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ORIGINAL CONTRIBUTION

Antagonistic yeasts competes for iron with winter wheat stem base pathogens

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Abstract *Aureobasidium pullulans* and *Sporobolomyces roseus* are a saprotrophic yeasts fungi commonly found on the leaves of winter wheat and on wheat kernels. The objective of this study was to compare the inhibitory effects of two species of yeasts fungi, *Aureobasidium pullulans* var. *pullulans* (de Bary) G. Arnaud and *Sporobolomyces roseus* Kluyver & van Niel, on the causal agents of stem base diseases, *Rhizoctonia cerealis* v. d. Hoeven, *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier, *Helgardia herpotrichoides* (Fron) Crous & W. Gams, *Fusarium oxysporum* (Schlecht) Snyd. et Hans.) and *Fusarium culmorum* (W. G. Smith). *A. pullulans* showed stronger inhibitory activity than *S. roseus*. Among the 70 *A. pullulans* isolates tested in the study, 25 were capable of suppressing the colony growth of *R. cerealis* under *in vitro* conditions. This is the first study to show that *A. pullulans* competes for iron with stem base pathogens, in particular with fast-growing *R. cerealis* and *F. culmorum.* Under greenhouse conditions, *A. pullulans* protected winter wheat seedlings against infection caused by *F. culmorum,* from two to four times compared with the control, and its protective effect was determined by the infection susceptibility of wheat cultivars and the time interval between the application of *A. pullulans* and inoculation with *F. culmorum.*

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Keywords Siderophores · *Aureobasidium pullulans* · Winter wheat · Stem base pathogens

Antagonistische Hefen konkurrieren mit Winterweizenhalmbasis-Pathogenen um Eisen

Zusammenfassung *Aureobasidium pullulans* und *Sporobolomyces roseus* sind saprotrophische Hefen, die auf Weizenblättern und -Korn weit verbreitet vorkommen. Das Ziel der Studie war es, die inhibitorische Wirkung von zwei Arten von Hefen *Aureobasidium pullulans* var. *pullulans* (de Bary) G. Arnaud und *Sporobolomyces roseus* Kluyver & van Niel mit den Halmbasis-Pathogenen: *Rhizoctonia cerealis* VD Hoeven, *Gaeumannomyces graminis* (Sacc.) Arx & E. Olivier, *Helgardia herpotrichoides* (Fron) Crous & W. Gams, *Fusarium oxysporum* (Schlecht) Snyd. und Hans, *Fusarium culmorum* (S. W. Smith) zu vergleichen. *A. pullulans* wies eine stärkere inhibitorische Wirkung als *S. roseus* auf. Unter den 70 getesteten *A. pullulans* Isolaten haben 25 unter *in vitro* Bedingungen den Zuwachs der Kolonie *R. cerealis* eingeschränkt. Dies ist das erste Anzeichen, dass den Beweis dafür liefert, dass *A. pullulans* mit den Halmbasis-Pathogenen, insbesondere mit den schnell wachsenden Arten, wie *R. cerealis* und *F. culmorum,* ums Eisen konkurriert. Unter Gewächshausbedingungen waren die mit *A. pullulans* behandelten und mit *F. culmorum* inokulierten Winterweizen-Sämlinge im Vergleich zur Kontrolle zwei- bis viermal weniger befallen. Die Schutzwirkung von *A. pullulans* war von der Empfindlichkeit der Weizensorten und dem Zeitintervall zwischen der Anwendung von *A. pullulans* und der Inokulation von *F. culmorum* abhängig.

Schlüsselwörter Siderophore · *Aureobasidium pullulans* · Winterweizen · Halmbasis-Pathogenen

