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Original Research

Activity of Fungicides Against Epiphytic Yeast-Like Fungi of Winter Wheat

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Abstract

In a field experiment the susceptibility of yeast-like fungi to selected fungicides with the active ingredients (a.i.) propiconazole, epoxyconazole, kresoxim-methyl, fenpropimorph, carbendazim, prochloraz and flusilazole, was analyzed. The direct effect of the fungicides on the colonies of yeast-like fungi was determined based on antibiograms – the diffusion method. The survival rate of the isolates of yeast-like fungi on the leaves of winter wheat seedlings was determined in a phytotron. The fungicides Alert 375 SC and Bumper 250 EC inhibited the growth of yeast-like fungi most effectively, both under field conditions and in laboratory tests. Juwel TT 483 SE significantly reduced the abundance of *Sporobolomyces roseus* on both upper and lower leaves of wheat plants under *in vivo* conditions. The isolate of *Pichia* sp. was resistant to the tested fungicides.

Keywords: yeast-like fungi, epiphytic fungi, fungicides, winter wheat

Introduction

Yeast-like fungi, along with bacteria, are the dominant epiphytic microorganisms isolated from winter wheat grown under moderate climate conditions [1-4]. These microorganisms do not grow as thread-like hyphae, but they form characteristic multicellular aggregates that can effectively adhere to leaf surface thanks to the presence of exogenous adhesins [5]. Yeast-like fungi belong to the phyla *Ascomycota* and *Basidiomycota* in the kingdom *Fungi* [6]. The phylum *Basidiomycota* is most often represented by members of the following genera: *Cryptococcus* [1, 7, 8]), *Rhodotorula* [8] and *Sporobolomyces* [2, 9]. The phylum *Ascomycota* is represented by the genera *Candida* and *Pichia* [6]. Yeast-like fungi colonizing leaves are usually considered to be potential antagonists of plant pathogens [7-12]. It has been documented that those fungi can effectively protect plants against pathogens, and therefore they are components of such commercial products as "Aspire" (*Candida oleophila*, Ecogen, www.agrobiologicals.com) and "Yield plus" (*Cryptococcus albidus*, Anchor Yeast, www.anchor.co.za) which provide biological control of *Penicillium* sp. and *Botrytis* sp. on citrus fruit, apples and pears. In Poland, yeast-like fungi are used as effective microorganisms (EM) to improve soil fertility [www.minrol.gov.pl]. They may induce systemic resistance of plants against pathogens [13, 14] and produce substances which are toxic to phytopathogens [15], thus inhibiting their growth and sporulation [8, 15]. Larger populations of certain species of yeast-like fungi indirectly contribute to increasing the yieldg capacity of crops [4]. A reduction in the population size of yeast-like fungi decreases the storage quality of grain and leads to the loss of exogenous amino acids, minerals and β-carotenes that are abundant in these fungi [16].

Yeast-like fungi are susceptible to xenobiotics which occur in the plant environment and are often used as bioindicators of environmental pollution [17]. Attention should be also paid to their varied sensitivity to fungicides [3, 11, 18-20]. The application of fungicides in winter

wheat fields may result in the selection of communities of epiphytic yeast-like fungi [20]. These fungi can also be used as model organisms to assess the ecotoxicity of new fungicides introduced to the market [21]. Despite their unquestionable advantages, yeast-like fungi are seldom described in Polish literature and their role is often underestimated. The effect of fungicides, including those containing new active substances, on this group of microorganism has also been poorly investigated.

The objective of this study was to analyze quantitative changes taking place in the communities of epiphytic yeast-like fungi under field conditions, to estimate the abundance of selected fungal isolates on wheat leaves treated with fungicides, and to determine their susceptibility to fungicides under *in vitro* conditions.

Materials and Methods

Field Experiment

A study on the susceptibility of yeast-like fungi to selected fungicides under field conditions was conducted in the years 2006-08. Experimental plots were located in Tomaszkowo near Olsztyn (NE Poland). Winter wheat cv. Tonacja was grown. The date and rate of sowing, the rates of mineral fertilizers and the timing of their application as well as cultivation measures, including herbicide application, corresponded to the recomentations. The experiment was performed in a randomized complete blocks design, in four replications. Plot area was 20 m^2. The following fungicides were applied during the stem elongation stage: Bumper 250 EC (active ingredient (a.i.) : propiconazole) at a dose of 0.5 l/ha, Juwel TT 483 SE (a.i.: epoxyconazole, kresoxim-methyl, fenpropimorph) at a dose of 1.5 l/ha, Karben 500 SC (a.i.: carbendazim) at a dose of 0.5 l/ha, Mirage 450 EC (a.i.: prochloraz) at a dose of 1 l/ha and Alert 375 SC (a.i.: flusilazole, carbendazim) at a dose of 1 l/ha. Control plants were sprayed with water. 200 l of water per hectare was used in all treatments.

