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SHORT COMMUNICATION

Efficacy and dose–response relationship in biocontrol of Fusarium disease in maize by *Streptomyces* spp.

Wellington Bressan · Jose Edson Fontes Figueiredo

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Abstract Two isolates of *Streptomyces* spp. DAUFPE 11470 and DAUFPE 14632 were evaluated to determine the antagonist–pathogen inoculum concentration relationship under greenhouse conditions. Pathogen and antagonist concentration, significantly ($P<0.05$) affected development of Fusarium disease in maize with a significant interaction between pathogen and antagonist concentration. Dose–response relationship also differed significantly ($P<0.05$) between the two isolates, but both isolates demonstrated effective control of Fusarium disease, regardless of pathogen concentration. The isolate DAUFPE 11470 provided the most effective control. The lowest value for disease incidence occurred at low pathogen (10^3 chlamydospore g^{-1} soil) and high antagonist concentration (10^6 cfu ml^{-1}) for both isolates. The disease incidence for control plants ranged from 19% to 76%. However, in relation to control the lowest disease reduction occurred at low pathogen (10^3 chlamydospore g^{-1} soil) and high antagonist concentrations (10^6 cfu ml^{-1}). These reductions were 10.6% and 13% for DAUFPE 14632 and DAUFPE 11470, respectively. The highest disease reductions, in relation to control plants, occurred at high pathogen (10^6 chlamydospore g^{-1} soil) and antagonist (10^6 cfu ml^{-1}) concentrations for both isolates. These values were 55% and 62.2% for DAUFPE 14632 and DAUFPE 11470, respectively. The chlamydospore germination of *Fusarium moniliforme* was affected by glucose addition, antagonist isolates and type of inoculum. The lowest chlamydospore germination was observed with bacterial suspensions of the isolates, for all glucose additions. The results suggested that both *Streptomyces* spp. isolates were effective at different doses as biocontrol agents against *F. moniliforme*. Also, there was evidence for at least two mechanisms of biocontrol and that apparently, both isolates showed the same mechanisms of biocontrol action related to production of bioactive compounds and competition for carbon. Further studies will be developed to improve the level and effectiveness of control by these isolates.

Keywords Biocontrol · *Fusarium moniliforme* · Maize · Seedborne diseases · *Streptomyces*

Fusarium moniliforme (*Giberella fujikuroi*), commonly infects a wide range of crops and is a major parasite of the Gramineae, particularly in tropical and subtropical regions. On maize (*Zea mays*) the fungus causes seedling blight as well as root, stalk, ear and kernel rot (Gauperin et al. 2003). A variable pathogenicity of the fungus has been reported with additional pathogen and stress factors (Velluti et al. 2000). Chlamydospores are survival structures for many soil fungi and are usually produced under certain environmental conditions. The fungus overwinters as

W. Bressan (✉) · J. E. F. Figueiredo
Microbiology Department, Embrapa Milho e Sorgo,
MG 424, Km 65,
35701-970 Sete Lagoas, MG, Brazil
e-mail: bressan@cnpms.embrapa.br

chlamydospore-like structures and mycelium on plant debris or crop residue (Nyvall and Kommedahl 1970) and mycelium on seed. The infection may occur early in the season (possibly at the seedling stage as a result of planting infected seed) and the fungus grows systemically, producing symptoms during the later stages of plant development or the infection occurs later in the growing season. The infection process occurs when the fungus invades host tissue directly or through wounds. Common points of entry are roots and stalks at the base of leaf sheaths.

