ORIGINAL ARTICLE

Biological control of *Fusarium graminearum* on wheat by antagonistic bacteria

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Abstract

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***Bacillus subtilis* strains 53 and 71, *Pseudomonas fluorescens* biov1 strain 32 and *Streptomyces* sp. Strain 3 were evaluated as potential biological agents for control of fusarium head blight (FHB) caused by *Fusarium graminearum*. Mycelial growth of the pathogen was reduced by cell free and volatile metabolites of bacterial antagonists by 37%-97%. *Streptomyces* sp. Strain 3 reduced disease severity of FHB 21 d after inoculation. The yield of wheat from plants treated with *Streptomyces* sp. strain 3 and *F. graminearum* was significantly greater than in controls inoculated with the pathogen alone. Treatments with *Streptomyces* sp. alone increased the yield of wheat compared to the uninoculated controls.**

Key words : *Fusarium graminearum*, fusarium head blight, biological control, wheat, *Bacillus subtilis*, *Streptomyces*, *Pseudomonas fluorescens*

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Fusarium head blight is a fungal disease of wheat found in both temperate and semi-tropical regions. A number of species of *Fusarium* may be responsible but generally *F. graminearum* and *F. culmorum* predominant. Both fungi cause root rot, food rot, crown rot, stem rot and head blight in wheat. Head blight causes reduced kernel set and kernel weight, destruction of starch granules and storage proteins and seed infection. The fungus first infects the extruded anthers and then ramifies throughout the developing caryopsis, floral bracts, and rachis. The severity of infection is correlated with the percentage of retained anthers .The fungus may also infect by direct penetration of glume, palea, or rachilla. Soon after infection, dark brown, water- soaked spots appear on the glumes of infected spikelets. Later, entire spikelets become blighted. Under favorable conditions, the fungus extends through the rachis, externally of internally, and infects other spikelets. If the weather is warm and humid, conspicuous aerial mycelium spreads externally from the spikelets originally infected to adjacent spikelets. If the fungus spreads internally, brownish chlorotic symptoms extends down into in culm and up to the top of spike, and as a results the entire spike may die and turn the color of straw. Visible pink mold appears on the spike when it is humid. Later in the season, perithecia of the fungus may appear as raised purplish black spot. Infected florets often fail to produce grain, or the grain they produce is poorly filled. Wheat is generally most susceptible to infection at the flowering stage, and susceptibility declines at later stages of caryopsis development. Some cultivars, however, may be most susceptible at the milk or soft dough stages. Head Infection in nature can occur anytime after beginning of flowering, when temperature and moisture are favorable. Anthesis may be period of greatest susceptibility, because the high level of choline and betaine produced in the extruded anthers stimulate the growth of *F. graminearum* and promote the infection of wheat spikes by the pathogen (Bai and Shaner,1994). The fusarium head blight fungi produce a large number of secondary metabolites, not all of which have been characterized including the trichothecenes deoxynivalenol (vomitoxin) and nivalenol. Fusarium toxins are harmful to human and animal health. The acute symptoms of trichothecene poisoning are characterized by skin irritation, food refusal, vomiting, diarrhea, hemorrhage, neural disturbance, miscarriage and death (Joffe, 1986). Human toxicosis due to ingestion of mycotoxin-contaminated food is well documented (Bhat *et al*., 1989). Chronic ingestion of small amount of trichothecens may result in an important secondary effect, the predisposition to infectious disease through suppression of the immune system (Miller and Atkinson, 1987). Precaution should be taken to avoid inhalation of mycotoxin- containing spores and dust and direct skin contact with infected kernels (Trenholm *et al*., 1989). The control of fusarium head blight has relied on the resistant varieties and use of fungicides. Resistant cultivars are very rare and application of fungicides may be used for control of fusarium head blight.

Biological control has been explored as an additional or alternative means of managing fusarium head blight. A range of bacterial isolates obtained from rhizosphere and kernel of wheat was reported in a previous study (Stockwell *et al*., 1997, 1999).

The aim of this research was to evaluate the potential of *Pseudomonas fluorescens* biov1 strain 32, *B. subtilis* strain 71 and 53 and *Streptomyces* sp. strain 3 for biological control of *F. graminearum*.

