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An Attempt to Protect Winter Wheat Against *Gaeumannomyces graminis* var. *tritici* by the use of Rhizobacteria *Pseudomonas fluorescens* and *Bacillus mycoides*

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Abstract

Pseudomonas fluorescens strains III107 and II21 and *Bacillus mycoides* strains JC192 and K184, stimulating growth of winter wheat, were chosen for the studies. The bacterial strains inhibited on agar nutrient medium the growth of *Gaeumannomyces graminis* var. *tritici* (*Ggt*) – the pathogenic fungus causing take-all on wheat. Both strains of pseudomonads synthesized relatively high amounts of Fe^{3+} chelators. The strains of bacilli were characterized by the very fast spreading on agar media. Furthermore, strain II21 was highly cyanogenic, and strain JC192 highly chitinolytic. Bacterization of winter wheat seeds (especially with strains III107 and JC192) significantly reduced the percentage of the plants infested with the pathogen in the 28 day glasshouse pot experiment. In the plot experiment, the winter wheat seeds were inoculated with a mixture of strains III107, II21 and JC192. Due to the bacterization the yield of wheat grain and straw was higher in comparison to the series with *Ggt* alone by 122% and 75%, respectively, but it amounted only to 45% and 43% of the control series not contaminated with *Ggt*. The decrease of percentage of wheat ears with weight less than 500 mg from 61% in *Ggt*-series to 25% in *Ggt*-bacterized-series, and especially the decrease of percentage of wheat ears with weight less than 200 mg from 43% to 14% additionally indicate the partial protection of the winter wheat against *Ggt* by the rhizobacteria. In the experimental series not contaminated with *Ggt* the percentage of these wheat ears fractions did not exceed 3% and 0.5%, respectively.

Key words: take-all biocontrol, winter wheat, *Pseudomonas fluorescens*, *Bacillus mycoides*.

Introduction

Take-all, caused by *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *tritici* J. Walker (*Ggt*) is the most significant root disease of wheat (*Triticum aestivum* L.) worldwide (Weller *et al.*, 1997). The increasing occurrence of this disease is a consequence of increasing area of winter wheat cultivation (Kuś and Mróz, 1996). There are no effective sources of cultivar resistance or chemical control (Sarniguet *et al.*, 1992). Biological control by naturally existing antagonistic microorganisms is an alternative strategy, which in certain circumstances might be integrated with other strategies (Hornby, 1998). There have been many reports that bacterial isolates from rhizosphere soil or plant roots (especially fluorescent pseudomonads, because of their excellent root colonization ability, and bacilli, because of their ability to survive in unfavourable conditions, and because of ability of both groups of bacteria to produce a range of compounds inhibiting *Ggt* growth) are able to control the plant disease or directly stimulate crop growth (Hornby, 1998; Kim *et al.*, 1997; Mariano *et al.*, 1997; Mróz *et al.*, 1994; Ryder *et al.*, 1999; Tsuchiya, 1997; Weller, 1988; Weller *et al.*, 1997; Wenhua and Hetong, 1997; Wong, 1994). Fluorescent pseudomonads may also have a role in natural form of biocontrol – take-all decline (Weller, 1988).

The purpose of our work was a preliminary evaluation of the potential of some strains of *Pseudomonas fluorescens* (Trevisan 1889) Migula 1895 and *Bacillus mycoides* Flügge, 1886, isolated from rhizosphere of winter wheat, to control *Gaeumannomyces graminis* var. *tritici* (*Ggt*) on wheat.

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Experimental

Materials and Methods

Inoculum of the pathogen. The pathogenic fungus, *Gaeumannomyces graminis* var. *tritici* (*Ggt*) was isolated from wheat infested with the pathogen. The inoculum of *Ggt* was prepared by growing the fungus on autoclaved oat grains. In a pot experiment the inoculum was mixed with the whole mass of soil at the rate of 1% (w/w). In a plot experiment the inoculum was mixed with the upper (20 cm) layer of the soil (75 g/m^2).

The source of the rhizobacterial strains. The rhizobacterial strains (*P. fluorescens* – III107 and II21, and *B. mycoides* – JC192 and K184) were isolated from the winter wheat roots according to the method describing by Kobus *et al.* (1993).

Metabolic activities of the rhizobacteria. Antagonistic activity of the isolated strains against *Ggt* was determined on Petri plates (9 cm diameter) with Difco potato-dextrose agar (PDA). The plugs of agar with *Ggt* (4 mm diameter) were placed in the middle of the plates, and the tested bacteria were inoculated at the distance of 3.5 cm from the middle of the plates. The zones of *Ggt* inhibition were measured after 7 days of the incubation at 28°C. Chitinolytic activity was determined in tubes with agar medium (Strzelczyk *et al.*, 1990) containing 0.5% (w/v) of colloidal chitin. The bacteria were inoculated at the top of the agar medium. The depth of clear zone indicating the rate of chitin hydrolysis was measured after 28 days of incubation at 28°C. Ability to synthesize Fe^{+3} complexing compounds was studied in mineral nutrient medium containing 2.5% (v/v) of glycerol (Księżniak and Kobus; 1993). After 21 day incubation at 28°C, to 1 cm^3 of the culture filtrates 0.3 cm^3 of 6% (w/v) $FeCl_3 \cdot 6H_2O$ in 0.1N HCl was added. After 1 hour the optical density of the mixture was analyzed at the wavelength of 520 nm, and the concentration of Fe^{+3} complexing compounds was estimated from the calibration curve, prepared for a synthetic iron chelator – deferoxamine mesylate USP (Desferal – CIBA-GEIGY) after its reaction with $FeCl_3$ (Jaroszuk-Ściseł and Kurek, 2001). The capability of the bacterial strains for HCN production was studied according to the method described by Paszkowski *et al.* (1996).

