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Original Research

Microorganisms as Biological Control Agents against *Fusarium* Pathogens in Winter Wheat

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Abstract

The effectiveness of bacteria of the genera *Sphingomonas* and *Bacillus*, and yeasts of the genera *Cryptococcus*, *Rhodotorula*, and *Saccharomyces* as biological control agents against pathogens colonizing winter wheat, was tested in laboratory conditions. All of the tested yeast isolates had an inhibitory effect on the development of *F. sporotrichioides* colonies. Under the same conditions, the *Sphingomonas* S 11 isolate was antagonistic against *F. avenaceum*, *F. culmorum*, *F. tricinctum*, and *F. graminearum*. The seedlings of winter wheat cv. Sumai treated with a suspension of *Sphingomonas* S 11 bacteria and inoculated with *F. culmorum* demonstrated significantly fewer infection symptoms than unprotected seedlings that were inoculated with the above-mentioned pathogen.

Keywords: bacteria, yeasts, winter wheat, *Fusarium*, biological control

Introduction

Fungi of the genus *Fusarium* are necrotrophic pathogens of winter wheat that infect crops in all regions of cultivation to cause root rot, foot rot, Fusarium seedling blight, and *Fusarium* head blight (FHB). The following *Fusarium* species are most frequently isolated from winter wheat grain: *F. culmorum*, *F. avenaceum*, *F. poae*, *F. graminearum*, *F. sporotrichioides*, *F. tricinctum*, *F. proliferatum,* and *F. langsethiae* are sporadically identified [1-4]. Those pathogens reduce wheat grain yield, and they may also colonize stored grain. Mycotoxins that pose a risk to human and animal health may accumulate in wheat grain contaminated by the above-mentioned fungi [5, 6]. Every *Fusarium* species produces one or more mycotoxins. The following mycotoxins are most often identified in winter wheat grain: deoxynivalenol (DON) [5, 6] and other trichothecenes, zearalenone (ZEA) [6], fumonisins, moniliformin and enniatins [1, 4, 7, 8]. The species *F. culmorum* causes systemic infections of wheat plants by attacking their roots, stems, and spikes [9]. The predominant role of *F. culmorum* in the pathogenesis of *Fusarium* root rot and FHB has been discussed extensively by various authors [9-11].

The main strategies that can be used to control *Fusarium* diseases include crop rotation schemes, the introduction of less sensitive cultivars, adequate soil tillage techniques, fertilization, and the use of benzimidazole, azole, and strobilurin fungicides [10, 12]. According to some reports, fungicides containing tebuconazole [12] and azoxystrobin [13] do not always effectively control FHB or reduce DON levels in wheat grain [12, 13]. Those shortcomings necessitate the search for alternative strategies, including biological methods, to control the spread of *Fusarium* pathogens. Biological products may be highly useful in organic farms where chemical substances cannot be applied due to certification requirements and consumer expectations. They also may be used to protect stored grain [14].

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Wiśniewska et al. [15] demonstrated that the antagonistic species of *Trichoderma harzianum* suppressed the growth of *Fusarium sporotrichioides* colonies and decreased the concentrations of type A trichothecenes in oat and wheat grain. Khan and Doohan [16] isolated *Pseudomonas fluorescens* strains MKB 158 and MKB 249 and *P. frederiksbergensis* strain 202 which reduced the intensity of *Fusarium* infections in spikes inoculated with *F. culmorum*, minimized a decrease in the thousand kernel weight, and lowered DON concentrations in winter wheat kernels. In the successive study, the above-mentioned authors demonstrated that commercial chitosan was more effective in reducing the symptoms of FHB and decreasing DON levels in winter wheat kernels than *Pseudomonas fluorescens* strain MKB 158 [17]. Zhang et al. [18] found that under greenhouse conditions, *Cryptococcus flavescens* had an antagonistic effect on wheat spike pathogens.

Despite numerous efforts, only a few commercial products containing microorganisms are available on the market, and they are used mainly for fruit protection [19, 20]. The aim of this study was to find isolates of bacteria and yeasts coming from various environments that inhibit the development of *Fusarium* pathogens.

