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RESEARCH ARTICLE

Biological control of the wheat root rot caused by *Fusarium graminearum* using some PGPR strains in Saudi Arabia

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Abstract

The aim of this study was to evaluate the efficacy of selected bacterial strains against the wheat soil-borne pathogen *Fusarium graminearum* under greenhouse conditions. The most potent isolates were 3 isolates out of 18 isolates, which have numbers 3, 9 and 10 with *in vitro* inhibition index 42.5%, 41.3% and 46.3% respectively. Isolates 3 and 10 were selected for the following experiments. Isolates 3 and 10 were identified as *Bacillus subtilis* MAA03 and *Pseudomonas fluorescens* MAA10, respectively according to International Identification Keys and, confirmed by using Biolog system and 16S rDNA where the strains exhibited more than 99.5% sequence identity. Their close taxonomic relationship was further documented by phenotypic similarities. The using of *B. subtilis* and *P. fluorescens* separately or in mixture as biocontrol agent against *F. graminearum* on wheat significantly increased the final germination percent, the mean daily germination and germination index of wheat cultivar, while the mean germination time was significantly decreased relative to infested control. The final infection percent, the mean daily infection and infection index were decreased significantly, while the mean infection time was significantly increased relative to infested control. The use of *P. fluorescens* as biocontrol agent was the most efficient than *B. subtilis* or in mixture and the best treatment was seed coating. The application of *B. subtilis* and *P. fluorescens* separately or in combination significantly affected the growth parameters of wheat cultivar Tabuki, the root length was significantly increased in seed coating and seed soaking treatments, while non-significantly decreased in case of soil drench treatment relative to infested control. Shoot length was significantly decreased in case of seed coating treatment relative to infested control. The shoot fresh and dry weights were significantly increased in seed coating and seed soaking treatments relative to infested control. The root fresh and dry weights were significantly increased in seed coating and seed soaking treatments relative to infested control. The number of leaves was significantly increased in all treatments relative to infested control.

Introduction

Fusarium spp., including *F. culmorum*, *F. graminearum*, *F. poae* and *F. avenaceum*, cause seedling blight, root rot and head blight diseases of cereals, resulting in yield loss (Fernandez & Jefferson, 2004; Nicholson *et al.*, 2004) in all regions of the world where these crops are cultivated. Pesticide use as a seed treatment has been shown to be effective in reducing the seed-borne inocula of *Fusarium* (Reddy *et al.*, 1999), but toxic residues associated with fungicides can be problematic. Commercial cereal cultivars with high resistance to *Fusarium* diseases are not yet available (Bello *et al.*, 2002).

Biological control involving microbial agents or biochemicals to control plant pathogens can be an eco-friendly and cost-effective component of an integrated disease management programme (Mao *et al.*, 1997).

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In wheat, seed treatment with several bacteria, including fluorescent pseudomonads, *Pantoea* sp., *Bacillus cereus* and the fungus *Trichoderma harzianum*, has shown promise for control of *Fusarium* seedling blight caused by *F. graminearum*, *F. culmorum* and *Microdochium nivale* in glasshouse and field studies (Bello *et al*., 2002; Johansson *et al*., 2003). Many studies have shown that fluorescent pseudomonads, *Pantoea* sp., *Bacillus* spp. and *T. harzianum* are among the most effective microbes in controlling a range of soil-borne diseases (Raaijmakers & Weller, 1998; Johansson *et al*., 2003). The biological control ability of selected fluorescent *Pseudomonas* spp. can be the result of competition for nutrients and space, siderophore-mediated competition for iron, antibiosis or induction of induced systemic resistance in the host plant (Pieterse *et al*., 2001; Berg *et al*., 2002). Strains of some *Bacillus* spp. have the ability to secrete chitinolytic enzymes and to induce systemic resistance in the host plant (Tsai *et al*., 2002). In agriculture, chitosan has been utilised as a soil amendment and foliar spray against plant pathogens (Hadwiger, 1994). It controlled infection of wheat seeds caused by *F. graminearum* under *in vitro* conditions (Reddy *et al*., 1999).