Preparation of the rhizobacterial inocula. In the pot experiment the inocula of the strains were prepared by rinsing off the bacterial cells after 48 hours growth on solid King's medium B, with 1% (w/v) solution of CM-cellulose. Before sowing, the seeds were soaked in the bacterial suspensions (10^9 CFU/ml) for 30 min. The seeds from the series non-inoculated with the bacteria were soaked for 30 min in 1% solution of CM-cellulose after rinsing off the plates containing the sterile nutrient medium.

In the plot experiment the inocula of the strains (III107, II21 and JC192) instead of the CM-cellulose solution were rinsed off the Petri plates with sterile water to obtain suspensions of the individual strains containing 10^9 CFU/ml. The winter wheat seeds were sprayed with the mixture (1:1:1 v/v) of the bacterial suspensions.

Characteristics of the soils. In the pot experiment a field black diluvial soil developed from sandy slight loam (pH_{KCl} 6.8; organic C content 0.96%; total N 0.09%; CEC 16.01 meq 100g^{-1}; clay 11%; silt 7% and sand 82%) was used. The soil, sieved through a 2 mm screen, was placed in the pots (1 kg per pot).

A brown soil developed from sandy loam (pH_{KCl} 6.0; organic C content 1.13%; total N 0.11%; CEC, 16.35 meq 100g^{-1}; clay 17%; silt 33% and sand 50%) was used in the plot (0.8 m^2 area) experiment.

Plant tests. The pot experiment was conducted in November in a glasshouse with additional electric lighting. In this experiment 20 seeds of winter wheat cv. Kobra per pot were sown. The number of emerged plants was determined everyday between the 7th and 14th day after sowing. The number of plants was reduced to 8 per pot on the 14th day after sowing. The plants were harvested after 28 day of growth. Then the dry weights of the plant shoots and roots, and the percentage of infested wheat plants and the percentage of roots infested with the pathogen were determined. These measurements were done with 4 replicates. After 21, 25 and 28 days of incubation the percentage of wheat plants with morbid symptoms (yellowing, browning and withering) on aboveground parts was determined from all 4 pots of each experimental series excluding replications.

In the microplot experiment the soil was enriched with multiple mineral fertilizer Azofoska (100 g per pot) and with potassium chloride (8 g per pot). Seeds of winter wheat cv. NAD 899 were sown in the amount of 200 per one microplot on the 15th of September. After the harvest, yield of the grain and the straw, number of ears and the weight of each individual ear were determined. All determinations were done with 4 replicates. The distribution of the ear weights of winter wheat was done for the ears of all plots of the individual experimental series.

Statistical evaluations. All the data (in 4 replicates) were subjected to analysis of variance and separated with Student's t-test (P = 95%). The values of percentage of healthy plants were transformed for statistical evaluation according to the equation $y = \text{arc sin } \sqrt{x}$

Results and Discussion

From among many various strains of fluorescent pseudomonads and bacilli isolated in our laboratory from winter wheat roots, the representatives of *Pseudomonas fluorescens* and *Bacillus mycoides* were chosen for the studies. The bacteria belonging to the former species were the most numerous group of isolates from the surface and the inside of the winter wheat roots and they were metabolically very active (Czaban, 2001). The endospore-forming bacteria belonging to *B. mycoides* are known as very fast growers, which spread rapidly on the solid nutrient media. These rhizobacteria were the fastest colonizers of the agar media from the pieces of soil free winter wheat roots (soil was rinsed off) put on the surface (Czaban, 2001). Based on this, we expected that strains belonging to these groups of bacteria would be good colonizers of wheat roots. The capability of bacteria for colonizing the plant roots is essential for biocontrol of root pathogens (Weller, 1988).

The four bacterial strains (*P. fluorescens* – III107 and II21, and *B. mycoides* – JC192 and K184) were chosen because of their ability to reduce the growth of *Ggt* on potato-dextrose agar medium (Table I) and to

Table I
Selected properties of the rhizobacterial strains chosen to the vegetation experiments with *Gaeumannomyces graminis.* var. *tritici* (*Ggt*)

Bacterial species	The strain symbol	Inhibition of *Ggt.* growth (inhibition zone in mm)	Fe^{3+} chelating compounds (μM Desferal l^{-1})	Chitinase synthesis (clearing zone in mm)	HCN synthesis (Scale 0–5)
Pseudomonas fluorescens	III107	10	125	0	0
	II21	17	190	0	5
Bacillus mycoides	JC192	0*	12	25	0
	K184	0*	18	0	0

* – the lack of the inhibition zone, but the growth of *Ggt* was very strongly inhibited by intensively expanding bacteria.

stimulate the growth of winter wheat (up to 44% in a case of *B. mycoides* K184) in sterile sand enriched with Hoagland's nutrient medium and in nonsterile soils, not contaminated with plant pathogens (Czaban, 2001).