Material and Methods

Origin of Antagonistic Microbial Isolates

Isolates of bacteria and yeasts obtained from the leaves and the rhizosphere of winter wheat cv. Tonacja were used in the experiment (Table 1). Isolates of *Sphingomonas* S 11 were obtained from the rhizosphere, and *Bacillus* B 1 bacteria were isolated from the phyllosphere. Their taxonomic status was validated by a molecular analysis [21]. Leaf fragments of 1 cm were placed in 250 ml flasks (15 per flask) filled with 15 ml of sterile water [22]. Fragments of winter wheat roots were placed together with 10 g of rhizosphere soil in 250 ml flasks filled with 90 ml of sterile water. Flasks containing leaves or roots were shaken for 30 minutes on a shaker table, 0.1 ml of the resulting suspension was transferred to Petri dishes and immersed in cooled Martin's medium [23] to obtain yeasts, King's B medium [24] to isolate *Sphingomanas* bacteria, and nutrient agar [20] to isolate *Bacillus* bacteria. Fungal colonies were stored in Eppendorf tubes at 4ºC. Isolates of yeast species *Cryptococcus albidus* var. *albidus* (CBS 2991), *Rhodotorula glutinis* var. *glutinis* (CBS 2367), and *Saccharomyces cerevisiae* var. *cerevisiae* (CBS 2451), obtained from the Utrecht GeneBank, were used for further *in vitro* tests.

Origin of *Fusarium* Isolates

Isolates of the genus *Fusarium* were obtained from the grain of winter wheat cv. Bogatka. Kernels were placed on PDA medium, and growing colonies were sub-cultured onto PDA. The isolates were identified to the species level based on their sporulation characteristics [24]. Their taxonomic status was validated by molecular analysis (Jędryczka – personal communications).

Table 1. The origin of isolates used in this study.

Isolate	Origin	Location in Poland	Year of isolation
Sphingomonas sp. (S 11)	Rhizosphere of wheat (cv. Tonacja)	Tomaszkowo 20º41'E, 53º72'N	2007
Bacillus sp. (B 1)	Phyllosphere of wheat (cv. Tonacja)		2005

Antagonistic and Competitive Effects of Microorganisms on *Fusarium* Pathogens

The inhibitory effects of *Bacillus* B 1 and *Sphingomonas* S 11 isolates and the isolates of three yeast species (*Cryptococcus albidus* var. *albidus* CBS 2991, *Rhodotorula glutinis* var. *glutinis* CBS 2367, and *Saccharomyces cerevisiae* var. *cerevisiae* CBS 2451) on pathogens of the genus *Fusarium* (*F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. sporotrichiodes*, *F. tricinctum*) were evaluated. Agar discs with a diameter of 5 mm overgrown with mycelium of 2-week-old *Fusarium* cultures were placed on PDA medium in the center of a Petri dish. Isolates of antagonistic yeasts and bacteria were plated at a distance of 2 cm from the pathogen colony. The dishes were kept in darkness at 24ºC. The competitive and inhibitory effects of selected isolates were assessed after four days. The inhibitory effects of bacteria and yeasts were evaluated in terms of the ellipticity coefficients of the tested phytopathogens, 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. Antagonist isolates, which changed the shape of phytopathogen colonies from a circle to a strongly elongated ellipse, were classified as active. In such cases, the ellipticity coefficient was below 0.69. The surface of colonies formed by *Fusarium* fungi was a measure of the competitive activity of the analyzed antagonists, determined with the use of ImageJ v. 3.0 software.

Survival Rates of Bacterial and Fungal Isolates at 35ºC

Isolate suspensions were cultured on agar-solidified PDA media. 0.1 ml of fungal or bacterial suspension (10^6) was plated on PDA at 35ºC. Colonies emerged after four days.