Lack of both stable formulations and knowledge of microbial adaptation to different environments means that the potential of biocontrol agents to control disease under field conditions has not been fully exploited (Emmert & Handelsman, 1999). In this respect, formulations based on spores of Gram-positive microorganisms such as *Bacillus* spp. and *Streptomyces* spp. have advantages over Gram-negative bacteria such as *Pseudomonas*, as they can withstand heat and desiccation stress (Emmert & Handelsman, 1999; Moussa, 2002; Moussa & Rizk, 2002). Vermiculite-based formulations have been shown to increase the survival rate of the biocontrol bacterium *Pseudomonas* sp. (Moenne-Loccoz *et al*., 1999). The addition of chitosan to a formulation of *B. pumilus* increased the effectiveness of this bacterium in controlling *Fusarium* wilt of tomato (Benhamou *et al*., 1998). Also, intra-specific communication between biocontrol *P. putida* strains increased their biocontrol activity (De Boer *et al*., 2003). Therefore, combining different biocontrol bacteria may increase disease control efficacy.

The main objective of this study was to evaluate the efficacy of selected bacterial strains against the wheat soil-borne pathogen *F. graminearum* under greenhouse conditions.

Materials and methods

Isolation and purification of bacteria

One gram of dry soil sample was added to 100 mL of nutrient broth medium (pH 6.6–7.0) in 125-mL Erlenmeyer flask. The mixture was shaken for 3 min and incubated for 6 h at 25°C, and then loopfulls of the resulting suspension were streaked into plates of nutrient agar medium plates and allowed to grow at 30°C. Colonies that were formed after incubation were selected and streaked again on nutrient agar medium to obtain pure cultures. All isolates were maintained on nutrient agar slants at 4°C for the further experiments. Bacteria were purified through the single colony technique used for isolation and purification of the bacterial culture (Salle, 1954). The isolated bacteria were identified according to the International Identification Keys (Parry *et al*., 1983; Stanley *et al*., 1989), and then confirmed by using Biolog system and 16S rRNA gene sequence.

Agar plate-based fungal inhibition assays

Bacterial strains were tested for pathogen inhibition on potato dextrose agar (PDA). *Fusarium graminearum* was maintained on 1× PDA. For inhibition assays, a 5-mm diameter agar plug of a 7-day-old culture of the pathogen was transferred to 1 cm from the edge of a plate of 1/4× PDA and incubated at 22–23°C in darkness. After 24 h, 5 μL from an exponentially growing bacterial culture at OD_{600} of 0.1 was spotted 1 cm from the other edge of the 1/4× PDA plate. The inhibition index was calculated according to the equation described by McSpadden & Weller (2001). Assays were conducted three times, with each strain replicated twice in each assay.

16 S rRNA gene sequence of the *Bacillus* and *Pseudomonas* isolates

The genomic DNA of the *Bacillus* isolate and *Pseudomonas* isolate were extracted following the standard protocol for bacterial genomic DNA preparations (Jasra, 2004). Eighteen hour old Luria–Bertani broth culture was used for the isolation of the DNA. The efficient isolates of *Bacillus* and *Pseudomonas* isolates were characterised and discriminated on the basis of 16S rRNA gene sequence amplified by polymerase chain reaction (PCR) using a set of universal primers; 27f 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492r 5′-GGTTACCTTGTTACGACTT-3′. PCR amplifications were performed in a 25 μL reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 20 pmol of primer, 0.5 U of *Taq* DNA polymerase and 50 ng of genomic DNA. Amplification conditions set for PCR in a thermocycler (Biometra UNO Thermoblock, Goettingen, Germany) were an initial denaturation at 94°C for 6 min, followed by 35 PCR cycles of 94°C for 45 s, annealing at 36°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 10 min was carried out for polishing the ends of PCR products.

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The amplified products were separated on 1.5% agarose gel in Tris borate Ethylenediaminetetraacetic acid (EDTA) buffer.

Greenhouse experiment

Five seeds of *Triticum aestivum* (wheat) cv. Tabuki were planted in plastic pot (25 cm diameter) containing 1.5 kg of sterilised soil. Twenty treatments with three replicates each were used. Seeds were planted in the soil infested with *F. graminearum* only at the rate $\approx 1.3 \times 10^7$ fungal propagules g^{-1} soil (infested control). The seeds were treated with antagonistic bacteria (*Bacillus subtilis* at the rate 7.4×10^8 CFU and/or *P. fluorescens* at the rate 1.4×10^9 CFU), in three forms as seed coating, seed soaking (3 min prior to planting) and soil drench (50 mL pot^{-1}) in absence and presence to fungal pathogen. Finally, seeds were planted free from any treatment with antagonists and/or fungal pathogen (non-infested control).

Seed germination and seedlings were checked daily to determine plant survival and recording any changes in seedling. At the end of the experiment (35 days) wheat plants were removed from the soil. Shoot and root fresh weight and dry weights were determined. Plant, root and shoot lengths also were calculated.