Materials and Methods

Fungal isolates and bacterial antagonist

Two isolates of *F. graminearum* 79-3 and 165 obtained from diseased wheat kernel in Mazendaran province of Iran were used *in vitro* but the mixture of the two isolates was used in glasshouse condition. These isolates were maintained on Potato Dextrose Agar (PDA). The healthy kernel samples that had been harvested in wheat field of Golestan province were used to screen the bacterial isolates. Twenty kernels of each field were mixed and were placed in 1 L water peptone (1%) in 2 L flask. The flasks were incubated on an orbital shaker at 100 rpm at 20ºC for 30 min. Bacterial isolates were isolated

with a serial dilution (Mclean and Cook, 1941). Two hundred µL aliquots of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions were plated out on selective media. For isolation of *Sreptomyces, Pseudomonas* and *Bacillus*, casein glycerol medium (CGM) (Kuster and Williams, 1964), King B and Nutrient agar (NA) were used, respectively. For Isolation of Bacillus, the bacterial suspensions were placed in a water bath at 70ºC for 10 min. before dilution (Shaad *et al*., 2001). In the primary test of 289 bacterial isolates which were isolated from wheat kernels, three bacterial isolates *Pseudomonas fluorescens* biov1 strains 32, *Streptomyces* sp. strain 3 and *Bacillus subtilis* strain 71 with inhibition zone of more than 15 mm against *F. graminearum* were selected and *B. subtilis* strain 53 with size of inhibition zone of 5 mm was also chosen for comparison. Based on the biochemical, physiological, nutritional characteristics and also the comparison of electrophoretic profiles of cell protein of bacterial isolates tested and the type strains, these four isolates of bacteria were identified. The bacterial isolates were maintained in sterilized distilled water (SDW) and incubated at 4ºC in darkness (Shaad *et al*., 2001).

Effect of *P. fluorescens, B. subtilis* and *Streptomyces* sp. *in vitro*

Dual culture (Dennis and Webster, 1971b) and cellophane overlays (Dennis and Webster, 1971a) were used to observe the effect of bacterial antagonist on *F. graminearum*. All antagonist-pathogen-pathogen combinations were examined on 10-15 mL of PDA in 9 cm petri dish with 4 replications. For dual culture, half of the agar surface was smeared with a suspension of one bacterial isolate in sterile distilled water using a sterile cotton bud. After incubation at 28ºC in the dark for 24 h for *Pseudomonas* and *Bacillus* and 48 h for *Streptomyces*, a plug (8 mm diam.) cut from the leading edge of 3 day-old culture of *F. graminearum* on PDA agar medium was placed on the other half of the plate. For control, PDA agar was inoculated with pathogen alone. The plates were incubated at 25ºC in the dark for 10 d when the inhibition zones were measured. The inhibition zone was a straight line measured from the bacterial antagonist to the edge of the *F. graminearum* colony. For the cellophane overlay technique, cellophane membranes (Australia Cellophane, Victoria) of 9 cm diameter were boiled in distilled water, interleaved with filter papers and autoclaved. A cellophane membrane was placed on the agar in each petri plate and dried in a laminar flow cabinet for 15 min. A 48-h old culture of bacterial isolate, suspended in SDW, was smeared over the entire surface of the cellophane. For the controls, SDW was applied. The plates were incubated in the dark at 25ºC for 48 h after which the cellophane membrane with adhering bacterial culture was removed. One 8-mm diameter plug of *F. graminearum* growing on PDA was placed in the center of the plate previously occupied by the antagonist. The Plates were incubated at 25ºC in the dark for 10 d when colony diameters were measured. The surface area of the *F. graminearum* colonies was recorded daily, compared with the controls and percentage inhibition of growth calculated. The pathogen colony diameter was the average of two measurements and the area was calculated. The percent growth inhibition was calculated using the formula $n = (a-b)/a * 100$ where n = % growth inhibition, a = colony area of uninhibited *F. graminearum* and b = colony area of treatment. For antifungal activity of volatiles, a petri dish containing PDA medium was inoculated by spreading about 200 µL of a suspension of bacterial cells, prepared from a 72 h nutrient agar. After inoculation at 25ºC for 24 h a second petri dish (containing PDA) was inoculated with a 5-mm diameter plug of the *F. graminearum* in the center of the plate, inverted over the bacterial culture. The two plates were sealed together with Nescofilm and further incubated at 25ºC. This ensured that both organisms were growing in the same atmosphere. Care was taken to ensure that the surface of the fungal culture did not become contaminated with the bacterium. For the control, instead of bacterium, SDW was spread on agar surface (Fiddaman and Rosall, 1993). The surface area of the colonies *F. graminearum* was recorded as above, compared with the controls and the