Introduction

The cereal stem base disease complex comprises eyespot (teleomorph: *Oculimacula yallundae* (Wallwork & Spooner) Crous & W. Gams*, Oculimacula acuformis* (Boerema & Hamers) Crous & W. Gams anamorph: *Helgardia yallundae* (Nirenberg) Crous & W. Gams*, Helgardia acuformis* (Nirenberg) Crous & W. Gams), Fusarium foot rot (*Fusarium culmorum* (W. G. Smith)*, Fusarium avenaceum* (Fr.) Sacc.*, Fusarium oxysporum* (Schlecht) Snyd. et Hans.), sharp eyespot (teleomorph: *Ceratobasidium cereale* Murray & Burpee, anamorph *Rhizoctoniacerealis* v. d. Hoeven) and take-all (*Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier) (Ray et al. 2006). Each year, stem base diseases cause a substantial decrease in total wheat yield, due to a reduced number of spikes per unit area and poor grain filling (Ray et al. 2006). The above diseases have proven to be difficult to control. Effective fungicide treatments have been developed only for pathogens of the genus *Helgardia* (Maliński 2008) and the species *G. graminis* (Bateman et al. 2006). The applied methods do not provide complete disease control in all cases, especially that some pathogens can evolve fungicide resistance (Frac Code list (FRAC) 2009). Therefore, alternative management strategies need to be developed to protect small grain cereals against stem base diseases.

Biological control offers a viable alternative for the management of fungal diseases, including stem base diseases. Attempts have been made to use bacteria and yeasts fungi as biocontrol agents against wheat pathogens during the growing season (Kita et al. 2004; Wachowska et al. 2003; Zhang et al. 2007; Liu et al. 2011) and throughout the grain storage period (Druefors and Schnürer 2004). Commercial biocontrol agents containing yeasts fungi, such as Boni Protect™ (*Aureobasidium pullulans*, DSM 14940 and DSM 14941 strains, Bio-ferm, Austria) are widely used today (Holb and Kunz 2013). Since biocontrol agents and fungicides have a different mechanism of action, their efficacy is difficult to compare. Beyond a doubt, biocontrol agents are environmentally-friendly and have a broad spectrum of activity against pathogenic species, including those difficult to control with fungicides (Zhang et al. 2007). Efforts made to date have focused on the use of selected bacterial strains as antagonists of take-all in winter wheat caused by *G. graminis* (Liu et al. 2011). The control of stem base pathogens, in particular in early stages of seedling development and in organic farming systems where the use of chemical crop protection agents is limited, has been insufficiently investigated.

Aureobasidium pullulans (de Bary) G. Arnaud is a saprotrophic, polymorphic yeast-like fungus. The inhibitory effect of this species on pathogens and plants has been widely discussed (Ippolito et al. 2000; Schena et al. 2003; Raspor et al. 2010), yet its exact mechanism of action remains unproven. *A. pullulans* is commonly observed on the surface and in the tissues of crops. It produces lytic enzymes, such as chitinase and beta-1,3-glucanase, which suppress the growth of phytopathogens (Vero et al. 2009; Gaur et al. 2010). *A. pullulans* can also produce aureobasidins, polypeptide substances showing fungicidal action, which inhibit the growth of species of the genus *Aspergillus* (Prasongsuk et al. 2013). Aureobasidins are a family of cyclic depsipeptide antibiotics with 18 known derivatives (aureobasidin A to R) that differ in their amino acid composition (Prasongsuk et al. 2013; Ikai et al. 1991) and are known to inhibit inositol phosphoacrylamide synthase, a key enzyme for fungal sphingolipid synthesis (Zhong et al. 2000).

One of the mechanisms of biological disease control in crop plants involves competition for iron between pathogens and antagonistic microorganisms (Vero et al. 2009). Iron is required in a number of fungal growth processes such as the synthesis of deoxynucleotides, respiration, tricarboxylic acid cycle and the synthesis of amino acids, lipids and sterols (Philpott 2006). Under iron deficiency, most microorganism produce low molecular weight, iron-chelating ligands—siderophores (Wang et al. 2009).

The aim of this study was to compare the inhibitory effects of selected yeasts fungi on the causal agents of stem base diseases in winter wheat under *in vitro* and under greenhouse conditions.

Material and Methods

Isolates of yeasts fungi used under *in vitro* conditions.