The final germination percent (FGP) and the final infection percent (FIP) were calculated as described by ISTA (1993, 1999). The mean daily germination (MDG), mean daily infection (MDI), the mean germination time (MGT) and mean infection time (MIT) were calculated as described by Moradi *et al*. (2008). The germination index (GI) and the infection index (II) were calculated as described by the Association of Official Seed Analysts (AOSA, 1983).

Statistical analyses

For all determinations, Statistical analyses were carried out including the calculation of the mean, standard deviation, standard error and *t*-value at level $P < 0.05$, according to the method of Armitage (1971). Analysis of variance (*F*-test) 'one-way analysis of variance'. The significance of the measured data was considered as follows, non-significant when $P > 0.05$ and significant when $P < 0.05$, where P is the probability (rejection of null hypothesis) details of formulae used were given by Armitage (1971).

Results

From the results of Table 1, the most potential isolates were the three isolates out of 18, which have numbers of 3, 9 and 10 with *in vitro* inhibition index 42.5%, 41.3% and 46.3%, respectively. The other isolates varied in inhibition index ranged from 0% to 35%.

Table 1 *In vitro* inhibition[a] of *Fusarium graminearum* by isolated bacterial strains[b]

Bacterial isolates	*In vitro* inhibition index[c] (%) ± SE
1	5.0 ± 1.2
2	5.0 ± 1.4
3	42.5 ± 5.0
4	30.0 ± 4.3
5	24.3 ± 2.3
6	35.0 ± 3.8
7	0.0 ± 0.0
8	13.5 ± 0.9
9	41.3 ± 3.6
10	46.3 ± 4.1
11	12.5 ± 2.2
12	25.0 ± 3.8
13	20.0 ± 2.8
14	15.0 ± 1.0
15	15.0 ± 1.1
16	2.5 ± 0.9
17	5.0 ± 0.7
18	25.0 ± 2.3

[a]Inhibition assayed on one-fourth strength potato dextrose agar (1/4 × PDA).
[b]Data are the means and standard errors of three replications.
[c]Inhibition index defined as $[y/(x+y)](100)$, where x is the distance from the centre of the plug to the leading edge of the fungus and y is the distance from the edge of the bacterial colony to the growing edge of the fungus.

On the basis of the above results, the authors selected isolates numbers 3 and 10 for the following experiments. Isolates 3 and 10 were identified according to International Identification Keys (Parry *et al*., 1983; Stanley *et al*., 1989) and was confirmed by using Biolog system and the 16S rRNA gene sequence, which showed that the isolate number 3 was high relatively similar to the published *Bacillus subtilis*. Also, the isolate number 10 was high relatively similar to the published *Pseudomonas fluorescens*. According to the nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and neighbour-joining phylogenetic trees (Saitou & Nei, 1987) (data not shown), the isolate number 3 was assigned as *B. subtilis* MAA03 and the isolate number 10 was assigned as *Pseudomonas fluorescens* MAA10.

Results in Table 2 showed that the using of *B. subtilis* as biocontrol agent against *Fusarium graminearum* on wheat significantly increased the FGP of wheat cultivar relative to infested control. Similarly, the MDG and GI were increased significantly, while the MGT was significantly decreased relative to infested control in all treatments. The FIP was decreased significantly relative to infested control. Also, the MDI and II were significantly decreased, while the MIT was significantly increased relative to

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Table 2 Effect of different treatments of *Bacillus subtilis*, *Pseudomonas fluorescens* and *Bacillus subtilis* + *Pseudomonas fluorescens* mixture on the final germination percent (FGP), mean daily germination (MDG), mean germination time (MGT), germination index (GI), final infection percent (FIP), mean daily infection (MDI), mean infection time (MIT) and infection index (II) of *Triticum aestivum* cv. Tabuki in non-infested and infested soil with *Fusarium graminearum*