Use of *Sphingomonas* Bacteria to Protect Winter Wheat Seedlings Against Infections Caused by *F. culmorum*

Kernels of spring wheat cv. Sumai were sown in pots with a diameter of 12 cm filled with 2:1 humus soil and sand. In every pot, 20 wheat kernels surface-disinfected

Table 2. Inhibitory effect of select yeasts and bacteria on *Fusarium* species.

Fusarium species	*F. avenaceum*			*Fusarium culmorum*	*Fusarium graminaeum*	*Fusarium poae*	*Fusarium sporotrichioides*	*Fusarium tricinctum*	Means of antagonist
Pathogens	Fa 69	Fa 70	Fa 9	Fc 36	Fg 88	Fp 24	Fs 74	Ft 65	
Antagonists	Ellipticity coefficient								
Control	nt	0.92jkl	0.97kl	0.94jkl	0.96kl	nt	1.00l	1.04l	0.97D
Bacillus sp. (B 1)	0.9f-j	0.74b-j	0.67a-i	0.67a-h	0.88f-j	0.77c-j	0.72b-j	0.48a-e	0.73A
Sphingomonas sp. (S 11)	nt	0.83f-j	0.63a-g	0.68a-k	0.66a-h	nt	0.81f-j	0.59a-d	0.69A
Cryptococcus albidus var. *albidus* (CBS 2991)	0.79d-j	0.86f-j	0.79d-j	0.87f-j	0.89f-j	0.9f-j	0.58a-d	0.58a-d	0.80B
Rhodotorula glutinis var. *glutinis* (CBS 2367)	0.77c-j	0.88f-j	0.79d-j	0.91g-j	nt	0.84e-j	0.55a-d	0.95j	0.78AB
Saccharomyces cerevisiae var. *cerevisiae* (CBS 2451)	0.8d-j	0.79c-j	0.93jkl	0.88f-j	nt	0.74b-j	0.52ab	0.94ij	0.76AB

Values followed by the same letters do not differ significantly according to the Newman-Keuls test (p=0.01).
nt – not tested

with 1% sodium hypochlorite solution were placed at a depth of 2 cm. After two weeks, the suspension of the *Sphingomonas* S 11 isolate (2×10^5 cells per cm^3 of water) was sprayed on leaves. Wheat seedlings were inoculated with 10 mm agar discs overgrown with 2-week-old cultures of *F. culmorum* Fc 38. The seedlings were infected with the pathogen 24, 48, 72, and 96 hours after the application of antagonistic bacteria. The pots were placed in a controlled environment chamber, and they were exposed to a 12-hour photoperiod at constant humidity of 98% and temperature of 22-23ºC. The experiment was performed in four replicates. Throughout the entire experiment, seedlings were watered with Hoagland's solution containing macronutrients and micronutrients. The experimental factors were time intervals between the application of bacteria and the pathogen. After two weeks, seedling health was evaluated on a 5-point scale:

0 – healthy plants
1 – pathological changes up to 3 cm long on coleoptile surface
2 – pathological changes 3-5 cm long on coleoptile surface
3 – pathological changes affecting coleoptile tissue
4 – dying seedlings

Statistical Analysis

The results were processed statistically using Statistica 8.0 software. The significance of differences between treatments was determined by the Newman-Keuls test (p=0.01, p=0.05).

Results

Microbial Antagonists and Competitors against Fungi of the Genus *Fusarium*

The studied isolates of antagonistic microorganisms inhibited the development of *Fusarium* colonies *in vitro* (Table 2). Isolates which gave rise to elliptically shaped pathogen colonies with ellipticity coefficient values below 0.69 were classified as biologically active. In such cases, the inhibitory effect of the antagonists was estimated at 31%, and the zones of inhibition between the antagonist and the pathogen were at least 10 mm in diameter. All of the applied yeast isolates had a satisfactory inhibitory effect only on the *F. sporotrichioides* Fs 74 isolate (Table 2). In most cases, yeasts significantly limited the growth of the studied phytopathogens (Table 3). The isolate of *F. avenaceum* Fa 69 proved to be least sensitive to the competition of the studied microorganisms, and none of the analyzed antagonists inhibited its growth to a satisfactory degree. The *Bacillus* B 1 isolate effectively reduced the development of *F. avenaceum* Fa 9, *F. culmorum* Fc 36, and *F. tricinctum* Ft 65 isolates (Tables 2 and 3).