percentage of growth inhibition calculated.

Biological control of *F. graminearum* on wheat in glasshouse condition

B. subtilis 71, *P. fluorescens* biov1 strain 32 and *Streptomyces* sp. strain 3 with great inhibition zone *in vitro* and *B. subtilis* with medium inhibition zone against *F. graminearum* were investigated for their ability to reduce the incidence of head blight in wheat. Ten seeds of susceptible wheat cultivar Falat were sown in autoclaved potting mix consisting of two parts compost and one part of field soil in 20 cm diameter plastic pots. Before sowing, seeds were surface-disinfected by soaking in 1% sodium hypochorite for 1 min then rinsed three times in sterile distilled water (SDW). There were 4 replicate pots per treatment, arranged in complete randomized design. Treatments were: *Streptomyces* strain 3 + *F. graminearum, B. subtilis* strain 71 + *F. graminrarum, B. subtilis* strain 53, *Pseudomonas fluorescens* biov1 strain 32 + *F. graminearum, Streptomyces* strain 3, *B. subtilis* strain 71, *B. subtilis* strain 53, *P. fluorescens* biov1, healthy control and *F. graminearum* only. Plants were maintained at constant temperature of 15ºC without supplementary lighting in October in Tehran, Iran, and then transferred to a glasshouse at constant temperature of 20ºC with a 14 h photoperiod of light. Bacterial antagonist were grown on PDA for 48 h then bacterial cells were washed with 10 mL of SDW. The concentration of bacterial suspension was adjusted to 10^9 using a spectrophotometer. Two isolates of *F. graminearum* were grown on PDA (90 mm diam.) for 14 d. Hyphae and conidia were harvested by pouring a few mL of sterile water (0.05% Tween 20) on the plates. The concentration of spores in the inoculum was approximately 2×10^5 spores/mL of the mixture of the two isolates of *F. graminearum*, but hyphal fragment concentration was not determined. Inoculation with bacterial antagonist began when the main spikes emerged from the boot and was repeated 10 d later.

Inoculation with *F. graminearum* began at six h after the bacterial inoculation and continued every other evening for 10 d. Immediately after each inoculation, the plants were misted with overhead mister. The inoculum of bacteria or *F. graminearum* was applied with a sprayer at about 7 mL per spike.

Head blight was evaluated by the severity, the number of necrotic spikelets in each spike divided into the number of spikelets in each spike 21 d after inoculation (Bekele *et al*., 1994). The weight of 100 grain per replication was determined at harvesting time.

The experiment was arranged as randomized complete design with 4 replications. Analysis of variance and Duncan's Multiple Range Test was used to determine differences among treatments (Little and Hills, 1978).

Results

Effect of bacterial strains on mycelial growth of *F. graminearum in vitro*

All bacterial antagonists tested inhibited mycelial growth of *F. graminearum* in dual culture. However, there were significant differences among the bacterial strains. Growth inhibition of *F. graminearum* by *P. fluorescens* biov 1 strain 32 was significantly greater than that of the other strains (Figure 1), but *B. subtilis* strain 53 had less effect on the growth of the pathogen.

Cell free metabolites of *B. subtilis* strain 71 and 53, *P. fluorescens* biov 1 strain 32, *Streptomyces* sp strain 3 and *B. subtilis* strain 53 reduced growth of *F. graminearum* by 97, 97, 87.5 and 51.5%, respectively (Figure 2).