The zones of *Ggt* growth inhibition on PDA (Table I) suggest the synthesis of some antibiotics by the examined pseudomonad strains. Both strains of *P. fluorescens* also produced relatively high amounts of Fe^{3+} complexing compounds, and *P. fluorescens* II21 produced high amount of HCN (Table I). Weller (1988) and Weller *et al.* (1997) claimed that the antibiotic production was one of the most important features of bacteria with regard to take-all control on wheat. The important role of siderophores in suppression of take-all was described by Hornby (1998), Tazawa-Isogami *et al.* (1997) and Wong and Baker (1984). Also, in the opinion of some authors, the bacterial synthesis of HCN may help to suppress the disease of cereals caused by *Gaeumannomyces graminis* (Hornby, 1998; Ross and Ryder, 1994; Paszkowski, 1998). Keel *et al.* (1990) suggested that the suppression of soil-borne pathogens by an effective biocontrol agent – *P. fluorescent* strain CHA0 was a multifactorial mechanism which was mainly due to the production of antibiotics, siderophores and HCN.

Strain JC192 of *B. mycoides* was capable for intensive degradation of chitin (Table I). Chitinolytic ability of this bacteria may play a role in control of *Ggt*, because antagonistic *B. mycoides* caused lysis of hyphae of *Ggt* (Bednářová-Civínová *et al.*, 1981; Campbell and Faul, 1979 – cited by Kim *et al.*, 1997).

In the pot experiment on soil not contaminated with the pathogenic fungus, bacterization of the wheat seeds with all tested bacterial strains had favorable influence on the seedlings emergence (Fig. 1) and on the plant biomass, especially the dry root weight (Fig. 2 and 3). These results confirm our earlier findings (Czaban, 2001) that these strains are PGPRs (plant growth promoting rhizobacteria).

Ggt significantly decreased winter wheat seedlings emergence (Fig. 1), reduced dry weight of plant shoots and roots (Fig. 2 and 3) and infested roots of almost all plants (Fig. 4). In the experimental series with *Ggt* the negative influence of the pathogen on winter wheat seedlings emergence was intensified by bacterization of wheat seeds until 9–10 day of the incubation (Fig. 1). It was probably caused by the synergistic effect of phytohormone-like substances produced by the fungus and the bacterial strains. At the end of the 28-day pot experiment the bacterial strains slightly increased the dry plant weight (except for *B. mycoides* K184) in comparison to series with *Ggt* alone, but this stimulation was statistically insignificant (Fig. 2 and 3). The abilities of the bacteria to protect wheat plants against *Ggt* were more distinctly visible in the shape of decreased percentage of plants with infested roots and in the shape of decreased percentage of infested roots of all plants (Fig. 4), as well as the percentage of plants with morbid symptoms on shoots (Fig. 5). The bacterial strains *P. fluorescens* III107 and *B. mycoides* JC192 were the best plant protectors against *Ggt*.

In the opinion of many authors, the use of bacterial strains in their combination will improve the effectiveness of biological control treatments against many plant pathogens, including *Ggt* (de Boer *et al.*, 1997; Duffy *et al.*, 1996; Lemanceau *et al.*, 1992; Pierson and Weller, 1994). Increasing the genetic diversity of the biological control system through the use of microbial mixtures may result in the treatments that persist longer in the rhizosphere and utilize a wider array of biocontrol mechanisms under a broader range of environmental conditions (Pierson and Weller, 1994).

A synergism between the antibiotic producing bacteria and the chitinase producing bacteria to inhibit *R. solani* was reported by Sung and Chung (1997). Moreover, Bednářová-Civínová *et al.* (1981) showed that while the population of the biocontrol agent – *P. putida* (introduced on wheat grain) declined with the age of the wheat plants, the population of the native chitinolytic *B. mycoides* increased in the rhizosphere (only in the bacterized series). This suggests that the *B. mycoides* having a mycolytical effect on *Ggt* could