The activity of selected isolates of two yeasts fungal species, *Aureobasidium pullulans* var. *pullulans* and *Sporobolomyces roseus,* was determined in the study (Table 1). *S. roseus* isolates were obtained from the leaves of winter wheat cv. Tonacja (List of Agriculture Cultivars 2008). Fifteen leaf segments, 1 cm in length, were placed in 250 ml flasks filled with 15 ml sterile water. *A. pullulans* isolates were obtained from the grain of winter wheat cv. Tonacja grown in Tomaszkowo (53°42'N, 20°26'E), protected and not protected with fungicides, and from winter wheat cv. Olivin (List of Agriculture Cultivars 2008) grown in Bałcyny (53°35'N, 19°51'), both localization in NE Poland (Table 1). Grain samples of 10 g were placed in 250 ml flasks filled with 90 ml sterile water. Flasks containing leaves or grain were shaken for 30 min (180 rpm) using a laboratory shaker, (358-S, Elpin +, Poland). The resultant suspensions, in the amount of 0.1 ml, were transferred to Petri dishes

Table 1 Origin of *A. pullulans* epiphytic isolates obtained from winter wheat kernels treated with various fungicides

Isolate code	Cultivar of winter wheat	Chemical treatments
Ap1÷11	Tonacja	Amistar 250 SC (25% azoxystrobin), Syngenta Crop Protection, Poland
Ap 12÷22	Tonacja	Mirage 450 EC (45% prochloraz), Makhteshim Agan, Poland
Ap 23÷34	Tonacja	without treatment
Ap 35÷45	Olivin	Siarkol Extra 80 WP (80% sulfur), Organika-Sarzyna Chemical Works, Poland
Ap 39÷55	Tonacja	Karben 500 SC (50% carbendazim), Bayer, Poland
Ap 56÷70	Tonacja	Bumper 250 EC (25% propiconazol), Makhteshim Chemical Works, Israel

(9 cm diameter, 0.1 ml in volume Margomed, Poland, and cooled Martin medium was poured into the dishes (Martin 1950). The colonies of yeasts fungi were transferred onto agar slants, and were identified to the species level by microscopic (Nikon E 200, Japan) methods (Barnett et al. 2000; Kurtzman et al. 2011) and to the variety level based on the ITS 1–5.8SrDNA–ITS 2 sequence regions by the method described in a previous study (Wachowska et al. 2013).

Isolates of pathogens causing stem-base diseases.

The isolates of *Helgardia herpotrichoides, Rhizoctonia cerealis, Gaeumannomyces graminis,Fusarium culmorum* and *F. oxysporum* were obtained from the stem bases of winter wheat cv. Tonacja. In order to obtain the colonies of the studied phytopathogens, infected stem base segments were cultured on PDA medium. *F. culmorum, F. oxysporum, G. graminis* and *H. herpotrichoides* were identified based on sporulation characteristics, and the number of nuclei in hyphal cells was determined for *R. cerealis*. The taxonomic affinity of the fungus *G. graminis* was confirmed by a pathogenicity test performed on wheat seedlings.

Activity of yeasts fungi under *in vitro* conditions.

At the first stage of the experiment, the inhibitory activity of two yeasts fungal species, *S. roseus* and *A. pullulans* var. *pullulans,* against the phytopathogens *F. culmorum*, *F. oxysporum, G. graminis, H. herpotrichoides* and *R. cerealis* was tested. A total of 70 *A. pullulans* isolates and one *R. cerealis* isolate were selected for further analyses. The antagonistic activity of all isolates was determined on Petri dishes. Five mm disks overgrown with seven-day-old pathogenic cultures were placed in the centre of Petri dishes. Forty-eight-h-old cultures of antagonistic yeast-like fungi were transferred to Petri dishes and were placed at a distance of 2 cm from the disks, on both sides of pathogen colonies. The microbial activity of yeast-like fungi was assessed after four days of growth. A measure of inhibitory activity was the elliptical shape factor calculated by dividing the length of the short (minor) axis by the length of the long (major) axis of the ellipse circumscribed around the colony. The fungal isolates in whose presence the colonies of *R. cerealis* assumed the elongated of an ellipse (elliptical shape factor -ESF-below 0.69) were considered to be biologically active.

Competition for iron between *A. pullulans* and pathogens.

The cultures of *A. pullulans,F. culmorum, G. graminis, H. herpotrichoides* and *R. cerealis* were placed on PDA medium containing different concentrations of iron chloride ($FeCl_3$) (0, 10, 100 and 500 µM), according to the method proposed by Vero et al. (2009). Agar disks overgrown with two-week-old pathogenic cultures were placed on Petri dishes, and *A. pullulans* cells were placed within a distance of 3 cm. After six days of incubation, the surface area of pathogenic colonies was measured using the ImageJ (v. 1.44n) application (Rasband 2011).