The *Sphingomonas* S 11 isolate demonstrated the strongest antagonistic effect against *F. avenaceum* Fa 9, *F. culmorum* Fc 36, *F. tricinctum* Ft 65, and *Fusarium graminearum* Fg 88 (Table 2). In comparison with control, the above-mentioned isolate had a significant inhibitory effect on the growth of pathogen colonies, but in comparison with the remaining microorganisms, *Sphingomonas* S 11 was the weakest biological control agent (Table 3). The S 11 isolate of the genus *Sphingomonas* sp. was used in further analyses to verify its potential protective activity against *F. culmorum*.

Survival Rates of Bacterial and Fungal Isolates at 35ºC

After four days of incubation at 35ºC, the isolates of *Rhodotorula glutinis* var. *glutinis* (CBS 2367) and *Saccharomyces cerevisiae* var. *cerevisiae* (CBS 2451) formed numerous colonies (Fig. 1), whereas no colonies of bacterial isolates *Bacillus* B 1 and *Sphingomonas* S 11 and the fungal isolate *Cryptococcus albidus* var. *albidus* (CBS 2991) were observed.

Table 3. Competitive effect of select isolates of yeasts and bacteria on *Fusarium* species (surface of the colony).

Fusarium species	*F. avenaceum*			*Fusarium culmorum*	*Fusarium graminaeum*	*Fusarium poae*	*Fusarium sporotrichioides*	*Fusarium tricinctum*	Mean for antagonist
Pathogens	Fa 69	Fa 70	Fa 9	Fc 36	Fg 88	Fp 24	Fs 74	Ft 65	
Antagonists	Area of pathogen colonies in mm2								
Control	nt	83.25cde	153.8f	108.7e	149f	nt	99.67de	183.4f	123.8C
Sphingomonas (S 11)	7.01a	73.32c-d	64.17c-d	44.9abc	54.26c-d	16.3ab	77.6b-d	65.99c-d	60.73B
Bacillus sp. (B 1)	7.01a	10.57ab	18.84ab	20.62ab	12.04ab	nt	14.97ab	26.17abc	15.82A
Cryptococcus albidus var. *albidus* (CBS 2991)	7.12a	13.92ab	13.82ab	19.89f-j	15.28ab	nt	13.35ab	26.69abc	15.44A
Rhodotorula glutinis var. *glutinis* (CBS 2367)	7.54a	14.03ab	17.69ab	22.29ab	nt	17.8e-j	26.43abc	16.85ab	18.63A
Saccharomyces cerevisiae var. *cerevisiae* (CBS 2451)	7.54a	14.13ab	16.54ab	21.14ab	nt	17.9b-j	26.69abc	19.05ab	18.71A

Values followed by the same letters do not differ significantly according to the Newman-Keuls test (p=0.01)
nt – not tested

Infection of Winter Wheat Seedlings Protected with the *Sphingomonas* S 11 Isolate

After 96 hours, the seedlings of winter wheat cv. Sumai treated with a cell suspension of *Sphingomonas* S 11 and inoculated with *F. culmorum* were significantly less infected than untreated seedlings inoculated with the analyzed pathogen (Fig. 2). In this treatment, the effectiveness of biological control with the *Sphingomonas* S 11 isolate reached 67%, compared with the control treatment. Bacteria of the genus *Sphingomonas* failed to protect the seedlings, which were infected with *F. culmorum* 24, 48, and 72 hours after their leaves had been inoculated with the antagonistic isolate.

