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Evaluation of Some *Bacillus* spp. Strains for the Biocontrol of *Fusarium graminearum* and *F. culmorum* in Wheat

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Abstract

Fusarium graminearum Schwabe - teleomorph Gibberella zeae (Schwein.) Petch. and F. culmorum (W.C.Smith) Sacc. are the most important head-blighting pathogens of wheat in many regions of the world, including Romania. The strategies developed for the control of Fusarium spp. infections on cereals include agrotechnical practices, resistant cultivars, and fungicides use. However, the toxicity of fungicides and the low efficacy of such products to control members of the Fusarium head blight (FHB) Complex led to the development of additional strategy that can be used as part of an integrated management of FHB (directed against fungal growth and/or mycotoxins biosynthesis and accumulation). The use of biocontrol agents is an important strategy for integrated management of fungal diseases in many economically important plat species. It was shown that Bacillus spp strains could suppress various soil borne diseases, their efficacy being associated with their antagonistic properties and competitiveness in the rhizosphere (including through sporulation). The aim of this study was the „in vitro" evaluation of biocontrol capacity of different Bacillus spp. strains isolated from soil and compost against strains of Fusarium graminearum and F. culmorum. The ability of four strains of bacilli (B1, B3, B5 and BIR) to inhibit fungal growth was established. For their identification BIOLOG system was used, coupled with molecular techniques (ARDRA). It was proved that the strains of interest belong to Bacillus amyloliquefaciens (BIR and B3) and Bacillus spp. (B1 and B5). The Bacillus amyloliquefaciens strains, BIR and B3, were also evaluated in pot experiments, in climatic chamber conditions, for their ability to stimulate wheat seeds germination and/or plantlet growth and reduce Fusarium infections in first stages of wheat vegetation. After 21 days of incubation significant differences were registered between variants infected with Fusarium and supplemented with Bacillus and control variants, not only in germination rate but also in wheat plantlets development. Further differences were observed in the number of Fusarium CFU/g of soil from the soils treated with bacteria in contrast with control variants.

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Keywords: bacillus, biocontrol, fusarium infections, wheat

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1. Introduction

Grains represent important food and feed crops around the world. Wheat in particular is grown on larger land area worldwide than any other crop (FAOSTAT, 2011). These economically and historically important crops are under attack by challenging plant diseases. Fusarium Head Blight (FHB or scab) is a fungal disease that affects wheat and other cereals, such as barley, oats, rye, maize, triticale, millets and some forage grasses. FHB infections results in billions of dollars of wheat yields and quality loss in the 90s and early 2000s (Nganje et al., 2004). Reduced yields and market prices produced by the FHB made other crops than wheat more attractive to growers.

A complex of closely related species from the Fusarium genus is responsible for this important disease of crops. From the Fusarium genus two members stand out as particularly dangerous to wheat: Fusarium graminearum and F. culmorum (Ittu et al., 2010). The mycotoxins produced by Fusarium species have negative effects on eukaryotic cells including protein, DNA and RNA synthesis inhibition, cell division, membrane and mitochondrial functions (Rocha et al., 2005).

Fusarium outbreaks can be prevented by avoiding maize as a previous culture, by growing wheat cultivars with resistance towards Fusarium species or by removing the plant debris of previous cultures. When infection conditions at the time of wheat anthesis are favorable, preventive measures these actions are not sufficient, and fungicides should be applied. However, some fungal populations are resistant to specific fungicide or can become resistant during its use, a process called acquired resistance (Dubos et al., 2011). Moreover, chemical fungicides are known to be toxic and can accumulate in time in soil and plant tissue.

Managing fungal diseases of plants with microbial antagonists either as an alternative or as a supplement to fungicides could be of real benefit. Biological control agents (BCAs) such as Bacillus spp. are attractive for disease control due to their positive environmental impact and due to their ability to reduce dependence on chemicals, thereby slowing the development of fungicide resistance in pathogen populations (Jochum et al., 2006).