Survival rates of *A. pullulans* at different temperatures.

The suspensions of *A. pullulans* isolates were cultured on solid PDA (Merck). 0.1 ml samples of *A. pullulans* at a concentration of 10^4 cells were placed in Petri dishes and incubated at 4, 14, 24 and 37 °C. The colonies were counted after four days of growth.

Susceptibility of winter wheat seedlings treated with the isolates of *A. pullulans* to infections caused by *F. culmorum*.

The biological activity of the *A. pullulans* isolate (denoted by the symbol Ap 6) was determined on the seedlings of winter wheat cvs. Roma and Sakwa (List of Agriculture Cultivars 2008). An isolate of *F. culmorum* characterized by the highest pathogenicity was selected among isolates obtained from the stem bases of winter wheat. Twenty surface-disinfected kernels were sown in pots with a diameter of 12 cm, filled with garden soil mixed with sand at the 1:1 ratio. The pots were placed in a growth chamber, and seedlings were watered with Hoagland's solution (Hoagland and Arnon 1950). The plants were grown at relative air humidity of 70–80%, temperature of 23 (±1) °C during the day and 16 (±1) °C at night, with a 12 h light/12 h dark photoperiod. After two weeks, yeast fungi at a concentration of 10^6–10^7 cells per ml were applied onto the leaves. Agar disks with a diameter of 10 mm overgrown with seven-day-old mycelium of *F. culmorum* were transferred to the coleoptiles of wheat seedlings on the medium surface. Inoculation with the pathogen was carried out 24, 48 and 96 h after the application of the *A. pullulans* isolate onto the leaves. Seedlings inoculated with the pathogen and not treated with a cell suspension of yeasts fungi, and seedlings not inoculated with the pathogen and treated with a cell suspension of *A. pullulans* served as control samples. The infection rate of winter wheat seedlings was assessed two weeks after inoculation with the pathogen. The health status of seedlings was evaluated on a four-point scale. Seedlings with single, small-sized spots on the leaf sheaths were considered to be mildly infected (1°), seedlings with lesions covering up to 50% surface area were considered to be moderately infected (2°), and seedlings with lesions covering

Table 2 Biocontrol activity of yeast-like fungi (*S. roseus*—Sr and *A. pullulans*—Ap6) expressed as values of elliptical shape factor. SE value are given in brackets

Species/isolate	Pathogens					
	G. graminis	*F. oxysporum*	*F. culmorum*	*R. cerealis*	*O. herpotrichoides*	Mean
Control	$0.94^{b\text{-}e}$	0.96^{de}	0.99^{de}	1.00^{de}	$0.94^{a\text{-}d}$	0.98^{i}
	(±0.05)	(±0.01)	(±0.23)	(±0.01)	(±0.11)	(±0.05)
Sr	0.83^{abc}	$0.77^{a\text{-}e}$	$0.94^{b\text{-}e}$	$0.63^{a\text{-}d}$	$0.95^{a\text{-}d}$	0.78^{ik}
	(±0.01)	(±0.03)	(±0.03)	(±0.04	(±0.02)	(±0.06)
Ap6	0.61^{abc}	$0.82^{a\text{-}e}$	0.84^{abc}	0.59^{ab}	$0.73^{a\text{-}e}$	0.71^{k}
	(±0.01)	(±0.03)	(±0.02)	(±0.06)	(±0.06)	(±0.04)
Mean	0.82^{xy}	0.88^{y}	0.89^{y}	0.71^{x}	0.77^{xy}	
	(±0.12)	(±0.04)	(±0.09)	(±0.10)	(±0.04)	

Values followed by the same letter do not differ significantly according to SNK test at $p<0.05$. *a-e*—for interaction, *i-k*—for mean values for antagonistis, *x-y*—for mean values for pathogens

more than 50% surface area were considered to be severely infected (3°). Dying out seedlings were considered to be most severely infected (4°). The average infection index was calculated based on the severity of symptoms. The experiment was conducted twice, in three replications.