Biological control of soilborne plant pathogens has been shown to be a potential alternative disease management strategy. These agents not only have the potential to protect seeds but they colonize the rhizoplane or rhizosphere when added as a seed treatment, and may protect the subterranean portions of growing plants from attack by plant pathogens (Ahmad and Baker 1987). Actinomycetes are naturally present in soils and when tested in *in vitro* condition strains of the genus *Streptomyces* have shown the potential to produce antibiotics which reduce or inhibit the growth and development of soilborne plant pathogens (Kim et al. 2000; Ouhdouch et al. 2001; Bressan 2003). The degree of disease control obtained depends on the density of the biocontrol agent, density of the pathogen, efficiency of the biocontrol agent in suppressing the pathogen, and proportion of the pathogen population that is potentially affected by the agent (Montesinos and Bonaterra 1996; Smith et al. 1997). Differences in the mechanism of action of the biocontrol agents also affect the dose–response relationship of the isolates (Larkin and Fravel 1999). Knowledge on the relationships between biocontrol agent and pathogen inoculum concentration can determine the population levels of the biocontrol agent that are required to achieve adequate disease control, as well as the pathogen population levels at which the control agent will or will not be effective. The objective of this work was to determine the biocontrol agent–pathogen inoculum concentration relationship on biocontrol of Fusarium disease in maize by two *Streptomyces* spp. isolates and elucidate the possible mechanism of biocontrol of these isolates.

The effect of the biocontrol isolates on chlamydospore germination of the pathogen *F. moniliforme* in soil was primarily assessed using a buried membrane filter technique according to Larkin and Fravel (1999). Chlamydospores were produced on casein hydrolysate (0.2%) liquid medium for 10 days on a rotary shaker at 135 rpm at 25°C and propagule suspensions were adjusted to 1×10^3 chlamydospores ml^{-1}. Biocontrol soil inoculum of *Streptomyces* spp. isolates DAUFPE 11470 and DAUFPE 14632 consisted of bacterial suspension (10^6 cfu ml^{-1}) and cell-free filtrate. The biomass of the biocontrol *Streptomyces* spp. isolates DAUFPE 11470 and DAUFPE 14632 was produced in a liquid culture. Biomass of each strain was prepared in five 250 ml Erlenmayer flasks containing 100 ml of medium (Kawamura et al. 1976). The flasks were placed on an orbital shaker at 180 rpm for 72 h at 28°C. After biomass inoculum production, one half of the bacterial inoculum volume of each strain was centrifuged, filtered through sterilized filter paper, collected in sterile tubes and used as a cell-free filtrate inoculum. The remaining half of the bacterial inoculum was used as a bacterial suspension inoculum. Bioassay tests carried out in plates containing the medium ISP2 (Pridham et al. 1956) showed that that no viable propagules were found in the cell-free filtrate. Glucose solution was added to sterilized soil at concentration ranging from 0 to 0.6 mg g^{-1} of soil to stimulate chlamydospore germination and to simulate the nutrients provided by root exudates. The inoculated filters were buried in 150 g of sterilized soil inoculated with 10 ml of cell-free filtrate or bacterial suspension and incubated in a moist chamber for 30 h at 25°C. Control consisted of filters inoculated with *F. moniliforme* buried in sterilized soil without *Streptomyces* spp. strains inoculation. After incubation the filters were removed, rinsed, and stained with trypan blue–lactophenol. The percentage of chlamydospore germination was counted under ×200 magnification. The trial was repeated twice.

Greenhouse evaluation of the dose–response relationship on biological control of Fusarium disease was made in pots containing sterilized sandy–loam soil arranged in a completely randomized design experiment with the following treatments: 4 seed antagonist treatments with *Streptomyces* spp. isolates (1×10^3, 1×10^4, 1×10^5 and 1×10^6 cfu ml^{-1}), 4 pathogen soil treatments (inoculation with *F. moniliforme* at 1×10^3, 1×10^4, 1×10^5 and 1×10^6 chlamydospore g^{-1} soil). Soil inoculum consisted of propagules of *F. moniliforme* on talc powder obtained by the method of Locke and Coulhoun 1974. Control included seeds treated only with *Streptomyces* spp. isolates planted in soil

inoculated with the pathogen. Five replicates were used for each treatment. Each replicate consisted of five plants. Maize (*Zea mays*), seeds of the commercial cultivar BR201 were surface disinfected by immersion in a solution of commercial bleach of 5.15% NaClO and 0.5% Tween 20 for 5 min and rinsed five times in sterile deionized water under agitation. *Streptomyces* spp. isolates inoculation was made by placing surface disinfected maize seeds in beakers containing each biomass inoculum, shaking continuously for 24 h at 28°C followed by air-drying of the seeds. Non-inoculated seeds were shaken under the same conditions but soaked in sterile deionized water. Disease was monitored for 30 days after planting and assayed as the total percentage of seedlings showing any symptoms of Fusarium disease (yellowing, dropping of leaves or vascular discolouration). Disease incidence was confirmed by plating slices cut from lower stem and roots from diseased seedlings, surface-disinfected in 1% sodium hypochlorite, on Komadas Fusarium-selective medium (Komada 1975). The experiment was repeated twice. All data were analysed using SAS (SAS Institute Inc. 1990). Statistical significance was determined at $P<0.05$.