There have been notable successes with Bacillus spp. as BCAs applied to soil (Pérez-García et al., 2011) whereas biological control of the fungal diseases by BCAs applied to aerial plant parts has been more challenging (Jacobsen et al., 2004). In light of these arguments the aim of this study was to test the capacity of some new Bacillus spp. isolates to inhibit the spreading of Fusarium spp., both in vitro and in vivo conditions.

2. Materials and methods

2.1. Isolation of pathogenic fungi:

Fusarium graminearum and Fusarium culmorum cultures were isolated from grains of wheat obtained in 2013 harvest by plating serial dilutions on PDA medium amended with chloramphenicol (30 mg/L) to suppress bacterial infections (Alwakeel, 2013). The cultures were identified based on colony aspect and microscopic particularities such as conidia characteristics and other morphological traits, using various keys for Fusarium spp. identification. The most aggressive cultures were selected based on pathogenicity tests (Grosu et al., 2014a). The cultures were maintained on PDA medium and stored at 4°C.

2.2. Bacterial isolates:

The bacterial strains were isolated from compost tea and selected according to their inhibitory action against Fusarium spp. (Grosu et al. 2014b). These bacteria were subjected to identification both by molecular methods and by using BIOLOG system and the results were compared with B. subtilis, B. amyloliquefaciens and B. licheniformis reference strains. All bacterial isolates were maintained on Luria-Bertani agar medium and stored at 4°C.

DNA extraction and PCR analysis: Total DNA isolation from selected bacterial strains was performed using the method described by Gevers et al. (2001). For PCR analysis, specie-specific primers were used in the experiments: BSL72 (CGTAGAGCCACTTGAGCG) and BSR328 (CTGCCGTTACAGTTCCTT) for B. subtilis, L100 (AAATCTGCCCGTATCGTCG) and R836 (GCGTCACGGCGR(AG)ATCT CAA) for B. amyloliquefaciens, L168 (TGG GATGACAAGTGATAAGC) and R514 (CTCCGTTGACAAGCAAGTTCG) for B. licheniformis and L354 (AGGGAAGAACAA GTGC(GA)AGAG) and R674 (GCTCCTCAG CGTCAGTTACA) for B. pumilus (Cao et al., 2008).

2.3. Wheat seeds:

Wheat seeds from Dropia cultivar were used in experiments. The seeds were surface sterilized using 2% of NaOCl solution for 1 minute (Knight and Sutherland, 2013) and seeded in pots.

2.4. Pot experiments:

The pot experiments were performed in environmental controlled conditions using Sanyo 351H plant growth chamber. Plastic pots were used, containing 70 grams of garden soil. In total, 12 variants in three replicates were used (Table 1).

Fungal strains were grown in PDB (potato-dextrose broth) medium at 26°C and continuous orbital shaking for 5 days. Cultures were then filtered through a sterile four layers cloth to separate the mycelia and harvest the conidial suspension. The final concentration of the inoculum was prepared at 106 CFU/g of soil, as recommended by Morsy et al. (2009).

The bacterial inoculum was prepared in 100ml of Nutrient Broth and incubated for 3 days at 30°C. Bacterial cells were recovered by centrifugation 10 min at 10000 rpm and suspended in sterile distilled water, to a final concentration of 108 CFU/ml.

Table 1. Pot experiment samples

Samples	Bacillus spp.		Fusarium graminearum	Fusarium culmorum
	B3	B8		
Control	–	–	–	–
C1	+	–	–	–
C2	–	+	–	–
C3	+	+	–	–
C4	–	–	–	+
C5	–	–	+	
V1	+	–	–	+
V2	+	–	+	–
V3	–	+	–	+
V4	–	+	+	–
V5	+	+	–	+
V6	+	+	+	–

Five wheat seeds were placed in each pot, two hours after the soil inoculation with bacteria / fungi. The experiments were monitored for 13 days after seeding in order to evaluate the germination and plantlet growth.

Determination of the treatment efficiency against fungal pathogens: In order to determine the treatment efficiency serial dilutions of soil samples from each experimental variant, collected at the end of the experiments were prepared, and plated on PDA supplemented with chloramphenicol (200 mg/L) and gentamicin (40 mg/L) (Marinach-Patrice et al., 2009). The inoculated plates were incubated at 26°C for five days. The fungal growth was evaluated according the following formula:

$$\text{Efficiency (\%)} = \frac{A - B}{A} \times 100$$

where: A stands for the number of colonies counted in the Fusarium sample and B is the number of colonies counted in the Bacillus-Fusarium interaction sample.