Discussion

The microorganisms used in the present study considerably inhibited the growth of *Fusarium* pathogens *in vitro*. In a greenhouse experiment by Zhang et al. [18], the use of an isolate of yeast *Cryptococcus flavescens*, OH 182.9, effectively reduced the severity of FHB symptoms in wheat. The above-mentioned authors observed that the defense response induced in plants by the analyzed isolate was not the key mechanism responsible for spike protection against *F. graminearum* infections [18]. Their results were validated by our study where isolates of *Cryptococcus albidus* var. *albidus* (CBS 2991), *Rhodotorula glutinis* var. *glutinis* (CBS 2367), and *Saccharomyces cerevisiae* var. *cerevisiae* (CBS 2451) had a clearly inhibiting effect on the development of *F. sporotrichioides*. The above-mentioned isolates were not effective biological control agents against the remaining *Fusarium* species. *Rhodotorula glutinis* var. *glutinis* (CBS 2367) and *Saccharomyces cerevisiae* var. *cerevisiae* (CBS 2451) isolates proliferated at 35ºC, therefore they were not recommended for testing in a field environment due to potential risks [26].

The use of *Bacillus* bacteria as control agents against pathogenic infections in winter wheat has been widely doc-

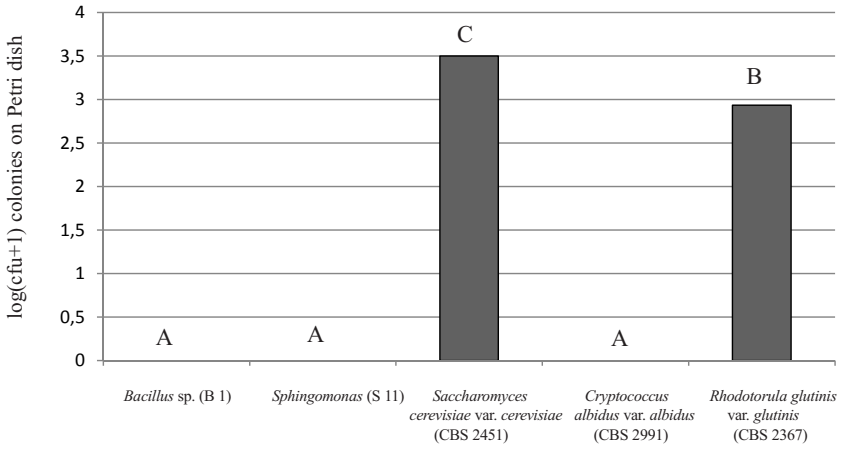

Fig. 1. Survival of microorganisms at 35ºC.
Values followed by the same letters do not differ significantly according to the Newman Keuls test (p<0.01).

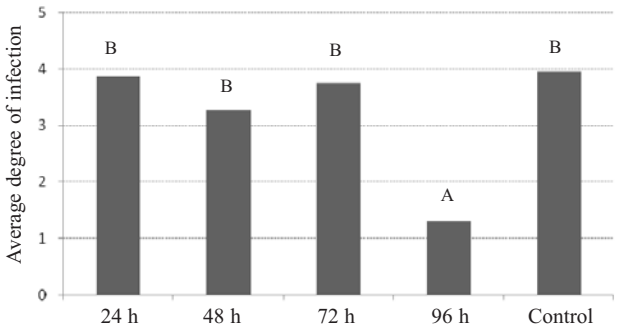

Fig. 2. The infection of winter wheat seedlings of cv. Sumai inoculated with the spore suspension of *Sphigomonas* S 11 and infected with *F. culmorum*.
Control – seedlings inoculated with *F. culmorum* but not inoculated with *Spingomonas* S 11.
24 h, 48 h, 72 h, 96 h – seedlings inoculated with the spore suspension of *Sphingomonas* S 11 and with *F. culmorum* at 24 h, 48 h, 72 h, 96 h time intervals.
Values followed by the same letters do not differ significantly according to the Newman-Keuls test ($p<0.01$)

umented [22, 27, 28]. Some authors have demonstrated that select *Bacillus* isolates may inhibit the development of *F. graminearum* on wheat spikes and limit the production of DON mycotoxin [28]. In this study, the B 1 isolate of *Bacillus* sp. had an antagonistic effect on three *Fusarium* species. In the work of Nourozian et al. [20], *Bacillus subtilis* isolates were potent antagonists of *F. graminearum*. Their metabolites had a much stronger fungicidal effect, and the metabolites of the *B. subtilis* 71 isolate inhibited the growth of *F. graminearum* colonies by 97% in comparison with control [20]. In our study, the B 1 isolate of *Bacillus* sp. mostly had an antagonistic effect on pathogens. *Fusarium* colonies growing in the presence of this isolate had the shape of a strongly elongated ellipse. The results of other studies indicate that bacteria of the genus *Bacillus* may produce lipopeptides with potential fungistatic activity [27].