In this study, both antagonist isolates of *Streptomyces* spp. demonstrated significant effects in reducing disease incidence at low and high antagonist and pathogen concentrations, respectively (Fig. 1). All pathogen and antagonist concentrations, significantly ($P<0.05$) affected development of Fusarium disease of maize with a significant interaction between pathogen and antagonist concentrations (P×A) (Table 1). Treatments with each of the two biocontrol isolates (DAUPPE 11470 and DAUFPE 14632) significantly ($P<0.05$) reduced disease compared with the control plants. When no antagonist was present, disease incidence ranged from 19% to 76% with increasing pathogen concentrations (Fig. 1). The level of disease control provided by the antagonist isolates differed between the two biocontrol isolates in relation to increasing pathogen concentration. Although the disease incidence increased with pathogen concentration for both antagonist isolates and concentrations, it was significantly lower than the control, regardless of the pathogen concentration. Differences in efficacy and dose–response relationship between isolates indicated the importance of using different antagonist and pathogen concentrations for the success of the

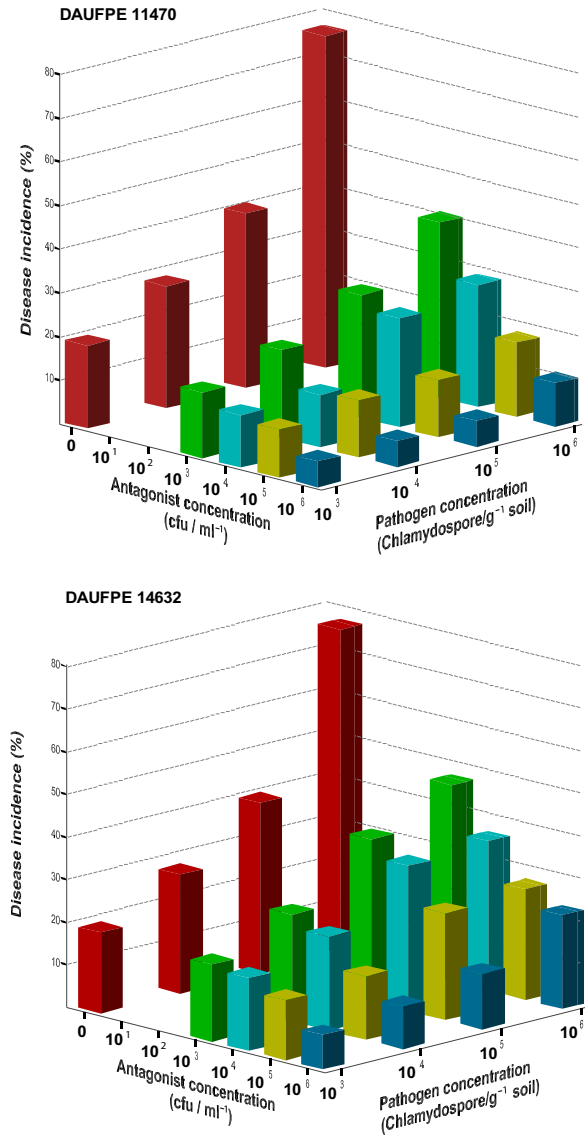

Fig. 1 Effect of varying pathogen (*F. moniliforme*) and antagonist (*Streptomyces* spp. isolates DAUFPE 11470 and DAUFPE 14632) inoculum concentrations on the development of Fusarium disease in maize

biological control under varying environmental conditions (Raaijmakers et al. 1995; Larkin and Fravel 1999).