Statistical analysis of the results was performed by XLSTAT add-in for Excel.

3. Results and discussions

Bacterial strains selected for their antifungal actions were identified, by BIOLOG system and molecular analysis. For these strains, the results obtained with both methods were similar: the strains B3 and B8 belong to B. amyloliquefaciens, and B1 is B. licheniformis. No amplification product was obtained with primers used in experiments for the strain B5 (figure 1).

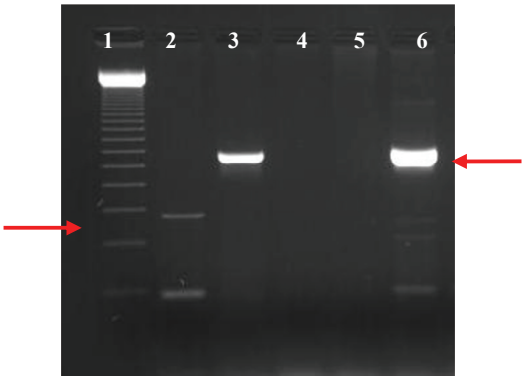

Figure 1. Electrophoretic pattern of amplicons:
1 – ladder 123 bp; 2 – B1; 3 – B3; 5 – B5; 6 – B8.

The wheat seeds germination ended after 7 days and differences of germination rates between variants were observed (Figure 2).

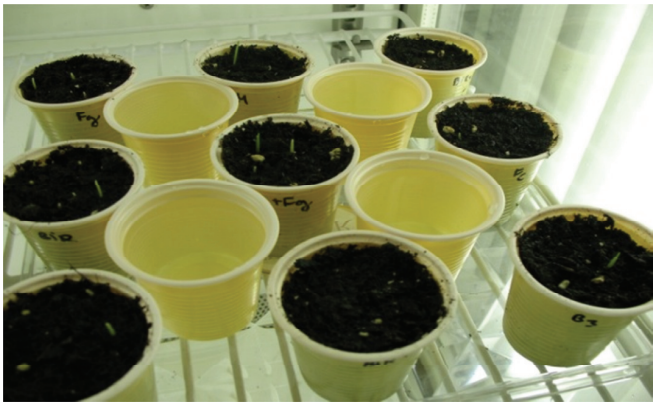

Figure 2. Seed germination in the experimental variants

Significant increases of the germination rates of seeds (3-3.5 times) in the presence of antagonistic bacteria were observed, comparing with the variants containing the pathogenic fungi. It was also shown that the bacterial mixtures positively affect the germination, the results suggesting synergic effects among the bacteria. However, in the presence of the fungi, the effects of mixture were lower than individual bacteria (Figure 3).

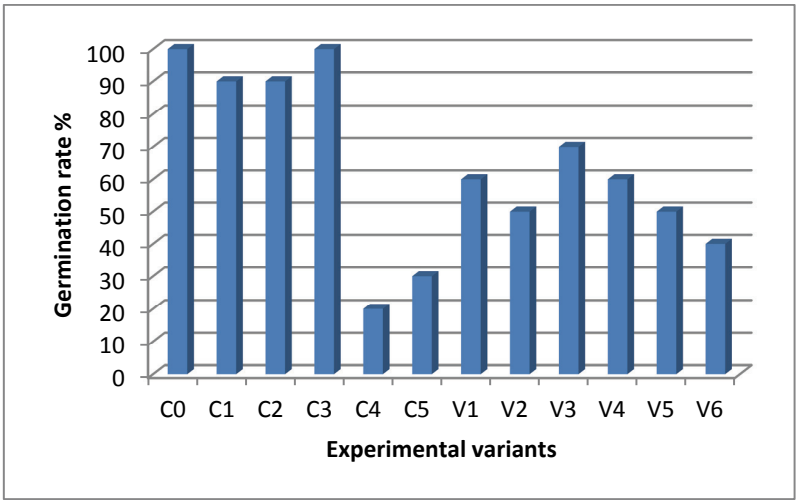

Figure 3. Seed germination rate

Best results against both fungal pathogens were obtained with Bacillus amyloliquefaciens B8, these results being in agreement with data from literature (Wang et al., 2013; Crane et al., 2013; Yuan et al., 2013).