Treatment	FGP	MDG	MGT	GI	FIP	MDI	MIT	II
	Bacillus subtilis							
Non-infested control	100.0	3.3	5.6	30.0	0.0	0.0	0.0	0.0
Seed coating	100.0	3.3	5.6	30.0	0.0	0.0	0.0	0.0
Seed soaking	93.0^{**}	3.1^{**}	6.1^{**}	27.6^{**}	0.0	0.0	0.0	0.0
Soil drench	86.0^{**}	2.9^{**}	6.5^{**}	25.6^{**}	0.0	0.0	0.0	0.0
Infested control	53.3	1.8	10.4	16.2	62.9	2.1	16.5	18.9
Seed coating	$80.0^{**\dagger\dagger}$	$2.7^{**\dagger\dagger}$	$7.0^{**\dagger\dagger}$	$24.0^{**\dagger\dagger}$	$25^{**\dagger\dagger}$	$0.8^{**\dagger\dagger}$	$28^{**\dagger\dagger}$	$7.5^{**\dagger\dagger}$
Seed soaking	$73.3^{**\dagger\dagger}$	$2.4^{**\dagger\dagger}$	$7.6^{**\dagger\dagger}$	$22.2^{**\dagger\dagger}$	$27^{**\dagger\dagger}$	$0.9^{**\dagger\dagger}$	$28^{**\dagger\dagger}$	$8.1^{**\dagger\dagger}$
Soil drench	$66.7^{**\dagger\dagger}$	$2.2^{**\dagger\dagger}$	$8.5^{**\dagger\dagger}$	$19.8^{**\dagger\dagger}$	$40.4^{**\dagger\dagger}$	$1.3^{**\dagger\dagger}$	21.5^{**}	$11.8^{**\dagger\dagger}$
1%	6.8	0.1	0.4	3.0	13.4	0.7	10.3	7.5
LSD 5%	4.5	0.07	0.3	2.1	9.2	0.5	7.1	5.1
	Pseudomonas fluorescens							
Non-infested control	100.0	3.3	5.6	30.0	0.0	0.0	0.0	0.0
Seed coating	100.0	3.3	5.6	30.0	0.0	0.0	0.0	0.0
Seed soaking	100.0	3.3	5.6	30.0	0.0	0.0	0.0	0.0
Soil drench	93.0^{**}	3.1^{**}	6.1^{**}	27.6^{**}	0.0	0.0	0.0	0.0
Infested control	53.3	1.8	$10.4^{**\dagger\dagger}$	16.2	62.9	2.1	16.5	18.9
Seed coating	$100.0^{\dagger\dagger}$	$3.3^{\dagger\dagger}$	$5.6^{\dagger\dagger}$	$30.0^{\dagger\dagger}$	$0.0^{\dagger\dagger}$	$0.0^{\dagger\dagger}$	$0.0^{\dagger\dagger}$	$0.0^{\dagger\dagger}$
Seed soaking	$100.0^{\dagger\dagger}$	$3.3^{\dagger\dagger}$	$5.6^{\dagger\dagger}$	$30.0^{\dagger\dagger}$	$0.0^{\dagger\dagger}$	$0.0^{\dagger\dagger}$	$0.0^{\dagger\dagger}$	$0.0^{\dagger\dagger}$
Soil drench	$73.3^{**\dagger\dagger}$	$2.4^{**\dagger\dagger}$	$7.6^{**\dagger\dagger}$	$22.2^{**\dagger\dagger}$	$0.9^{\dagger\dagger}$	$0.9^{**\dagger\dagger}$	$28^{**\dagger\dagger}$	$8.1^{**\dagger\dagger}$
1%	6.8	0.1	0.4	3.0	13.4	0.7	10.3	7.5
LSD 5%	4.5	0.07	0.3	2.1	9.2	0.5	7.1	5.1
	Bacillus subtilis + *Pseudomonas fluorescens*							
Non-infested control	100.0	3.3	5.6	30.0	0.0	0.0	0.0	0.0
Seed coating	100.0	3.3	5.6	30.0	0.0	0.0	0.0	0.0
Seed soaking	100.0	3.3	5.6	30.0	0.0	0.0	0.0	0.0
Soil drench	100.0	3.3	5.6	30.0	0.0	0.0	0.0	0.0
Infested control	53.3	1.8	10.4	16.2	62.9	2.1	16.5	18.9
Seed coating	$100.0^{\dagger\dagger}$	$3.3^{\dagger\dagger}$	$5.6^{\dagger\dagger}$	$30.0^{\dagger\dagger}$	$0.0^{\dagger\dagger}$	$0.0^{\dagger\dagger}$	$0.0^{\dagger\dagger}$	$0.0^{\dagger\dagger}$
Seed soaking	$93.0^{**\dagger\dagger}$	$3.1^{**\dagger\dagger}$	$6.1^{**\dagger\dagger}$	$27.6^{**\dagger\dagger}$	$21.8^{\dagger\dagger}$	$0.7^{**\dagger\dagger}$	$28^{**\dagger\dagger}$	$6.4^{*\dagger\dagger}$
Soil drench	$80.0^{**\dagger\dagger}$	$2.7^{**\dagger\dagger}$	$7.0^{**\dagger\dagger}$	$24.0^{**\dagger\dagger}$	$33.3^{\dagger\dagger}$	$1.1^{**\dagger\dagger}$	$21.5^{**\dagger\dagger}$	$9.8^{**\dagger\dagger}$
1%	6.8	0.1	0.4	3.0	13.4	0.7	10.3	7.5
LSD 5%	4.5	0.07	0.3	2.1	9.2	0.5	7.1	5.1