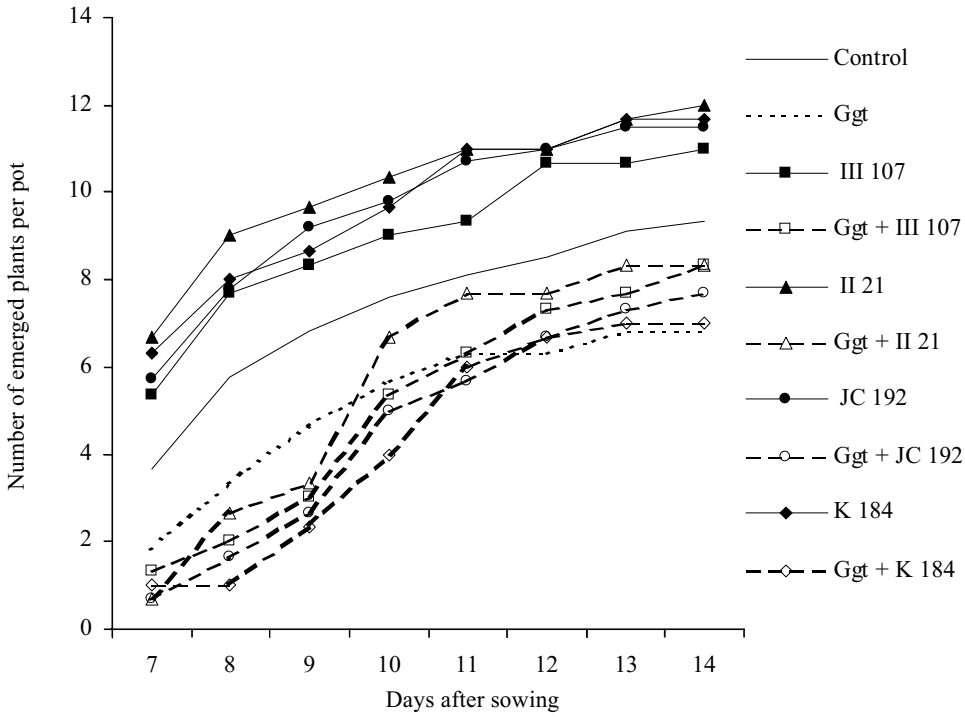

Fig. 1. Winter wheat seedlings emergence as an effect of soil contamination with *Ggt* and wheat seed inoculation with rhizobacteria: *Pseudomonas fluorescens* (strains III107 and II21) and *Bacillus mycoides* (strains JC192 and K184) in the 28 day glasshouse pot assay

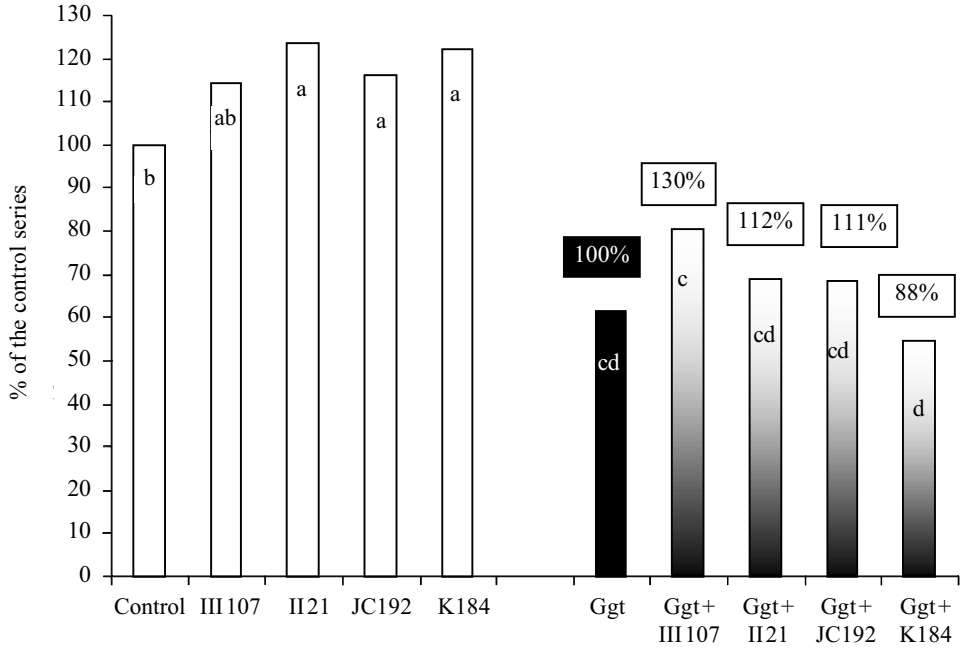

Fig. 2. Dry weight of winter wheat shoots as an effect of soil contamination with *Ggt* and wheat seed inoculation with rhizobacteria: *Pseudomonas fluorescens* (strains III107 and II21) and *Bacillus mycoides* (strains JC192 and K184) in the 28 day pot experiment.
The mean weights of the shoots expressed in columns as percentage of the control value [350 mg per pot] with different letters are significantly different at $P<0.05$.

protect the wheat against take-all in the later stages of the plant growth following the initial protection by *P. putida*. Also Wong (1994) suggested that there might be a role for combining species of *Pseudomonas* and *Bacillus* to extend the period of biocontrol of take-all.