Isolation of Epiphytic Fungi

The abundance of epiphytes was determined 7 and 14 days after the protective treatment. The third leaves (counting from the top to the bottom) were collected randomly from winter wheat plants in each plot, except from the edge. A total of 20 fully developed leaves were collected. Fragments of leaf blades, 1 cm in length, were cut out of the lower part of the leaf (2 cm from the base). In each treatment, 15 fragments of leaf blades were used to determine the abundance of filamentous and yeast-like fungi, and 24 specimens were used to determine the abundance of fungi-forming balistospores.

15 ml of sterile water was poured into 250 ml flasks, and 15 leaf fragments were placed in each flask [3, 20]. The flasks were shaken for 30 minutes in a shaker, type 358S (170 rpm, amplitude 8). 0.1 ml of microbial suspension was taken from each flask and transferred on PDA medium amended with rose bengal and streptomycin (pH 6.5) [4]. The experiment was performed in four replications (four Petri plates, 9 cm in diameter). The plates were incubated in darkness, at a temperature of 25ºC (± 1ºC). The number of colonies in dishes was counted and converted to the number per cm^2 of leaf area (taking into account leaf width).

In order to estimate the number of colonies of balistospore-forming fungi, leaf fragments (24 in each treatment) were stuck to the top of a Petri plate with petroleum jelly [20]. Six leaves, three placed on the lower surface of the blade and three placed on the upper surface of the blade, were stuck to each dish. The bottom of the plate was filled with PDA medium. The experiment was performed in four replications. The plates were incubated in darkness, at a temperature of 25ºC (± 1ºC). The number of colonies in plates was counted and converted to the number per cm^2 of leaf area (taking into account leaf width). White and red colonies were counted.

Experimental data, i.e. the number of colony-forming units per cm^2, were verified statistically by analysis of variance with the use of Statistica 8.0 software. The significance of differences between mean values was estimated by Newman-Keuls test. Prior to analysis, numerical data were transformed according to the formula: log (cfu+1).

Determination of Selected Strains of Yeast-Like Fungi

Selected isolates of yeast-like fungi were identified based on such characteristic morphological features as the size and shape of vegetative cells, the mode of germination and the presence of pseudofilaments. The physiological features of particular isolates were also determined, using API 20 C AUX strips (Biomĕrieux). The results were read after 24-48 hours of incubation [22]. The first microtube, containing no medium, served as control. A positive response was noted in microtubes with a more turbid suspension of yeast-like fungi, as compared to the control sample. Fungal isolates were identified to genus or species based on identification strips and indicating chart [22, 23] and available monographs [6, 24, 25].

Effect of Fungicides on Yeast-Like Fungi under *in Vitro* and *in Vivo* Conditions

The direct effect of fungicides on the colonies of yeast-like fungi was determined based on antibiograms - the diffusion method. Pure cultures of three isolates of yeast-like fungi were introduced into Petri dishes, 9 cm in diameter, filled with a Sabouraud medium. Isolates of *Sporobolomyces roseus* (code 10/5) and of *Candida tropicalis* (code 167) were obtained from the leaves of winter wheat cv. Tonacja. The isolate of the species *Pichia anomala* (code W) was obtained from grapevine fruit. The cells were evenly distributed over the surface of the plate with a spreader (surface culture), and after incubation the entire surface was covered by fungal colonies [26]. Before incubation, 5 mm

Table 1. Activity of fungicides against epiphytic yeast-like fungi in diffusion test.