*, significant at 5% relative to non-infested control; **, significant at 1% relative to non-infested control; †, significant at 5% relative to infested control; ††, significant at 1% relative to infested control. LSD, Least Significant Difference.

infested control in all treatments. Seed coating was the best treatment followed by seed soaking and finally soil drench.

The using of *P. fluorescens* as biocontrol agent against *F. graminearum* significantly increased the FGP of wheat cultivar Tabuki relative to infested control. Similarly, the MDG and GI were increased significantly, while the MGT was significantly decreased relative to infested control in all treatments. The FIP was completely suppressed in seed coating and seed soaking treatments, while significantly decreased in case of soil drench treatment relative to infested control. Therefore, MDI and II were null in the two treatments (seed coating and seed soaking), and significantly decreased in case of soil drench treatment. Also, MIT was null at both treatments (seed coating and seed soaking), and significantly increased in case of soil drench treatment relative to infested control. Seed coating was the best treatment followed by seed soaking and finally soil drench (Table 2).

Also, results in Table 2, revealed that the using of *B. subtilis* and *P. fluorescens* as a mixture for control of *F. graminearum* infection on wheat significantly increased the FGP of wheat cultivar relative to infested control. Similarly, the MDG and GI were increased significantly, while the MGT was significantly decreased relative to infested control in all treatments. The FIP was completely suppressed in seed coating treatment, while significantly decreased in case of seed soaking and soil drench treatments relative to infested control. Therefore, MDI and II were null in case of seed coating treatment,

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and significantly decreased in case of seed soaking and soil drench treatments, also, MIT was null in seed coating treatment, and significantly increased in case of seed soaking and soil drench treatments relative to infested control. Seed coating was the best treatment followed by seed soaking and finally soil drench.

The application of *B. subtilis* significantly affected the growth parameters of wheat cultivar Tabuki, the root length was significantly increased in seed coating and seed soaking treatments relative to infested control and significantly decreased in case of seed soaking treatment relative to non-infested control, while non-significantly decreased in case of soil drench treatment, but significantly increased in relation to non-infested control. Shoot length was significantly decreased in case of seed coating treatment relative to infested control. Subsequently, the root/shoot (R/S) ratio varied with different treatments of *B. subtlilis*, R/S was increased in seed coating treatment and decreased in case of seed soaking and soil drench treatments. The shoot fresh and dry weights were significantly increased in all treatments in relation to infested control, but decreased in relation to non-infested control. The root fresh and dry weights were significantly increased in all treatments in relation to infested and non-infested control. The number of leaves was significantly increased in all treatments relative to infested control (Table 3).

The application of *P. fluorescens* significantly affected the growth parameters of wheat cultivar Tabuki, the root length was significantly increased in all treatments relative to infested control and significantly decreased in case of seed soaking and soil drench treatments relative to non-infested control. Shoot length was significantly decreased in case of seed coating and seed soaking treatments relative to infested control. Subsequently, R/S ratio varied with varying *P. fluorescens* treatments, R/S was increased in seed coating treatment and decreased in case of seed soaking and soil drench treatments. The shoot fresh and dry weights were significantly increased in case of seed coating and seed soaking treatments in relation to infested and non-infested controls, but decreased in relation to infested and non-infested controls in case of soil drench treatment. Also, the root fresh and dry weights were significantly increased in case of seed coating and seed soaking treatments in relation to infested and non-infested controls, but in case of soil drench treatment was significantly decreased in relation to infested and non-infested controls. The number of leaves was significantly increased in all treatments relative to infested control (Table 3).