The results shown in Figure 3 indicated that the antifungal activity of volatile metabolites of the bacterial strains was variable. Percentage reduction in growth of *F. graminearum* with *B. subtilis* strain 53 was significantly greater than that of the other bacterial antagonists tested. Antifungal activity of *Streptomyces* strain 3 was less than the others.

Biological control of *F. graminearum* on wheat in glasshouse condition

Disease severity in plants inoculated with the pathogen and *Streptomyces* strain 3 was significantly less than the pathogen control, but there were no significant differences between

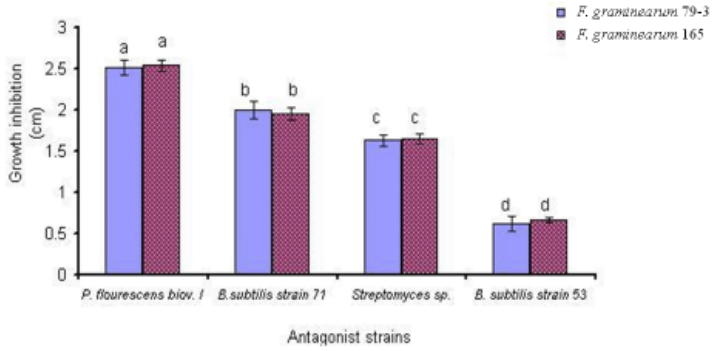

Figure 1. Effect of antagonist strains on growth inhibition of *Fusarium graminearum* isolates 79-3 and 165 (dul culture). Treatments with the same letters do not differ significantly (p<0.05) according to Duncan's Multiple Range Test. The vertical bars represent standard deviation with four replicates.

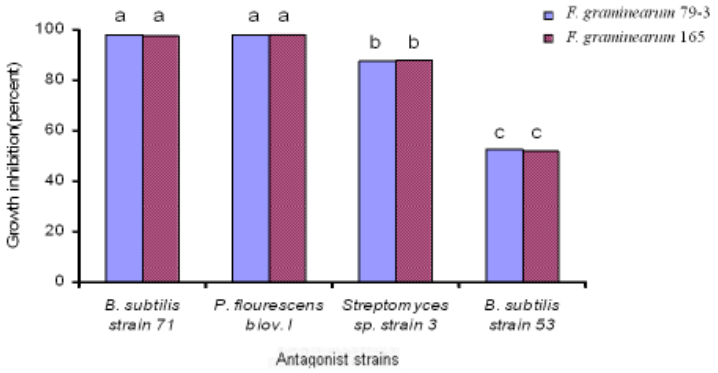

Figure 2. Effect of cell-free metabolites of bacterial strains on growth inhibition of *Fusarium graminearum* isolates 79-3 and 165 (cellophane method). Treatments with the same letters do not differ significantly (p<0.05) according to Duncan's Multiple Range Test. The vertical bars represent standard deviation with four replicates.

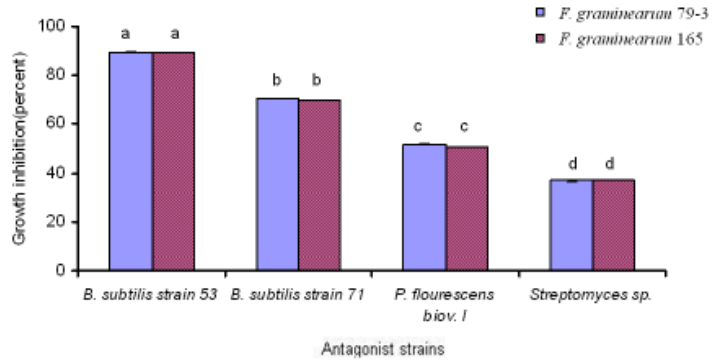

Figure 3. Effect of volatile metabolites of bacterial strains on growth inhibition of *Fusarium graminearum* isolates 79-3 and 165. Treatments with the same letters do not differ significantly (p<0.05) according to Duncan's Multiple Range Test. The vertical bars represent standard deviation with four replicates.

treatment with pathogen control and treatments with other bacterial strain plus *F. graminearum* (Figure 4). The yield of wheat from plants treated with *F. graminearum* and *Streptomyces* strain 3 was significantly greater than of the control inoculated with pathogen alone. Treatments with *Streptomyces* sp. strain 3 alone increased the yield of wheat compared with the uninoculated control (Figure 5).