Statistical analysis.

The data were verified statistically by analysis of variance (ANOVA), using Statistica 9.0 software (StatSoft, Inc. 2009). The significance of differences between means in laboratory analyses was estimated by the Student–Newman–Keuls test.

Results

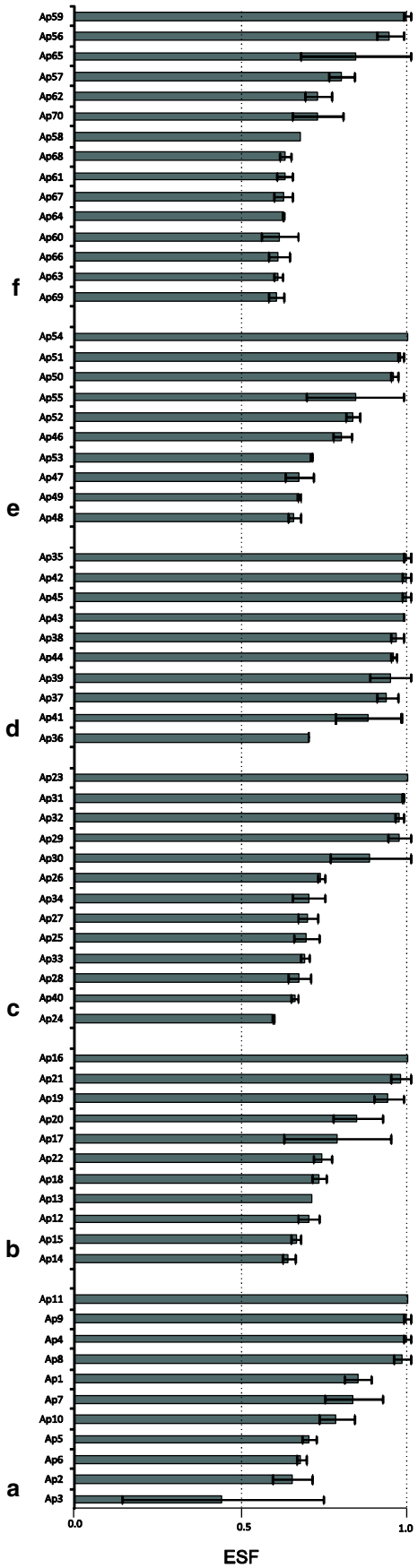
Numerous populations of *S. roseus* and *A. pullulans* were isolated from the leaves of winter wheat cv. Tonacja. The inhibitory activity of both fungal species was compared at the first stage of the study. *S. roseus* was isolated from leaves, and its inhibitory effect on the causal agents of stem base diseases was unsatisfactory, particularly with respect to *F. culmorum* (Table 2). The elliptical shape factor calculated for this phytopathogen in the presence of *S. roseus* reached 0.94, and it did not differ significantly from the values reported for the control treatment.

A. pullulans var. *pullulans* formed characteristic, fast growing cream-colored colonies. Under *in vitro* conditions, the selected isolate of *A. pullulans* (Ap 6) showed a strong suppressing effect on the tested phytopathogens, in particular *R. cerealis* and *G. graminis* (Table 2). The elliptical shape factor of *R. cerealis* colonies in the presence of the antagonistic species reached 0.59, and it was significantly lower than in the control treatment. At such a value of the shape factor, at least 8 mm inhibition zones were formed between the pathogen and the antagonist colonies. The pathogen colonies became more elliptical in shape and the values of their shape factors decreased along with an increase in the number and activity levels of inhibitors produced by the antagonistic species. *R. cerealis* was most sensitive to the presence of the yeast fungus. The antagonistic activity of *A. pullulans* against *G. graminis* was slightly weaker. Since the elliptical shape factor of this pathogen was 0.61, the inhibitory effect exerted by *A. pullulans* was considered satisfactory. The colony development of the other tested pathogenic species was suppressed to a low degree. In the presence of *A. pullulans*, the elliptical shape factors of *F. culmorum* and *H. herpotrichoides* reached 0.84 and 0.73, respectively. Therefore, the activity of the antagonistic species was found to be relatively weak (Table 2).