The application of different treatments of mixture of *B. subtilis* + *P. fluorescens* significantly affected the growth parameters of wheat cultivar Tabuki, the root length was significantly increased in all treatments relative to infested control and significantly decreased in all treatments relative to non-infested control. Shoot length was significantly decreased in case of seed coating treatment and increased in seed soaking and soil drench relative to infested control. Subsequently, R/S ratio varied with varying mixture of *B. subtilis* + *P. fluorescens* treatments; R/S was increased in all treatments relative to infested control. The shoot fresh and dry weights were significantly increased in case of seed coating and seed soaking treatments in relation to infested control, where significantly decreased in relation to non-infested control. They were decreased in relation to infested control and non-infested controls in case of soil drench. The root fresh and dry weights were significantly increased in case of seed coating treatment in relation to infested and non-infested controls, but in case of seed soaking and soil drench treatments were significantly decreased in relation to infested and non-infested controls. The number of leaves was significantly increased in all treatments relative to infested control (Table 3).

Discussion

Naturally occurring antagonistic microorganisms may play an important role in suppressing plant diseases. Several fungi and bacteria have been tested for their effectiveness to control a variety of plant pathogens (Moussa, 2002). Bacteria, including *Pseudomonas*, *Pantoea* and *Bacillus*, have been used as seed bacterisation with reasonable effect against wheat seedling blight caused by *F. graminearum* and *F. culmorum* (Johansson *et al*., 2003; Khan *et al*., 2006).

The most potential isolates were 3 out of 8 isolates, which have numbers 3, 9 and 10 with *in vitro* inhibition index 42.5%, 41.3% and 46.3%, respectively. The other isolates varied in inhibition index ranged from 0% to 35%.

All rhizobacterial isolates exhibited strong antifungal activities against maize root pathogens (Pal *et al*., 2001). However, inhibitory effects were more prominent on PDA than on NA. The nutrient constituent of the medium plays a significant role in influencing the production of a particular antifungal metabolite (Hebbar *et al*., 1992*b*) by the antagonistic rhizobacteria. The differences in the inhibitory effect on the fungal pathogens might be due to the nutritional differences of the two media. Similar observations were also reported earlier with *Pseudomonas cepacea* (Hebbar *et al*., 1992*a*). The possible mechanisms by which fluorescent *Pseudomonas* and bacilli exhibit biocontrol have been reported (Bull *et al*., 1991; Dowling & O'Gara, 1994; Cartwright *et al*., 1995; Emmert & Handelsman, 1999).

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Table 3 Effect of different treatments of *Bacillus subtilis*, *Pseudomonas fluorescens* and mixture of *Bacillus subtilis* + *Pseudomonas fluorescens* on the different growth parameters of *Triticum aestivum* cv. Tabuki in non-infested and infested soil with *Fusarium graminearum*

Treatment	Length (cm)			R/S	Shoot wt. (g)		Root wt. (g)		Average no. leaves/plant
	Plant	Root	Shoot		Fresh	Dry	Fresh	Dry	
	Bacillus subtilis								
Non-infested control	24.7	17.3	7.4	2.34	0.66	0.09	0.16	0.07	3.3
Seed coating	23.7*	16.7**	7.0**	2.39	0.95**	0.13*	0.37**	0.17**	3.3
Seed soaking	23.7*	12.0**	11.7**	1.03	0.87**	0.10	0.32*	0.03**	3.3
Soil drench	20.0**	9.0**	11.0**	0.82	0.63	0.07	0.26*	0.15**	3.0*
Infested control	22.0	11.0	11.0	1.0	0.45	0.04	0.15	0.04	2.7
Seed coating	24.7††	17.0††	7.7**††	2.21	0.57**††	0.08†	0.42**††	0.16**††	3.3††
Seed soaking	23.7*††	12.7**†	11.0**	1.15	0.49**†	0.07†	0.40**††	0.21**††	3.3††
Soil drench	22.5††	10.3**	11.7**	0.88	0.61*††	0.08†	0.30*†	0.17**††	3.0*†
1%	1.4	1.6	3.1	–	0.07	0.05	0.2	0.03	0.5
LSD	0.9	1.0	2.0	–	0.04	0.03	0.1	0.02	0.3
5%									
	Pseudomonas fluorescens								
Non-infested control	24.7	17.3	7.4	2.34	0.66	0.09	0.16	0.07	3.3
Seed coating	26.7**	19.3	7.4	2.61	0.91**	0.11	0.28*	0.11**	3.3
Seed soaking	23.3**	16.0*	7.3	2.19	0.81*	0.09	0.18	0.08	3.3
Soil drench	21.7**	14.0**	7.7**	1.82	0.49*	0.08	0.21	0.08	3.0*
Infested control	22.0	11.0	11.0	1.0	0.45	0.04	0.15	0.04	2.7
Seed coating	24.7††	18.0*††	6.7††	2.69	0.87**††	0.11††	0.51**††	0.18**††	3.7*††
Seed soaking	22.3**	14.3**††	8.0**††	1.79	0.86**††	0.11††	0.55**††	0.28**††	3.3†
Soil drench	26.7**††	15.0**††	11.7**	1.28	0.28**†	0.03**	0.07	0.02**††	3.0*†
1%	1.4	1.6	3.1	–	0.2	0.05	0.2	0.03	0.5
LSD	0.9	1.0	2.0	–	0.1	0.03	0.1	0.02	0.3
5%									
	Bacillus subtilis + *Pseudomonas fluorescens*								
Non-infested control	24.7	17.3	7.4	2.34	0.66	0.09	0.16	0.07	3.3
Seed coating	23.7*	16.3**	7.4**	2.20	0.80**	0.11	0.20**	0.14**	3.3
Seed soaking	21.7**	14.3**	7.4	1.93	0.61*	0.08	0.17*	0.09*	3.0*
Soil drench	21.3**	10.0**	11.3**	0.89	0.52**	0.06*	0.12**	0.04**	3.0*
Infested control	22.0	11.0	11.0	1.0	0.45	0.04	0.15	0.04	2.7
Seed coating	23.3**†	16.3**	7.0**	2.33	0.57**††	0.07†	0.19**††	0.08††	3.3††
Seed soaking	22.3**	14.0**††	8.3†	1.69	0.55**††	0.07†	0.15*	0.06†	3.0*†
Soil drench	21.0**	11.3*††	9.7*††	1.16	0.27**††	0.03**	0.02**††	0.02**†	3.0*†
1%	1.4	1.6	3.1	–	0.07	0.05	0.02	0.03	0.5
LSD	0.9	1.0	2.0	–	0.04	0.03	0.01	0.02	0.3
5%									