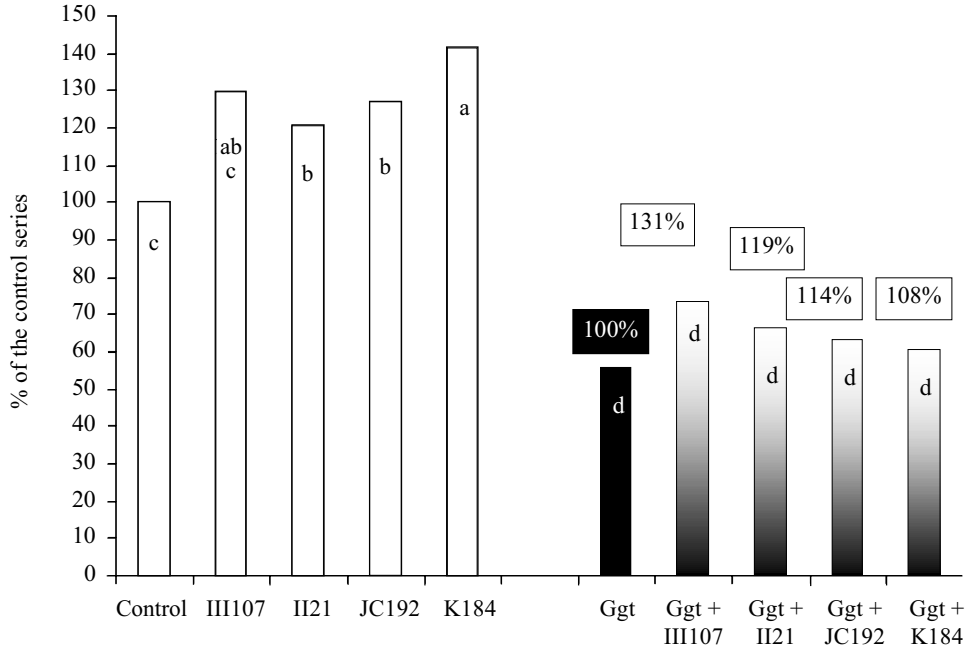

Fig. 3. Dry weight of winter wheat roots as an effect of soil contamination with *Ggt* and wheat seed inoculation with rhizobacteria: *Pseudomonas fluorescens* (strains III107 and II21) and *Bacillus mycoides* (strains JC192 and K184) in the 28 day pot experiment.
The mean weights of the roots expressed in columns as percentage of the control value [86 mg per pot] with different letters are significantly different at $P<0.05$.

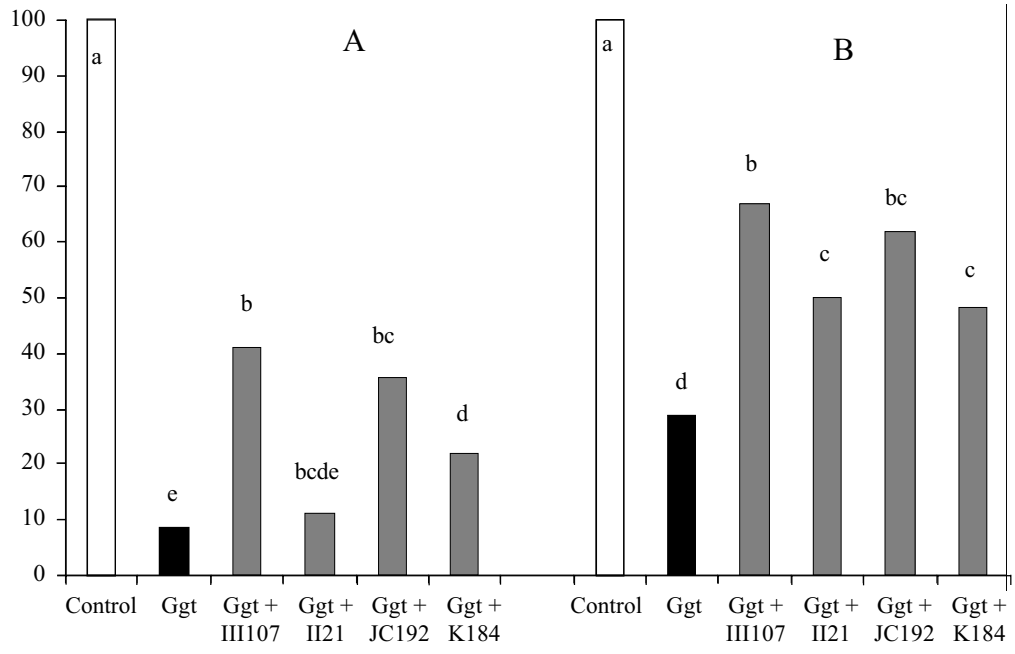

Fig. 4. The ability of rhizobacteria: *Pseudomonas fluorescens* (strains III107 and II21) and *Bacillus mycoides* (strains JC192 and K184) to protect winter wheat against *Ggt* expressed by means of: (A) percentage of plants with all healthy roots, and (B) percentage of healthy roots of all plants in the 28 day pot experiment.
Values of any columns with different letters are significantly different at $P<0.05$.

The earlier studies (Czaban, 2001) showed that after 4 weeks the number of strains III107 and II21 in unsterile nonrhizosphere soil decreased 112000 and 25000 times, respectively, but the number of strain JC192 remained at the initial level, and the K184 number decreased only 6 times. Moreover, after 4 weeks