The inhibitory activity of 70 *A. pullulans* isolates obtained from wheat kernels against *R. cerealis* was determined at the second stage of the experiment. Only 25 *A. pullulans* isolates (35.71%) inhibited the growth of *R. cerealis* (Fig. 1). Most of those isolates were obtained from the grain of winter wheat cv. Tonacja, seven isolates came from unprotected plants, and nine isolates were obtained from wheat plants treated with propiconazole (Table 1). Other active strains were isolated from the kernels of wheat plants treated with products containing carbendazim, prochloraz and azoxystrobin.

One *A. pullulnas* isolate (Ap 6), was selected for further analyses. An attempt was made to investigate its mechanism of action in plant pathogens and crops. This isolate is safe to use as it is able to grow only in a narrow temperature range. The optimum growth temperature of the above isolate on the PDA medium (Merck) is 24°C, and it is unable to grow at 4 and 37°C.

The analyzed pathogen colonies developed in Petri dishes in the presence of *A. pullulans*. The larger surface areas of the tested pathogen colonies in Petri dishes containing 500 µM ferrous chloride were an indirect indicator of the degree of competition for iron between the pathogens and *A. pullulans* in the medium with lower levels of Fe^{3+} ions. A faster growth rate of *F. culmorum, R. cerealis* and *H. herpotrichoides* colonies was observed in agar media with ferrous chloride than in media containing no this compound (Table 3). The fastest growth rate was reported for *F. culmo-*



◀ **Fig. 1** Biocontrol activity of *A. pullulans* epiphytic isolates (Ap1–Ap70) against *R. cerealis* colony growth expressed in values of elliptical shape factor (ESF). Error bars represent SE. Origin of isolates: A—Amistar 250SC, B—Mirage 450 EC, C—Siarkol Extra 80 WP, D—Karben 500 SC, E—Bumper 250 EC

rum colonies cultured in media containing 500 µM ferrous chloride. After six days of incubation, the surface area of *F. culmorum* colonies was 45 % larger than the surface area of respective control colonies. The fast growing species *R. cerealis* developed most rapidly in PDA containing 100 µM ferrous chloride, and the surface area of its colonies was significantly larger than in PDA without ferrous chloride.

The protective effect of the studied *A. pullulans* isolate against *F. culmorum* infections was analyzed using seedlings of winter wheat cv. Roma and Sakwa. A series of experiments performed in a growth chamber revealed no significant differences in infection rates of seedlings of booth cultivars (Fig. 2). Seedlings of cv. Roma treated with *A. pullulans* cell suspensions and inoculated with *F. culmorum* after 96 hours were significantly less severely infected than control, unprotected seedlings inoculated with the pathogen. The protective effect of *A. pullulans* on wheat seedlings of cv. Roma was significant only at the longest time interval. Wheat seedlings of cv. Sakwa were significantly less severely infected than unprotected seedlings inoculated with *F. culmorum* at 24-h and 96-h time intervals between the application of *A. pullulans* cell suspensions and inoculation with *F. culmorum*. Seedlings of cv. Sakwa, compared with cv. Roma, responded faster to inoculation with *F. culmorum* following the application of *A. pullulans* cells onto the leaves.

Discussion

Biological control strategies for wheat diseases have been extensively studied. The most commonly tested biologically active microorganisms are bacteria (Jochum et al. 2006; Kita et al. 2004; Nourozian et al. 2006; Liu et al. 2011; Wachowska et al. 2013) and filamentous fungi of the genera *Trichoderma* (Brožová 2004; Brunner et al. 2005) and *Gliocladium* (Teperi et al. 1998; Roberti et al. 2008). The activity of epiphytic yeast and yeast-like fungi against cereal phytopathogens remains poorly investigated (Raacke et al. 2006; Wachowska et al. 2003; Zhang et al. 2007), although they abundantly colonize grain crops (Bashi and Fokkema 1977; Dik et al. 1992).