Fungicide	*Sporobolomyces roseus*			*Candida tropicalis*			*Pichia anomala*		
	active ingredient in ml/l								
	10	100	1000	10	10	1000	10	10	1000
	inhibition zone in mm^2								
Bumper 250 SC	91.8 *ab*	64.9 *ab*	159.2 b	0.0 *a*	0.0 *a*	150.0 *bc*	0.0 *a*	0.0 *a*	0.0 *a*
Average	105.3 *b*			50.0 *a*			0.0 *a*		
Alert 375 SC	21.0 *ab*	40.2 *ab*	61.0 *ab*	112.5 *abc*	434.4 *d*	548.8 *d*	0.0 *a*	0.0 *a*	0.0 *a*
Average	40.7 *ab*			365.2 *c*			0.0 *a*		
Juwel TT 483 SE	0.0 *a*	0.0 *a*	84.5 *ab*	0.0 *a*	50.8 *ab*	232.7 *c*	0.0 *a*	0.0 *a*	0.0 *a*
Average	28.2 *a*			94.5 *a*			0.0 *a*		
Karben 500 SC	0.0 *a*	0.0 *a*	518.6 *c*	23.1 *ab*	493.4 *d*	727.1 *e*	0.0 *a*	0.0 *a*	0.0 *a*
Average	172.9 *c*			414.5 *c*			0.0 *a*		
Mirage 450 EC	0.0 *a*	86.3 *ab*	30.3 *ab*	0.0 *a*	217.1 *c*	488.0 *d*	0.0 *a*	0.0 *a*	0.0 *a*
Average	38.9 *ab*			235.0 *b*			0.0 *a*		

Mean values followed by the same letters are not significantly differentin within a column according to SNK test (p=0.01).

filter paper disks moistened with the following fungicides: Bumper 250 EC (a.i.: propiconazole), Juwel TT 483 SE (a.i.: epoxyconazole, kresoxim-methyl, fenpropimorph), Karben 500 SC (a.i.: carbendazim), Mirage 450 EC (a.i.: prochloraz) and Alert 375 SC (a.i.: flusilazole, carbendazim) were placed on the surface of each plate. This allowed fungicides to diffuse into the medium during incubation [26]. The fungicides were applied at three concentrations: 10, 100 and 1000 mg of active substance in 1 l of water. The surface areas of inhibition zones were measured with the use of computer image analysis (ImageJ 1.37v). Numerical data were verified statistically by analysis of variance using Statistica 8.0 software. The significance of differences between mean values was estimated by Newman-Keuls test.

The survival rate of the isolates of yeast-like fungi on the leaves of winter wheat seedlings was determined in a phytotron. Seeds of winter wheat cv. Tonacja were sown in glass beakers filled with sterile soil aggregates (90 g) and sand (14 g). Kernels were surface disinfected with sodium hypochlorite for 3 minutes, rinsed three times with sterile water. After 24 hours kernels were sown at a depth of 0.5 cm. The experiment was conducted in a phytotron (temperature 23 (± 1)ºC (day) and 16 (± 1)ºC (night), relative humidity 70-80%, photoperiod 12/12 h). Plants were irrigated with Hoagland's solution [27]. Two weeks after sowing, cells of yeast-like fungi were introduced onto wheat seedlings by immersing leaves in a cell suspension culture at a concentration of 1 - 1,3 x 10^6 cells. After 48 hours, the bottom leaves of seedlings were immersed in a fungicide solution. The fungicides analyzed in a field experiment were applied at concentrations corresponding to the recommended doses and the approved amount of spray liquid was 200 l per ha. After the next 48 hours the population of yeast-like fungi was determined on the lower (treated with fungicides) and upper (non-treated with fungicides) leaves of wheat plants. Fungi were isolated from leaf surface by the previously described technique which involved washing off epiphytic microbes [3, 20]. Fungal suspensions were introduced into Petri plates. The experiment was performed twice in three replications. The results were analyzed statistically by the procedure applied to analyze microbial populations isolated from wheat leaves under field conditions.

Results

The investigated fungicides had a significant effect on the abundance of epiphytic fungi in winter wheat under field conditions (Fig. 1). Filamentous fungi and all analyzed yeast-like fungi were more abundant on leaves protected with Karben 500 SC than in the control treatment. This difference was statistically significant with respect to filamentous fungi. The fungicides Alert 375 SC, Bumper 250 EC and Mirage 450 EC had an inhibitory effect on the communities of filamentous and yeast-like fungi, and on the population of *Sporobolomyces roseus*. However, statistically significant differences in the abundance of yeast-like fungi between treated and non-treated leaves were noted only for the first two fungicides. The population size of yeast-like fungi was lower on leaves protected with Juwel TT 483 SE, compared to control.

In the diffusion test, the isolate of the species *Candida tropicalis* (code 167) was found to be more susceptible to fungicides than the isolate of *Sporobolomyces roseus* (code 10/5) (Table 1). The surface area of inhibition zones around filter paper disks moistened with fungicides was on average threefold higher in Petri dishes in which fungi of the species

C. tropicalis were cultured. The fungus of the species *Pichia anomala* was resistant to all of the analyzed fungicides. Regardless of the concentration of active substances, the growth of its colonies was not inhibited.