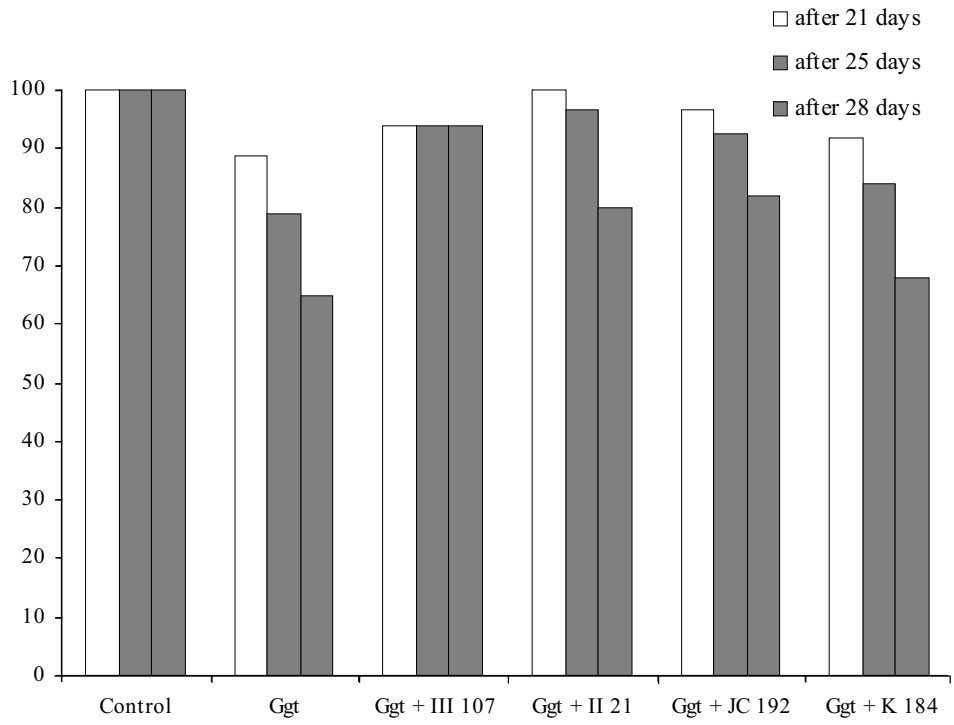

Fig. 5. The ability of rhizobacteria: *Pseudomonas fluorescens* (strains III107 and II21) and *Bacillus mycoides* (strains JC192 and K184) to protect winter wheat against *Ggt*, expressed by means of percentage of plants not possessing visible morbid symptoms on shoots in the 28 day pot experiment.

of the winter wheat growth the strains of bacilli colonized rhizosphere soil and the plant roots at the same degree as the pseudomonad strains.

Considering the information cited above a mixture of three strains of rhizobacteria (*P. fluorescens* III107 and II21 and *B. mycoides* JC192) was used in the plot experiment.

The bacterization of winter wheat seeds in the series not contaminated with *Ggt* did not exert statistically significant influence on the yield of grain and straw of the plants (Fig. 6), but it increased the mean weight of one ear (Fig. 7). *Ggt* very significantly reduced the yield of grain and straw (Fig. 6) as well as the number of wheat ears and the mean weight of one ear (Fig. 7). The mixture of the rhizobacteria distinctly protected the plants against the pathogen which was evident from the increase of grain and straw yield (Fig. 6) and the increase of mean ear weight (Fig. 7). The rhizobacteria had much weaker (in the *Ggt* contaminated series statistically significant) influence on the weight of 1000 wheat kernels than on the mean ear weight, which means that the ears from bacterized series comprise more kernels than corresponding not bacterized ones (Fig. 7).

The percentages of ears with weight less than 500 mg and (especially) less than 200 mg were very good indicators of *Ggt* infestation level. In the experimental series not contaminated with *Ggt* the percentage of ears with weight less than 500 mg was lower than 3% (Fig. 8A) and the percentage of ears with weight less than 200 mg was lower than 0,5% (Fig. 8B), but in the series with *Ggt* the former value exceeded 60% (Fig. 8A) and the latter exceeded 40% (Fig. 8B). This suggests that the pathogen-infested wheat plants which survived to maturity, had ears with weight less than 500 mg and even less than 200 mg. The inoculation of wheat seeds with the mixture of rhizobacteria markedly decreased the percentage of these ears (Fig. 8). Comparing the distribution of the wheat ears with weight higher than 500 mg, it is visible that the ears from the bacterized experimental series, both, not contaminated and (especially) contaminated with *Ggt*, were composed in higher extent of heavier fractions than the ears from corresponding not bacterized ones (Fig. 9).

All the presented results of both pot and plot experiments show that the used strains of rhizobacteria protected to some extent the winter wheat against *Ggt*. However, this protection was incomplete – the wheat grain and straw yield reached the level of only 45% and 43% of the control series comprising healthy plants. In comparison, Wütrich and Défago (1990) obtained complete wheat protection against *Ggt* after inoculation with *P. fluorescens* CHA0, but the level of infestation by the pathogen in their experiment was much