Regarding the influences of the microbial treatments on plantlets growth differences in plantlets length between experimental variants were observed, starting the 9th day of cultivation but the most significant results were revealed after 12 days of cultivation (Table 2).

Table 2. Average plantlet length

Sample	Plantlet length (cm) at different times of cultivation				
	7 days	8 days	9 days	12 days	13 days
C0	9,28	12,28	14,01	15,93	17,66
C1	10,23	13,49	15,50	16,84	18,83
C2	8,87	12,02	14,10	17,95	19,60
C3	10,34	13,89	15,99	18,50	20,76
C4	10,15	14,20	16,15	20,35	23,25
C5	8,80	13,00	14,75	18,20	20,50
V1	9,33	12,83	14,98	19,00	20,50
V2	11,00	13,86	16,32	19,67	21,75
V3	9,94	13,82	15,78	20,62	23,92
V4	10,17	12,98	15,06	20,10	23,38
V5	10,23	13,83	15,93	19,83	23,07
V6	10,05	12,17	13,87	16,17	21,00

Considering the only the bacterial treatments, noticeable plant stimulation effects were obtained compared to the uninoculated control. In this respect, the highest growth rate, with an increase of 17.5 %, was encountered when the bacterial mixture was used, after 13 days of monitoring. Surprising results were observed in the presence of fungal pathogens: significant increased lengths of plantlets, with 16.08 % for Fusarium culmorum to 31.65 % for F.graminearum (Figure 4).

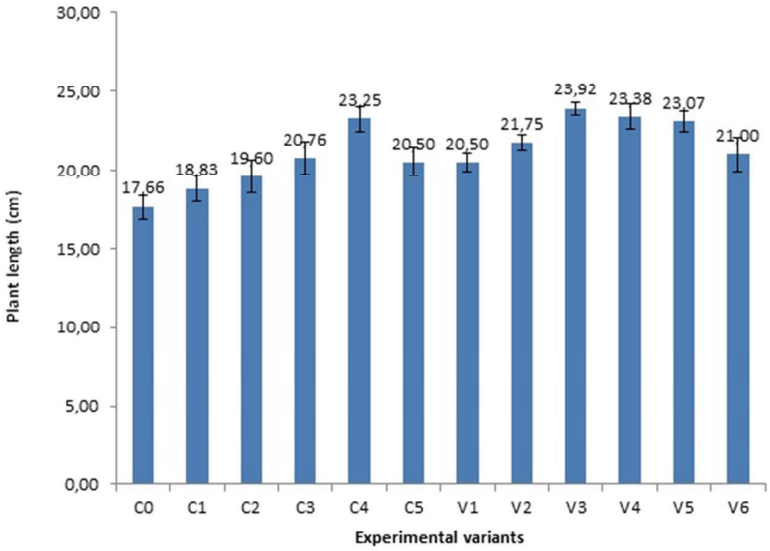

Figure 4. Wheat plantlet length at 13 days after seeding

These results could be explained by the ability of the fungal strains to produce phytohormones, like gibberellins (Ma et al., 2013). This phytohormone increases internode elongation in wheat plants (Zhang et al. 2007). However in the presence of higher quantities of this compound the plantlets are higher as length but their vigor is reduced. Similar stimulation is produced by bacteria due to other phytohormones synthesis (indole-3-acetic acid) (Duca et al., 2014; Shokri and Emtiazi, 2010; Sicuia et al., 2012) but the plant vigor was not affected in these cases. Moreover, in the variants where combination of antagonistic bacteria and fungi were used, both the increase of plantlets length and vigor (Figure 5) were revealed.