The highest disease reduction was obtained when the seeds were inoculated with antagonist isolate DAUFPE 11470 regardless of the pathogen concentration (Fig. 1). In considering the pathogen concentration in the soil, the lowest disease reduction, in relation to control plants (soil inoculated with low pathogen concentration and no antagonist inoculation)

Table 1 Factorial analysis of variance for pathogen *F. moniliforme* and antagonist *Streptomyces* spp. isolate concentrations and interactions on development of Fusarium disease in maize

Source of variation	*df*	MS	
		Antagonist isolate	
		DAUFPE 11470	DAUFPE 14632
Pathogen concentration (P)	3	2409.0*	3266.0*
Antagonist concentration (A)	4	3416.48*	2307.7*
P×A	12	469.77*	381.08*
Error	80	8.61	13.91

Probabilities <0.05 are followed by single asterisks.
df: degrees of freedom, *MS*: mean square.

was 13% and the highest was 62.2% when the soil and the seeds were inoculated with high concentrations of pathogen and antagonist, respectively. However, the lowest Fusarium disease incidence was observed at the lowest pathogen concentration in the soil and at highest antagonist concentration (Fig. 1). At the lowest pathogen concentration only the seed inoculation with antagonist at 1×10^3 did not differed significantly ($P<0.05$) from the control plants. Increasing soil pathogen concentration from 1×10^3 to 1×10^4 chlamydospore g^{-1} soil and antagonist a concentration from 1×10^4 to 1×10^5 cfu ml^{-1} resulted in no significant differences ($P<0.05$) in disease incidence. However, the incidence of Fusarium wilt was lower than the control in this treatment. At the highest pathogen concentration all seed treatments with antagonists differed significantly ($P<0.05$) from the control plants in disease incidence (Fig. 1).

For isolate DAUFPE 14632, the lowest reduction in Fusarium wilt in relation to control plants (soil inoculated with low pathogen concentration and no antagonist inoculation) was 10.6% (Fig. 1). The highest reduction (55%) occurred at high antagonist and pathogen concentration when the control consisted of soil inoculation with high pathogen concentration and no antagonist-inoculated seeds. However, the lowest disease incidence was observed in the treatment with high antagonist concentration and low pathogen concentration (Fig. 1). At pathogen concentration of 1×10^3, 1×10^4 and 1×10^5 chlamydospores g^{-1} soil, no significant ($P<0.05$) reduction in disease incidence, in relation to control plants, was observed when increasing antagonist concentration from 1×10^3 to 1×10^4 cfu ml^{-1}. At the highest pathogen concentration of 10^6 chlamydospore g^{-1} soil no significant differences ($P<0.05$) was observed in disease incidence at antagonist concentration of 1×10^5 and 1×10^6.