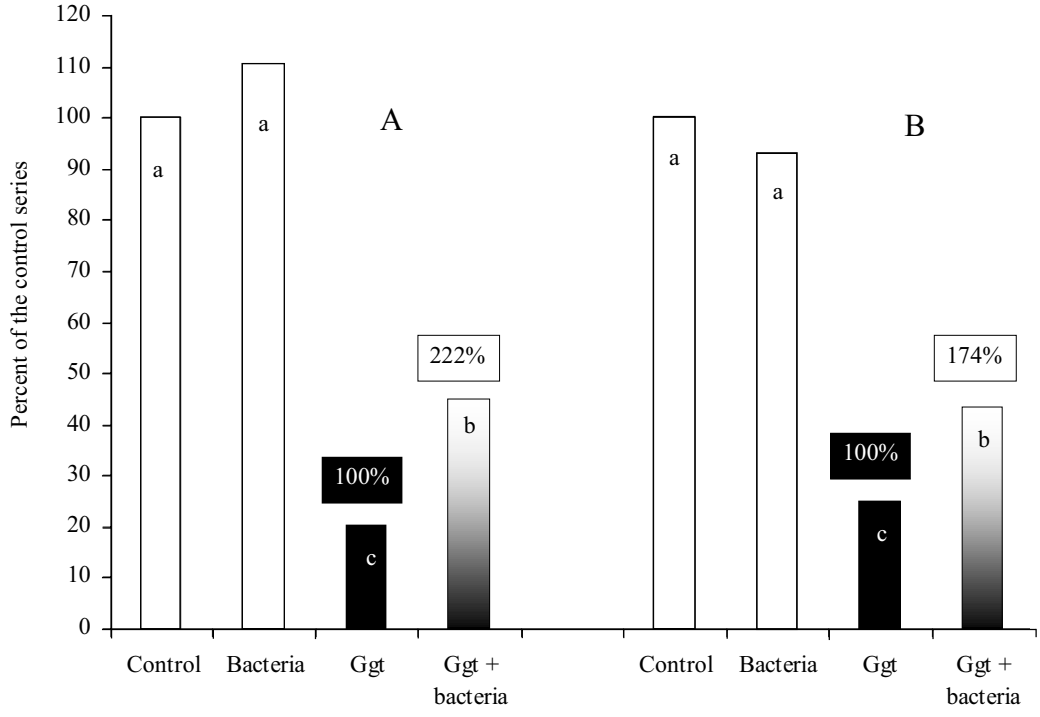

Fig. 6. The yield of winter wheat grain (A) and straw (B) as an effect of soil contamination with *Ggt* and wheat seed inoculation with the mixture of rhizobacteria: *Pseudomonas fluorescens* (strains III107 and II21) and *Bacillus mycoides* (strain JC192) in the plot experiment.

The yield of grain and straw in the control series amounted to 506 g and 660 g, respectively. Means expressed in columns as percentage of the control values with different letters are significantly different at $P<0.05$.

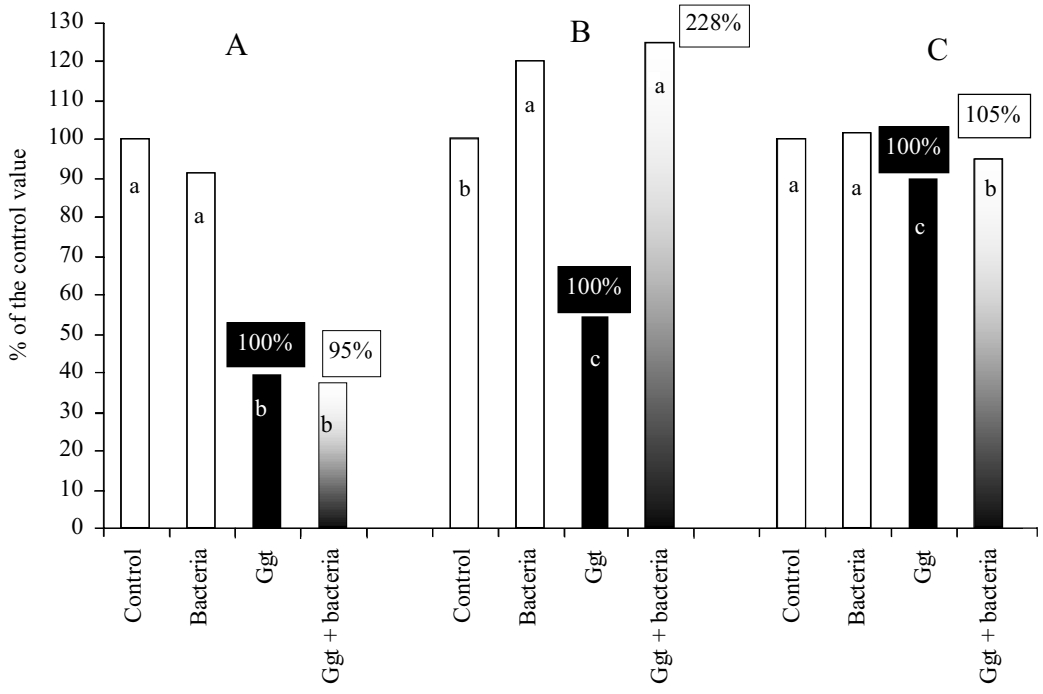

Fig. 7. The number of winter wheat ears (A), mean weight of 1 ear (B) and weight of 1000 kernels (C) as an effect of soil contamination with *Ggt* and wheat seed inoculation with the mixture of rhizobacteria: *Pseudomonas fluorescens* (strains III107 and II21) and *Bacillus mycoides* (strain JC192) in the plot experiment.

The number of ears, mean weight of 1 ear and the weight of 1000 kernels in the control series amounted to 402, 1.56 g and 55 g, respectively. Means expressed in columns as percentage of the control values with different letters are significantly different at $P<0.05$).