In our study *in vitro* tests indicate that yeasts fungi can considerably inhibit the growth of the causal agents of stem base diseases. Antibiotic and/or lytic substances produced by the tested isolates of yeasts fungi diffused into the medium and affected the shape of pathogen colonies. In the present experiment, *A. pullulans* exhibited higher activity than *S. roseus* under *in vitro* conditions. The inhibitory

Table 3 Inhibition of pathogen radial growth in dual cultures with *A. pullulans* at different concentrations of iron expressed as area of colonies (in cm^2). SE is given in brackets

Concentrations of iron (ferric chloride in M)	*G. graminis*	*F. culmorum*	*R. cerealis*	*H. herpotrichoides*	Mean
0	6.46 ^{bc}	9.13 $^{c-g}$	7.89 ^{cde}	7.35 ^{cde}	7.71^{A}
	(±0.13)	(±0.26)	(±0.51)	(±0.13)	(±1.34)
10	5.84 ^{b}	9.65 $^{d-g}$	9.46 $^{d-g}$	7.58 ^{cde}	8.13^{B}
	(±0.02)	(±0.11)	(±0.77)	(±0.22)	(±0.26)
100	6.91 ^{cde}	11.53 ^{gh}	11.32 ^{fgh}	10.09 ^{efg}	9.96^{C}
	(±0.12)	(±0.17)	(±0.82)	(±1.26)	(±0.55)
500	6.88 ^{cde}	13.24 ^{h}	10.06 ^{efg}	8.11 $^{c-f}$	9.57^{C}
	(±0.31)	(±0.99)	(±0.96)	(±0,31)	(±0.22)
Mean	5.32 ^{x}	10.88 ^{w}	9.68 ^{z}	8.28 ^{y}	
	(±0.33)	(±0.14)	(±0.35)	(±0.01)	

Values followed by the same letter do not differ significantly according to SNK test at $p<0.05$: *a-g*—for interaction, *w- z*—for mean values for antagonistic species, *A—C*—for mean values for $FeCl_3$ concentrations

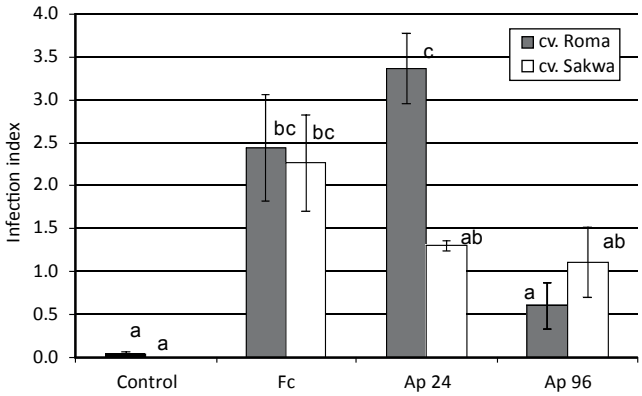


Fig. 2 Susceptibility of winter wheat seedlings of cvs. Sakwa and Roma treated with A. pullulans to infection caused by F. culmorum. Values followed by the same letter do not differ significantly according to SNK test at $p<0.05$. C—untreated control, Fc—control with F. culmorum, Ap 24, Ap 96–24, 96– h time intervals between the application of A. pullulans cell suspensions and inoculation with F. culmorum. Error bars represent SE

effects of yeasts fungi have been also observed in previous research (Marquina et al. 2002; Walker et al. 1995; Vero et al. 2009). The studies of other authors have shown that *A. pullulans* isolates may produce the enzymes glucanase and chitinase in the presence of *Penicillium expansum* cell walls (Vero et al. 2009).

In our experiment, the *A. pullulans* isolate obtained from winter wheat grain inhibited the growth of all analyzed pathogens under low iron availability. In a study by Chi et al. (2013), the marine-derived *A. pullulans* HN 6.2 isolate grown in a medium without added Fe^{3+} produced hydroxamate-type siderophores at 92.1 mg per g of cell dry weight, and when iron was added to the medium, the production of siderophores dropped to 28 mg. The available literature provides no information on the production of siderophores by *A. pullulans* isolates obtained from the growing environment of crop plants. Apart from antibiosis and competition, empirical evidence suggests that *A. pullulans* and other saprotrophs can induce defense mechanisms in plants (Ippolito et al. 2000; Droby et al. 2002; Gary and Goldmann 2004; Raacke et al. 2006; Wees et al. 2008). A few studies have demonstrated that iron levels in the environment can modify fungal pathogenicity, and that reactive ferric iron mediates defensive H_2O_2 production in plants (Greenshields et al. 2007).