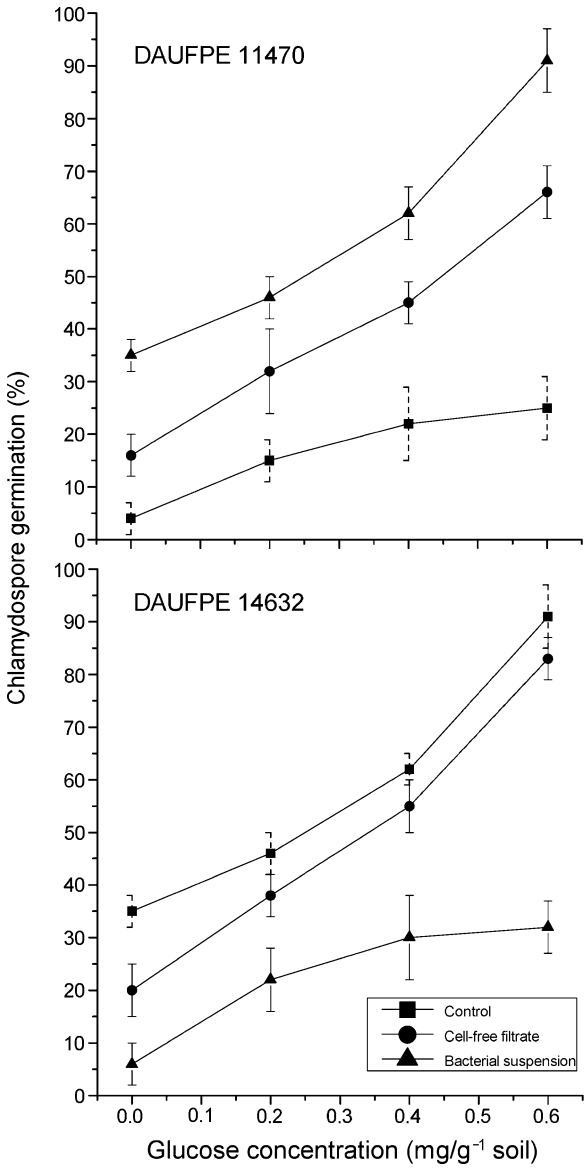

Fig. 2 Effect of cell-free filtrate and bacterial suspension of the *Streptomyces* spp. isolates, DAUPE 11470 and DAUFPE 14632, on chlamydospore germination of *F. moniliforme* at various glucose concentrations. Data represent combined results from two experiments. Bars indicate 95% confidence limits

Addition of glucose to the soil increased chlamydospore germination in all treatments. The germination of the chlamydospores of *F. moniliforme* was higher in the control treatment (non-inoculated soil) than inoculation with bacterial suspension or cell-free filtrate in all glucose treatments for both *Streptomyces* spp. isolates. These isolates inhibited *F. oxysporum* chlamydospore germination in all glucose concentrations compared with the control treatment. Cell-free suspension of the isolate DAUFPE 14632, however, did not differ significantly ($P<0.05$) from the control at glucose concentrations ranging from 0.2 mg g^{-1} soil to 0.6 mg g^{-1} soil. The lowest chlamydospore germination occurred with the bacterial suspension of the biocontrol DAUFPE 11470 or DAUFPE 14632 isolates (Fig. 2). Compared to the control treatment the reduction in chlamydospore germination by DAUFPE 11470 ranged from 32% to 65% for 0.0 and 0.6 mg of glucose g^{-1} soil, respectively. This reduction by DAUFPE 14632 ranged from 28% to 60% for the same glucose concentration. Although a difference in biocontrol efficacy has been observed between the isolates DAUPE 11470 and DAUFPE 14632, both apparently showed the same mechanisms of action. Research has indicated the importance for glucose competition and other carbon compounds as an active mechanisms of biocontrol (Larkin and Fravel 1999; Raaijmakers et al. 1995). However, *Streptomyces* spp. isolates have shown the potential to produce bioactive compounds (Huddleston et al. 1997; Mukhopadhyay et al. 1999; Ouhdouch et al. 2001; Gesheva 2002) and to reduce or inhibit many seed fungi (Bressan 2003). The data obtained indicated that production of bioactive compounds contributed to the mode of action for *Streptomyces* spp. isolates as observed in Fig. 2. However, in the treatment with bacterial suspension the chlamydospore germination was lower than the cell-free filtered. The addition of glucose should have contributed to production of bioactive compounds by isolates DAUFPE 11470 and DAUFPE 14632. The substrates that support production of bioactive compounds or diffusible inhibitors are either transiently available or highly localized, and the inhibitors themselves can be absorbed onto soil components, degraded or inactivated when they diffuse away from the producer organisms. It follows that, irrespective of the precise mode of action, interactions between biocontrol agents and pathogens are likely to occur at close range.

The results for both isolates showed that the control of Fusarium disease in maize is affected by the pathogen/antagonist interaction and the dose–response study used in this work was useful for characterizing the differences in the inoculum concentration relationships between the biocontrol isolates and to evaluate the effectiveness of the isolates in controlling Fusarium disease in maize. DAUFPE 11470 was the most desirable biocontrol isolate for Fusarium disease and will be used in further studies for the improvement of its effectiveness as a biocontrol agent. The data suggest that production of bioactive compounds by *Streptomyces* spp. isolates and competition for nutrients contributed to the biocontrol action. The results indicated that this study may be applied to biocontrol situations, in which the most important criteria for characterizing disease and biological control are the inoculum concentration of the pathogen and biocontrol agent.v

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