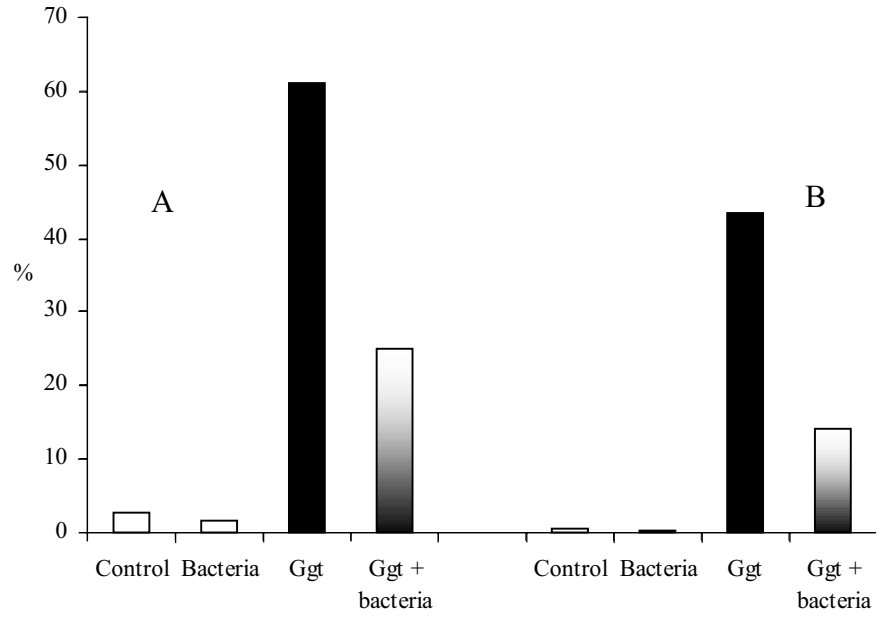

Fig. 8. The ability of the rhizobacterial mixture: *P. fluorescens* (strains III107 and II21) and *B. mycoides* (strain JC192) to protect winter wheat against *Ggt* expressed by means of percentage of ears with weight less than 500 mg (A) and with weight less than 200 mg (B) in the plot experiment.

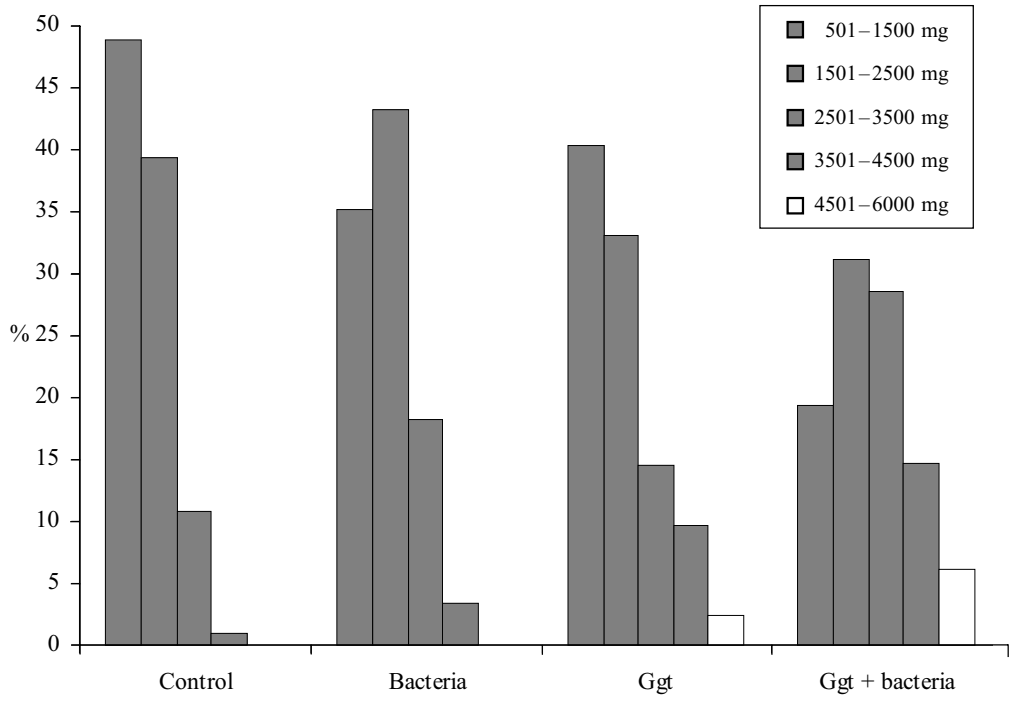

Fig. 9. The distribution of winter wheat ears with weight bigger than 500 mg as an effect of soil contamination with *Ggt* and wheat seed inoculation with the mixture of rhizobacteria: *Pseudomonas fluorescens* (strains III107 and II21) and *Bacillus mycoides* (strain JC192) in the plot experiment
The number of ears with weight bigger than 500 mg per 1 plot amounted to 391, 361, 62, 113 in the control series, the bacterial series, the *Ggt* series and in the *Ggt* + bacteria series, respectively.

lower than in ours. We would like to emphasize that the winter wheat grain yield in *Ggt*-bacterized series exceeded the yield in the series with *Ggt* alone by 120%. The yield increase due to the plant protection against take-all by inoculation with various bacteria according to many studies presented by Hornby (1998) and Wong (1994) ranged from 5 to 114%.

In the future, ability of bacterial strains to protect wheat against *Ggt* should be verified in field conditions on different soils and with other strains of *Ggt*. Wong (1994) in his review stated that the results of such field studies are inconsistent, with good responses in some years and on some sites but not on others, and Mazzola *et al.* (1995) found that different strains of *Ggt* varied in their sensitivity (from very sensitive to insensitive) to antibiotics produced by fluorescent *Pseudomonas* spp. The search of other bacterial strains more effective in take-all control on wheat than those presented in this paper is also necessary.

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