In this experiment, the *Sphingomonas* sp. S 11 isolate demonstrated a strongly antagonistic effect against four pathogenic *Fusarium* species. Innerebner et al. [29] reported that isolates of the genus *Sphingomonas* could protect *Arabidopsis thaliana* plants against *Pseudomonas syringae* pathogens. The above-mentioned authors demonstrated the competitive and antagonistic effects of the analyzed isolates on pathogens colonizing leaf surfaces [29]. In their study, the predominant role was played by substances with antibacterial properties, which suppressed the growth of pathogens. Bacteria of the genus *Pseudomonas* ssp. produce several such compounds, including phenasin and 2,4-diacetylphloroglucinol [20]. The antagonistic and competitive effects of the *Sphingomonas* S 11 isolate used in our study against *Fusarium* pathogens were demonstrated *in vitro*. The results of experiments performed in a controlled environment chamber suggest that the *Sphingomonas* S 11 isolate could elicit defensive responses in plants. In these studies, seedlings of winter wheat cv. Sumai, which is characterized by high resistance to pathogenic infections, were inoculated with *Sphingomonas* S 11 and infected with *F. culmorum* in temporal and spatial isolation. The antagonist was applied to the leaves, and the pathogen to the coleoptile. It can be deduced that the antagonist elicited induced systemic resistance (ISR) in plants. As shown by earlier studies, plant-growth promoting rhizobacteria (PGPR) of the genus *Pseudomonas* spp. elicit ISR in the host [30-35] with the involvement of cell membrane components, lipopolysaccharides (LPS), flagellin, or secreted compounds such as Fe^{3+}-chelating siderophores [23-37]. ISR relies mainly on the priming mechanism [30-36]. Plant cells produce an intensified defensive response due to the accumulation or the posttranslational modification of one and/or more signaling proteins that undergo expression and/or modification and become deactivated. The plant is able to activate a faster and stronger defense response only when a signal is received from the infecting pathogen [38].

Petti et al. [39] observed that bacteria *Pseudomonas fluorescens* significantly affected the accumulation of 1,203 transcripts and primed 74 to positively and 14 to negatively respond to the pathogen ($P = 0.05$). In the present study, plants inoculated with *F. culmorum* exhibited the lowest sensitivity to pathogen infection 96 hours after the application of the *Sphingomonas* S 11 isolate. In plants infected with the pathogen, the symptoms of *F. culmorum* infection were severe 24, 48, and 72 hours after the application of the antagonist to the leaves, rendering the analyzed biological control agent ineffective. The symptoms of *F. culmorum* infection were effectively reduced only after 96 hours. The *Sphingomonas* isolate probably induced changes at the level of gene expression in jasmonic acid/ethylene-dependent defense signaling pathways. The above-mentioned change was confirmed in *Pseumodomonas* bacteria, and it enabled the plant to generate a more effective defense response against pathogenic infections without inducing significant metabolic changes [30, 31, 36-38]. The response could be used to improve the growth and yield of winter wheat crops under field conditions.

Conclusions

All of the tested yeast isolates inhibited the growth of *F. sporotrichioides* colonies to a satisfactory degree. In the greenhouse environment, the *Sphingomonas* S 11 isolate had an antagonistic effect on *F. avenaceum*, *F. culmorum*, *F. tricinctum*, and *F. graminearum*. It reduced the symptoms of an infection caused by *F. culmorum* in the seedlings of spring wheat cv. Sumai.

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