Karben 500 SC showed the highest inhibitory activity. This fungicide effectively inhibited the growth of the fungus of the species *C. tropicalis*, particularly when applied at a concentration of 1000 mg/l which was also sufficient to suppress the growth of *S. roseus*. The fungus of the species *C. tropicalis* was also highly sensitive to the fungicides Alert 375 SC and Mirage 450 EC. The latter inhibited the growth of this fungus when applied at a concentration of 100 and 1000 mg of active substance per l. Bumper 250 EC reduced the growth of *S. roseus* regardless of concentration. Juwel TT 483 SE showed the weakest inhibitory activity against the isolates of *S. roseus* and *C. tropicalis*.

During *in vivo* studies conducted in a phytotron, the population of *S. roseus* (symbol 10/5) developed at a slightly faster rate on the upper leaves of winter wheat seedlings cv. Tonacja in the control treatment (Fig. 2). Most of the applied fungicides significantly decreased the cell count of fungi washed from the upper leaves. Juwel TT 483 SE significantly reduced the abundance of *S. roseus* on both upper and lower leaves of winter wheat plants. A significant inhibitory effect of Mirage 450 EC was observed only on lower leaves.

In the control treatment, the population size of the isolate of the species *C. tropicalis* (code 167) was substantially higher on the lower leaves of seedlings than on the upper leaves (Fig. 3). All fungicides significantly suppressed the growth of this fungus on lower leaves. Mirage 450 EC inhibited the growth of this isolate also on upper leaves.

The abundance of the fungus of the species *P. anomala* (code W), obtained from grapevine fruit, was significantly higher on the lower leaves of winter wheat seedlings than on the upper leaves which were not treated with fungicides. This isolate was resistant to the tested fungicides (Fig. 4).

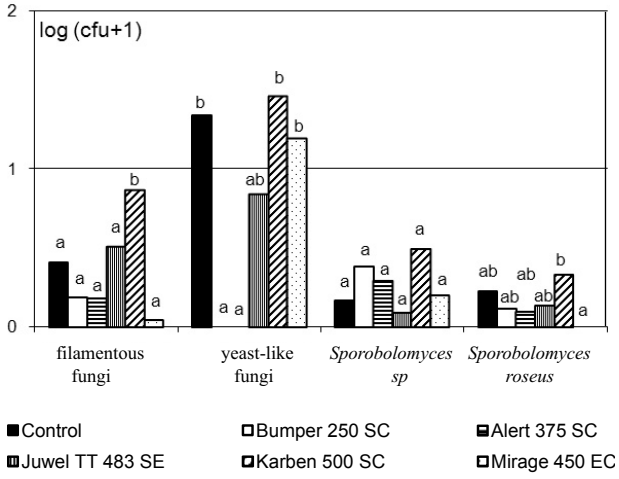

Fig. 1. Number of population of epiphitic fungi on winter wheat growth in field conditions (mean with 2006-08).
Mean values followed by the same letters are not significantly different according to SNK test (p=0.01).

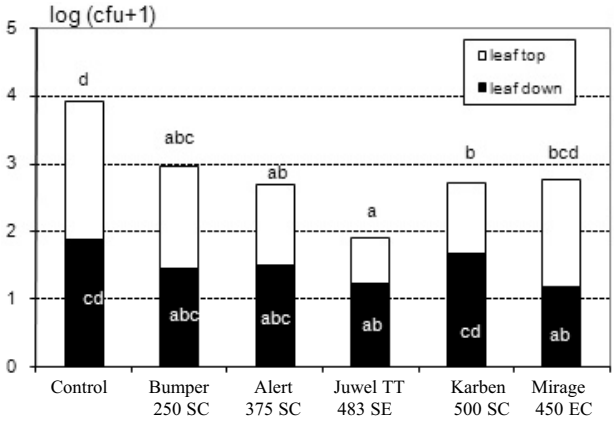

Fig. 2. Number of yeast-like fungi cells *Sporobolomyces roseus* (isolate 10/5) on 1 cm^2 of winter wheat leaf fungicide treatment. Mean values followed by the same letters are not significantly different according to SNK test (p=0.01).