Discussion

In the dual culture experiments, all bacterial isolates tested inhibited the growth of *F. graminearum*. Zones of inhibition were observed between the colonies of pathogen and bacteria. The inhibition zone could be due to the effect of diffusible inhibitory substances produced by the bacteria, which suppressed the growth of *F. graminearum*. The presence and size of the zone of inhibition have been used as evidence of the production of antibiotics by the bacteria (Rothrock and Gottlieb, 1981, Jackson *et al*., 1991, Crawford *et al*., 1993). Another possibility is that the bacterial isolates depleted the nutrient in the agar surrounding them and thereby inhibited the growth of *F. graminearum*. Hsu and Lockwood (1969) found that the inhibition zone produced by none

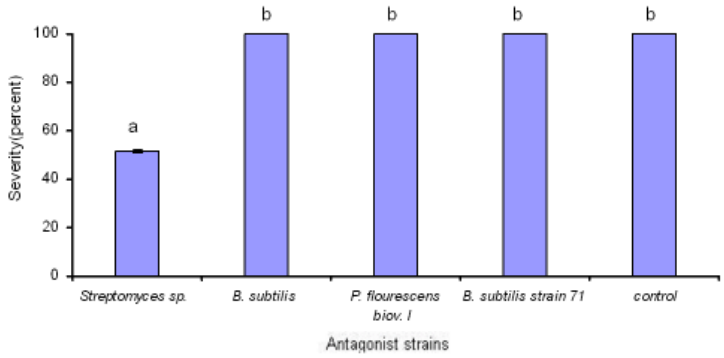

Figure 4. Effect of bacterial strains on fusarium head blight severity. Treatments with the same letters do not differ significantly (p<0.05) according to Duncan's Multiple Range Test. The vertical bars represent standard deviation with four replicates.

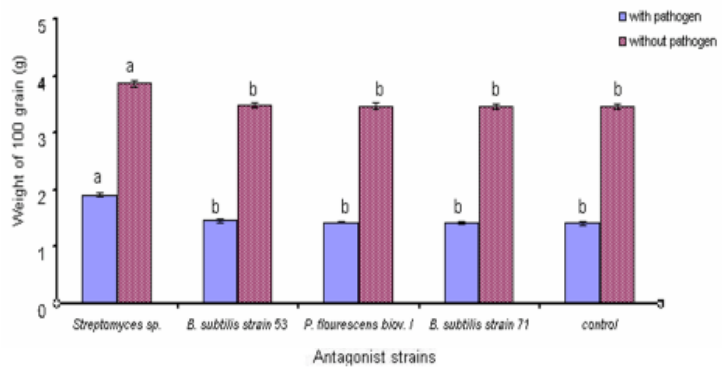

Figure 5. Effect of bacterial strains plus *Fusarium graminearum* and bacterial strains only on weight of 100 wheat grains. Treatments with pathogen or without pathogen with the same letters do not differ significantly (p<0.05) according to Duncan's Multiple Range Test. The vertical bars represent standard deviation with four replicates.

antibiotic-producing *Streptomyces* were due to the rapid depletion of glucose and glutamic acid to suboptimal level in agar media adjacent to the *Streotomyces* colonies. Cell-free metabolites produced by four different isolates of bacteria reduced colony area of the pathogen by 51.97-98.16%. Even though the cellophane overlay technique has been used mainly for investigating non-volatile metabolites of bacteria (Jackson *et al*., 1991) and also fungi (Dennis and Webster, 1971a), preliminary evidence suggested that the involvement of nutrient depletion should be considered. It was not possible to extract and determine the antibiotic substances from *P. flourescens, B. subtilis* and *Streptomyces* tested in this investigation, but other investigators reported some antibiotics produced by these antagonists. For examples, the mode of action of *Streptomyces* spp. appeared to be antagonism by the production of tubercidin, phosphlactomycin and candicidin (Hwang *et al*., 1994; Fushimi *et al*., 1989; Lechevalieet *et al*., 1953). *P. flourescens* produces a wide range of antibiotic such as phenasin (Mazzolla *et al*., 1992), 2,4-diacetylphloroglucinol (Shanahan *et al*., 1992).