*, significant at 5% relative to non-infested control; **, significant at 1% relative to non-infested control; †, significant at 5% relative to infested control; ††, significant at 1% relative to infested control. LSD, Least Significant Difference.

The authors selected isolates numbers 3 and 10 for the following experiments. Isolates 3 and 10 were identified as *B. subtilis* MAA03 and *P. fluorescens* MAA10, respectively according to International Identification Keys (Parry *et al.*, 1983; Stanley *et al.*, 1989) and was confirmed by using Biolog system and the 16S rRNA gene sequence.

The Biolog system performed best with oxidase-positive fermenters and biochemically active non-fermenters and had the most problems with unreactive non-fermenters (Holms *et al.*, 1994). The most valuable advantage of the Biolog technique in ecological research is the rapid differentiation of large numbers of isolates by means of their metabolic profiles (Rüger & Krambeck, 1994). The Biolog system was efficient in the identification of the isolates to the species level, as 53 of the 56 (94.6%) isolates of *Pseudomonas syringae* pv. *tomato* were identified as *P. syringae* (Shenge *et al.*, 2008).

These strains exhibited more than 99.5% sequence identity and within experimental uncertainty could be regarded as identical. Their close taxonomic relationship was further documented by phenotypic similarities. A PCR test based on the 16S rRNA gene was set up that could identify any of the five species of the '*Bacillus subtilis* group' (*B. subtilis, B. pumilus*, *B. atrophaeus*, *B. licheniformis* and *B. amyloliquefaciens*). The test was directly applicable to single colonies and showed excellent specificity (Wattiau *et al.*, 2001). 16S rDNA-based molecular identification could achieve identification, for reasons including its universal distribution among bacteria and the presence of species-specific variable regions (Drancourt *et al.*, 2000).

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16S rRNA genes have been applied as one of several analytical methods in a polyphasic study of the pseudomonads. Nearly complete sequences have been determined of the PCR-amplified 16S rRNA genes of 21 species of the genus *Pseudomonas* (*sensu stricto*), including multiple strains of most species (Moore *et al*., 1996*a*,*b*). For bacteria belonging to the genus *Pseudomonas*, data collected on 16S rDNA genes make taxonomical studies possible, including those in the rhizosphere. As a result of such studies, many organisms classified as species of this genus have been reclassified (Kersters *et al*., 1996).

Application of the compatible plant growth-promoting rhizobacteria either singly or in combinations effectively suppressed the disease severity, or increased the percent disease reduction, although there was no variation among the treatments (Pal *et al*., 2001).