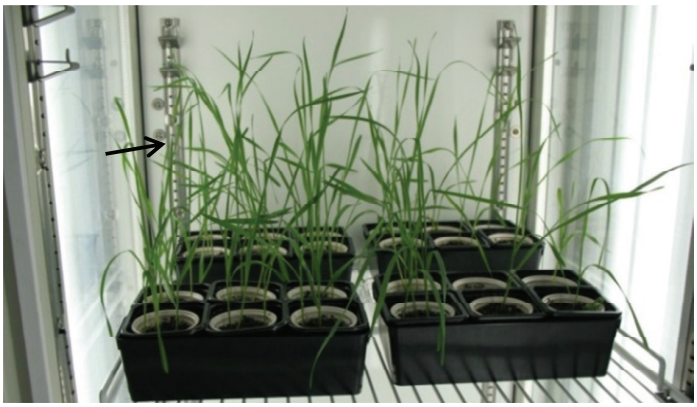

Figure 5. Plant stimulation effects of bacterial treatments (arrow)

Related to the influence of antagonistic bacterial treatments on total soil fungal load it was observed that, at the end of monitoring, significant reduction of fungal CFU/g of soil was produced, ranging from 41% in V6 to 82% in V1 (Table 3).

Table 3. Efficiency of bacterial treatments on total fungal load

	Fc	Fg	B3-Fc	B3-Fg	B8-Fc	B8-Fg	Mix-Fc	Mix-Fg
CFU/g	$72x10^4$	$104 x10^4$	$13 x10^4$	$29 x10^4$	$10 x10^4$	$23 x10^4$	$42 x10^4$	$61 x10^4$
Reduction of fungal load (%)	-	-	**82%**	**72%**	**86%**	**78%**	**42%**	**41%**

where: Fc – Fusarium culmorum, Fg – Fusarium graminearum, B3 – Bacillus amyloliquefaciens B3 strain, B8 – Bacillus amyloliquefaciens B8 strain, Mix – mixture of Bacillus amyloliquefaciens B3 and B8.

These results are in concordance with the effectiveness found in the germination step of the experiment. The best results were registered in the case of B. amyloliquefaciens B8 treatment that reduces the fungal load with 86% for F. culmorum and 78% for F. graminearum, respectively (Figure 6).

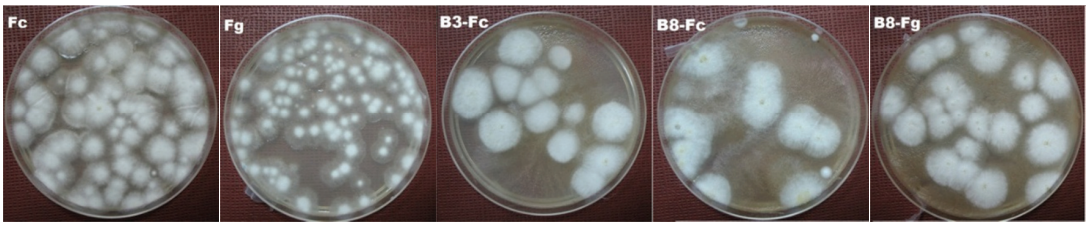

Figure 6. The effects of bacterial treatment on fungal load detected on plates with PDA (10-4 dilution)

In contrast, the bacterial mix registered the lowest inhibiting rates probably due to a competition between bacteria, but further analyses are necessary. Moreover, it is known that the interactions between bacteria, fungi and plants are very complex and could involve symbiosis-like relationships (Borriss, 2011; Zhao et al., 2014). Additionally, root colonization by bacteria can be related to the chemotaxis response to different root exudates (Compant et al. 2010). Our results could contribute to the knowledge of these complex natural relationships among soil microorganism.

4. Conclusions

The results obtained during the experiments allowed the following conclusions:

By applying biochemical and molecular techniques it was possible to identify the bacterial isolates as Bacillus amyloliquefacies and B. licheniformis.

Two out of the bacterial strains, designated as B3 and B8 were proved to stimulate both wheat seed germination and plantlet growth.

Inhibitory effects of bacterial treatments at soil level against Fusarium culmorum and F.graminearum were detected, best results being obtained with B.amyloliquefaciensB8.

These results suggest that the selected bacterial strains could be used as BCAs to reduce Fusarium spp. contamination of wheat through soil applications.

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