The results of volatile metabolite activity indicated that percentage reduction in the growth of *F. graminearum* with *B. subtilis* strains ranged from 51% to 89%. These results closely parallel those of Fiddaman and Rossal (1993) on *Bacillus* volatile antagonizing *Rhizoctonia solani* and *Pythium ultimum*. Two groups of antibiotics were produced by *B. subtilis*, one group consisting of several antibacterial compounds, and the other consisting of only two antifungal components. One of these antifungal compounds is known to be bacillomycin (Landy *et al*., 1948) .The other antibiotic produced by *B. subtilis* is fengymcine and proved to be different from the other compounds. It was less toxic to the test plants and protected them better from Rhizoctonia disease (Loefler *et al*., 1986)

Inoculation of wheat in the greenhouse showed that treatments with *Streptomyces* reduce fusarium head blight severity. In addition to antibiotics production by *Streptomyces*, Bochow and Fritzche (1991) reported that the effectiveness of *Streptomyces* isolates against *Phtophthora infestans* was do to the induction of host resistance by the antagonists. *Streptomyces* spp. have been implicated as effective biological control agents (Jones and Samac 1996; Liu *et al*., 1995; El-abyad *et al*., 1993; Etebarian *et al*., 2003).

The results of head blight test showed that *Bacillus* strains did not reduce disease severity. However, a recent published work (Luz, 2000) showed that the best isolates of *B. megatherium* (Embr.9790) and *B. subtilis* (Embr.9786) significantly diminished the disease incidence and severity up to 50% and 67%, respectively. The reasons for these differences in sensitivity are not immediately apparent. It is possible that different isolates of fungus may respond to different strains of *Bacillus*, or that variation in antifungal activity may reflect differences in the site of action or the ability of fungi to detoxify the metabolites. It may have also been due to the inactivation of the antibiotics in the environment and application method because *Bacillus* strain is applied as vegetative cells.

Streptomyces isolates significantly reduced the percentage of scabby spikelets when tested under greenhouse conditions and frequently reduced the disease in field trails as well, even though they may not have inhibited growth in the laboratory assay. This suggests that greenhouse evaluation for biocontrol of scab is more reliable as an early selection method than *in vitro* assays in large-scale screening of bioprotectants (Luz, 2000).

Some workers have been investigating antagonists to control FHB under greenhouse or field conditions and some bacterial strains have reduced FHB severity and significantly reduced vomitoxin contamination in grains (Stockwell *et al*., 1997, 1999).

In the absence of *F. graminearum, Streptomyces* isolates significantly increased the yield of wheat, perhaps due to the production of growth regulators such as gibberellin-like substance and auxines (Katznelson and Cole, 1965).

Future research will involve studies on the application of biological agents to the ears of wheat

at flowering such as the timing of the application, inoculation technology, physiological state of the organisms, spike colonization, survival of organism under the harsh environmental conditions, variability of biocontrol from year to year, fermentation, formulation, and storage of the antagonists. Since wheat grains are usually stored for a long time after harvesting, it seems that the antibiotics produced by the antagonists are inactivated and may not be hazardous to consumers. Several microbial agents have been used for biological control of postharvest diseases, even on fresh fruits (Janisewiz and Kartsten, 2002). For example, *P. fluorescens* isolate A506 is a commercially available microbial pesticide registered on pome fruit for the control of fire blight caused by *Erwinia amylovora* (Stockwell *et al*., 2002). The other commercial biocontrol products were registered in the US and are sold as Aspire with a yeast active ingredient for biological control of postharvest disease of apple (Droby *et al*., 1998). Some strains of *Streptomyces* spp are also commercially available as microbial pesticides registered on some crops for biological control of some disease (Jones and Samac, 1966). However, biological agents tested in this study should be investigated extensively for food safety before commercialization.

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