Our results indicate that the pathogen under the given inoculum concentration (1.3×10^7 fungal propagules g^{-1} soil) may not cause the severe damage of the seed embryos until 2 days. Galli *et al*. (2005) also observed that the germination was not reduced, but that seed vigour decreased under laboratory conditions after maize seeds had been incubated on agar plates colonised by *F. graminearum* for 32 h. It has been reported that most *Fusarium* spp. may not reduce seed germination rate, but cause decay of the seed and arrest the development of the radicles (Munkvold & O'Mara, 2002). However, there is also a report showing that *F. graminearum* infection reduces germination rate in cereals (Brodnik, 1975).

The application of *B. subtilis* and *P. fluorescens* separately or in mixture as biological control agents (BCAs) against *F. graminearum* on wheat significantly increased FGP, MDG and GI of wheat cultivar, while MGT was significantly decreased relative to infested control. FIP, MDI and II were decreased significantly, while MIT was significantly increased relative to infested control.

A plant growth-promoting isolate of a fluorescent *Pseudomonas* sp. EM85 and two bacilli isolates MR-11(2) and MRF, isolated from maize rhizosphere, were found strongly antagonistic to *F. moniliforme*, *F. graminearum* and *Macrophomina phaseolina*, causal agents of foot rots and wilting, collar rots/stalk rots and root rots and wilting, and charcoal rots of maize, respectively (Pal *et al*., 2001). Abo-Elyousr *et al*. (2009) concluded that by combining the BCAs with resistance inducers, there was increased consistency of suppression of root rot of cotton seedlings caused by either *F. oxysporum* or *P. debaryanum*.

Cropping systems and soil properties influence both detrimental and beneficial microorganisms in the rhizosphere, which subsequently impact root health, plant vigour and crop yield (Rovira *et al*., 1990; Hornby & Bateman, 1997). Strains of rhizobacteria with ability to reduce severity of root diseases of cereal crops have been selected for field application to increase crop productivity (Lemanceau & Alabouvette, 1993; Hornby & Bateman, 1997). However, performance of selected rhizobacteria introduced into some field soils for disease suppression has been very inconsistent (Thomashow & Weller, 1996).

Despite several reports on the degree of suppression of maize root diseases caused by *F. graminearum*, *F. moniliforme* and *M. phaseolina*, by different rhizobacteria like *Bacillus* spp. (Kommendahl & Chang, 1975), *P. fluorescens* (Raju *et al*., 1999), *Pseudomonas* spp. (Chen *et al*., 1999), *P. cepacea* (Hebbar *et al*., 1992*a*,*b*) and *Burkholderia cepacea* UPR5c (Sanchez *et al*., 1994).

The application of *B. subtilis* and *P. fluorescens* separately or in combination significantly affected the growth parameters of wheat cultivar Tabuki, the root length was significantly increased in seed coating and seed soaking treatments relative to infested control. Shoot length was significantly decreased in seed coating treatment relative to infested control. The shoot fresh and dry weights were significantly increased in seed coating and seed soaking treatments relative to infested control. The root fresh and dry weights were significantly increased in seed coating and seed soaking treatments relative to infested control. The number of leaves was significantly increased in all treatments relative to infested control.

Growth parameters were adversely affected confirming the infection process. After 60 days of planting the root rot disease severity had progressed as a result of infection with *Fusarium* isolates. Plant height, fresh weight and number of branches were significantly reduced after inoculation with most of *Fusarium* species (Hashem *et al*., 2010). A clear correlation was observed between plant fresh weight and the degree of disease severity suggesting that infection with *F. oxysporum* was the main growth limiting factor in all inoculated plants (Reuveni *et al*., 2002).

Infection of the roots with pathogenic necrotrophic fungi of the genus *Fusarium*, in contrast, leads to necrotised roots and severe reduction of root and shoot biomass. Upon infestation with *P. indica*, roots were protected from *Fusarium* infections as evidenced by reduced root rot symptoms. *In vitro* analysis of the interaction of *P. indica* and *F. graminearum* under axenic culture conditions did not reveal reciprocal growth inhibition suggesting that retardation of *Fusarium* in roots is mediated by a plant response rather than by antibiosis (Deshmukh & Kogel, 2007).

Conclusion

The combinations of fluorescent pseudomonas and bacilli could suppress the wheat root invading pathogens efficiently. Fluorescent pigment and antifungal antibiotics (or metabolites) of *P. fluorescens* and antifungal antibiotics

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of *B. subtilis* coupled with successful root colonisation of the BCAs might be involved in biological suppression of the pathogens.

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