. The species composition of yeast communities on grapes can be the key factor determining the quality of wine. In a study by Garcia et al. (2004), the residues of three fungicides (cyprodinil, fludioxonil and pirimethanil) influenced the aromatic composition (acids, alcohols and esters) of white wine made of *Vitis vinifera* inoculated with three strains of *S. cerevisiae*.

In our study, the isolates from winter wheat kernels subjected to intensive protection were less sensitive to the tested fungicides in vitro than the isolates from control plots or treatments where fungicides were applied in combination with chitosan. Buck and Burpee (2002) demonstrated that yeast isolates that had not been previously exposed to fungicides were more sensitive to chlorothalonil, propiconazole, flutolanil and iprodione than yeasts isolated from protected grass. The cited authors suggested that yeasts colonizing the phylloplane of grasses are commonly resistant to fungicides. Casalone et al. (2010) reported that fungicide-resistant mutants are present in fungal populations before exposure to xenobiotics. Resistance to strobilurin is also conditioned by mutations in the gene encoding cytochrome b (*CYTb*) (Hnatova et al., 2003). In the current study, the A143 mutation was detected in the following yeast species: *A. pullulans*, *C. albicans*, *C. sake* and *D. hansenii.* The A143 mutation has been found in the strobilurin-producing basidiomycete *Mycena galopoda* and in plant pathogens, whereas its presence in yeasts naturally colonizing wheat grain has not been documented to date (Ishii and Holloman, 2015).

The agrochemicals tested in our study were divided into four classes of toxicity in yeasts. Tebuconazole and a commercial mixture of flusilazole and carbendazim were found to be most toxic to yeast isolates. In some cases, pesticide toxicity classes were correlated with the information found in product safety cards. For instance, carbendazim is also highly toxic to *Daphnia magna* (lethal concentration LC_{50} 0.15 mg/l). The allocation of tebuconazole to class 1 was also consistent with product safety data (LC_{50} for *D. magna* 5.4 mg/l, half-life DT_{50} of 60 days). Azoxystrobin, identified as a class 4 fungicide in this study, is characterized by low persistence (Wang et al., 2015). Its toxic influence on environmental microorganisms may also be limited. The above was confirmed by Hooser et al. (2012) in whose study, lethal concentration LC_{50} (mg/l) values in treatments where *Bufo cognatus* had been exposed to strobilurin fungicides for 72 h were determined at 0.0037 (pyraclostrobin) and 1.02 (azoxystrobin). A toxicity assessment of chitosan, assigned to class 3 in our study, demonstrated that LD_{50} values for mice exceeded 16 g/day/kg body weight, and were similar to salt and sugar (Singla and Chawla, 2001).

5. Conclusions

Yeasts abundantly colonize winter wheat grain, in particular the surface of kernels. Yeast abundance increases during grain storage. The size of yeast communities on wheat grain to some extent depends on the quality and quantity of the fungicides used in agricultural practices. Agrochemicals exert varied inhibitory effects on yeast isolates. Tebuconazole is four-fold more toxic than azoxystrobin to the majority of tested species, and propiconazole is highly toxic to all *C. albicans* isolates. Resistant forms of yeasts, belonging to various species, develop in yeast communities exposed to selection pressure exerted by agrochemicals. The majority of yeast isolates obtained from fungicide-protected plants are resistant to most of the tested agrochemicals. The frequency of azoxystrobin-resistant yeast isolates is particularly high; these isolates contain a point mutation in the *CYTb* gene, which is responsible for azoxystrobin resistance in yeasts.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2018.06.042.

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