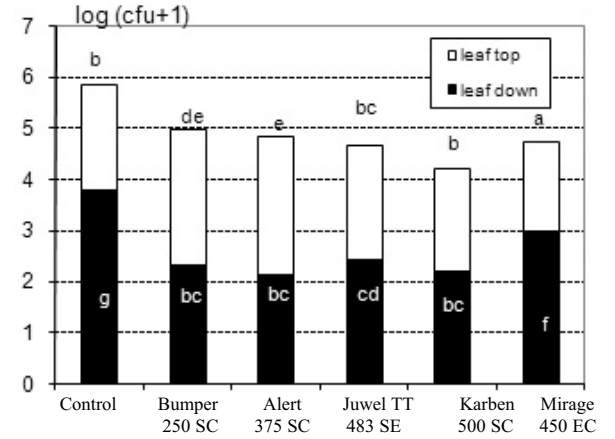

Fig. 3. Number of yeast-like fungi cells *Candida tropicalis* (isolate 167) on 1 cm^2 of winter wheat leaf fungicide treatment. Mean values followed by the same letters are not significantly different according to SNK test (p=0.01).

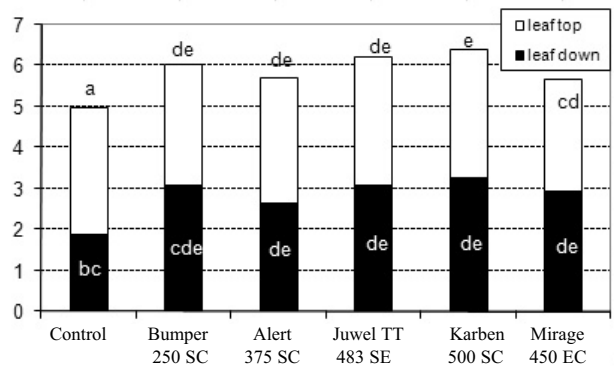

Fig.4. Number of yeast-like fungi cells *Pichia anomala* (isolate W) on 1 cm^2 of winter wheat leaf fungicide treatment.
Mean values followed by the same letters are not significantly different according to SNK test (p=0.01).

Discussion

Epiphytic yeast-like fungi dominated on winter wheat cv. Tonacja under field conditions. However, their abundance varied widely, which is typical of such ecosystems [28]. Similarly as in studies carried out on grasses [29], species of the genus *Sporobolomyces*, forming white colonies, were more frequent than *Sporobolomyces roseus*.

Epiphytic yeast-like fungi are believed to perform the role of a natural buffer against necrotrophic plant pathogens, and the great potential of particular isolates may be used for effective crop protection [13, 15]. It follows that the application of protective agents which negatively affect this group of microorganisms may promote the growth of pathogenic or allergenic fungi. In the present study, fungicides usually reduced the abundance of yeast-like fungi on leaf surface, which is consistent with the findings of Jenkyn and Prew [30]. This phenomenon is considered undesirable due to its negative effect on the quality of wheat grain which is deprived of natural protection against pathogens of the genera *Penicilium* [7] and *Fusarium* [31] during storage.

A stimulating effect of Karben 500 SC (active ingredient: carbendazim) was also noted in field investigations. Most probably, this fungicide acted as a strong selection pressure in the communities of yeast-like fungi. The populations of fungi that were less susceptible or resistant to carbendazim could redevelop to a similar size. Buck and Burpee [29] observed such a phenomenon on the leaves of grasses with respect to white yeast colonies. These authors reported the presence of yeast colonies resistant to fungicides classified as sterol biosynthesis inhibitors, respiration inhibitors and benzimidazoles.

In the current experiment, 14-day-old isolates of yeast-like fungi were well adapted to survive on the surface of wheat leaves. The population of the fungus of the species *Pichia anomala*, whose cells multiplied at a fast rate, reached very high densities. It seems that the cells of the tested fungi adhered to and remained attached to the leaf surface by means of viscous or gelatinous material produced at the sites of bud formation [5]. The cited authors detected a specific adhesin, mannoprotein, on the cell surface of *R. glutinis*, which helped to overcome hydrophobic forces between the fungus and leaf surface. The adhesive ability of *R. glutinis* is not permanent, and it may affect the colonization process and diverse biological activities, as observed by other authors. Environmental conditions (including nutrient availability) influence blastospore development, thus promoting adhesion [5].

Much higher population densities of *Candida tropicalis* were recorded on older, lower leaves of winter wheat seedlings growing in a phytotron. Most probably, these microbes showed greater affinity for older cells whose surfaces contain larger amounts of nutrients [32]. It appears that yeast-like fungi better utilize nutrients found in older leaves.

Under *in vivo* conditions, *Sporobolomyces roseus* was sensitive to most of the tested fungicides, of which Juwel TT 483 SE exhibited the strongest inhibitory activity, also against fungal cells on the upper, non-treated leaves. This could result from the unique transport properties of kresoxim-methyl [33, 34] and from the quasi–systemic mode of action of this compound, which spreads in gaseous form within wheat seedlings [www.minrol.gov.pl].

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