In our study, *A. pullulans* cells were applied onto the leaves of wheat plants, and the coleoptyles were inoculated with *F. culmorum*. It was found that the tested *A. pullulans* isolate protected wheat seedlings against infection. The antagonist and the pathogen were spatially separated during the course of the experiment. Seedlings inoculated with the pathogen 24 h after treatment with the antagonist yeast-like fungus were severely infected, and the noted infection rates were comparable with those reported for the control treatment. The lowest infection rates were observed in seedlings treated with the antagonist cell suspension 96 hours before the inoculation with *F. culmorum.* Our results corroborate the finding of authors who found that biological methods require longer time intervals between the application of the antagonist and the pathogen (Ippolito et al. 2000; Ghaouth et al. 2002; Zhang et al. 2007). The above phenomenon has been well documented. In studies investigating the induction of defense responses in apple fruit (Ippolito et al. 2000; Ghaouth et al. 2002), the highest activity levels of chitinase and glucanase were noted after 96 hours. The yeast-like fungus *A. pullulans* also provided more effective control of *Botrytis cinerea* storage rots on fruits after longer time intervals (Ippolito et al. 2000). Yao and Tian (2005) applied the antagonist *Cryptococcus laurentii* to control postharvest diseases (*Monilinia fructicola* and *Penillium expansum*) in peach fruit. The cited authors noted the maximum activity levels of 1,3-glucanase and peroxidase (POD) and enhanced

activity of phenylalanine ammonia-lyase (PAL) after 24 hours at 25 °C. Resistance induction in plants treated with non-pathogenic microorganisms has been observed in cereals (Roberti et al. 2008; Jochum et al. 2006; Zhang et al. 2007) and stored fruits (Ippolito et al. 2000; Ghaouth et al. 2002; Droby et al. 2002; Yao and Tian 2005).

In our study, seedlings of two wheat cultivars showed different susceptibility to *F. culmorum* infection, which confirms that proper cultivar selection is an important consideration in organic and integrated farming systems where the use of chemical crop protection agents is limited. The defense responses of different plant varieties have been widely discussed in literature. Westhuizen et al. (1998), who studied the defense responses of several wheat cultivars to aphid infestation, found that peroxidase activity increased in resistant varieties after 2 days. A rapid increase in chitinase activity levels was also observed in some resistant wheat varieties as soon as after 1 day. In susceptible cultivars, peroxidase activity did not increase or increased slightly within a few days. An increase in chitinase activity in the tissues of susceptible wheat cultivars was observed after 6–12 days, depending on cultivars, and it was substantially lower than in the resistant cultivars. In a study by Mohammadi and Kazemi (2002), peroxidase and polyphenol oxidase activities were higher in resistant non-inoculated winter wheat cultivars, compared with susceptible cultivars. The activity levels of the above compounds increased also after wheat head inoculation with the pathogen *Fusarium graminearum,* particularly in resistant cultivars. Wiwart et al. (2013) investigated the response of three wheat species to head inoculation with *F. culmorum* and reported that the protein content of grain derived from inoculated heads was on average 7.64 % higher, compared with control grain. Raacke et al. (2006) studied the expression of systemic acquired resistance-related genes in *Arabidopsis* and reported that plant treatment with an autoclaved yeast suspension activated the salicylate pathway and induced the accumulation of camalexin. Salicylic acid and jasmonic acid pathways were differently activated by bacterial peptide flagellins. The activation of defense mechanisms involving no camalexin accumulation was observed in *Arabidopsis* by Gomez-Gomez et al. (1999).

The results of our study indicate that the *A. pullulans* var. *pullulans* Ap 6 isolate obtained from winter wheat grain inhibits the growth of stem base pathogens and protects winter wheat seedlings against *F. culmorum*. *A. pullulans* exerts varied and long-term effects on pathogens and crop plants, including the production of compounds that suppress pathogen growth, compete for iron with pathogens and modify plant susceptibility to infections. However, the numerous disadvantages of the presented method include different responses of cultivars to the applied biocontrol agent, and a strong correlation between biocontrol efficacy and the time of application of the fungal antagonist.

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