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Spectrum of biocontrol bacteria to control leaf, root and vascular diseases of dry bean

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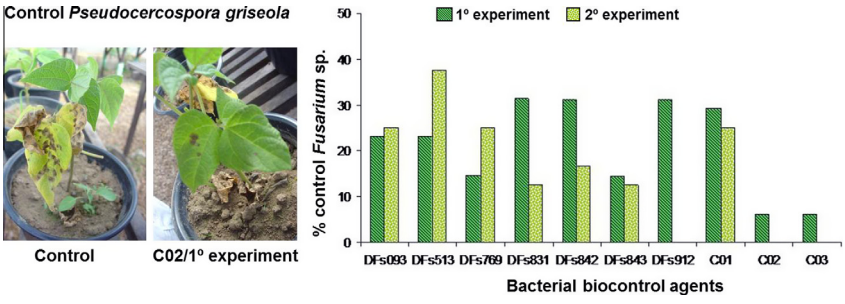
HIGHLIGHTS

- The bacteria studied have the potential to control fungal and bacterial diseases.
- The bacteria studied have the potential to control foliar and stem/vascular diseases.
- The combination of these bacteria diversifies and intensifies the effect that bacteria produce individually.

GRAPHICAL ABSTRACT



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ABSTRACT

Bacterial biocontrol agents, previously selected to control bacterial blight and anthracnose, were evaluated for the control of bacterial wilt, fusarium wilt, charcoal rot and angular leaf spot of dry beans. The seeds were microbiolized with these bacteria singly or in combinations. The microbiolization resulted in reduction of severity of all four diseases, showing wide spectrum of diseases control by these bacteria. However, the severity reduction of all four diseases by combination C01 composed of isolates DFs093 and DFs769 of *Bacillus cereus* and DFs831 of *Pseudomonas fluorescens* was significantly higher than if the seeds were microbiolized by a single isolate of a biocontrol agent.

1. Introduction

Brazil is one the major dry bean (*Phaseolus vulgaris* L.) producers with annual production of nearly 3 million tons harvested from about 2 million ha. The crop has high social and economical significance, especially for subsistence farmers. The yield per unit area is very low due to high incidence pathogens and their of several leaf, root and vascular diseases. Major diseases that attack bean crops in Brazil are anthracnose, leaf rust, angular leaf spot, common bacterial blight, bean golden mosaic, sclerotinia white rot, charcoal rot, and fusarium and bacterial wilt. Although, growing resistant cultivars is recommended to control these diseases, most of the cultivars are susceptible, and sometimes the resistance is short lived due to high variability many of these pathogen's (Maringoni and Lauretti, 1999). The chemical control of leaf diseases is effective, but small farmers do not use it frequently due to resource constraints. The integrated management of the plant diseases aims to increase yields at reduced cost, and biological control to complement existing control measures (Mader et al., 2002).

Efficient control of some bean diseases such as common bacterial blight (Zanatta et al., 2007; Corrêa, 2007), rust (Centurion and Kimati, 1994; Mizubuti et al., 1995), halo leaf blight (Garret and Schawrtz, 1998), anthracnose (Corrêa et al., 2008), angular leaf

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spot (Garcia, 2008), charcoal rot and fusarium wilt (Martins et al., 2003) by bacterial biocontrol agents has been reported in greenhouse trials. However, results in the field are generally inconsistent especially due to sensitivity of the biocontrol agents to climatic fluctuations (Pierson and Weller, 1994).

The use of a mixture of compatible biocontrol agents with multiple mechanisms of action, can be effective under a wider range of climatic condition, thus reducing some of limitations of biocontrol activity in the field. Such mixtures can potentially have synergistic effects, which may result in higher level of protection and wider spectrum of diseases that can be controlled (Guetsky et al., 2001; Boer et al., 2003).

The previous studies showed the potential of select bacterial isolates to control *Xanthomonas axonopodis* pv. *phaseoli* (Smith) Vauterin and of *Colletotrichum lindemuthianum* (Sacc & Magn.) Lams & Scrib. on dry bean (Zanatta et al., 2007; Corrêa et al., 2008). The following study was done to evaluate the same isolates, individually and also in combination to control bacterial wilt (*Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Hedges) Collins & Jones), angular leaf spot (*Pseudocercospora griseola* (Sacc.) Ferraris), charcoal rot (*Macrophomina phaseolina* (Tassi) Goidanich) and fusarium wilt (*Fusarium oxysporum* Schlecht. sp. *phaseoli* Kendrick & Snider) through seed microbiolization.

2. Materials and methods

2.1. Bacterial isolates

The bacterial isolates used in this study were previously selected to control of *X. axonopodis* pv. *phaseoli* (Zanatta et al., 2007) and *C. lindemuthianum* (Corrêa et al., 2008), and are part of the culture collection of the Plant Protection Department, Pelotas Federal University – Brazil (Table 1).

2.2. Cultivation of bacteria and seed microbiolization

All bacterial isolates were grown for 24 h on the medium 523 (Dhingra and Sinclair, 1995), and then the cells were suspended in sterile saline solution (NaCl 0.85%). The suspension was not washed or centrifuged and the cell concentration was adjusted to absorbance of $A_{540} = 0.50$ (10^{-8} UFCmL^{-1}).

The combinations of isolates were prepared by mixing equal volumes of the individual bacterial suspensions, and the cell concentration was adjusted to absorbance of $A_{540} = 0.50$. The combination C01 consisted of isolates DFs093 + DFs769 + DFs831, the C02 of isolates DFs093 + DFs769 + DFs842, and C03 contained isolates DFs769 + DFs348 + DFs831.

The bean seeds 'BRS Valente' were microbiolized by immersing and agitating for five hours at 10 °C in the cell suspension (20 seeds/20 mL). Control seeds were treated similarly in sterile saline solution without bacterial cells. Five seeds from each treatment were then planted in non-sterile soil in pots (2L), and after seedling emergence only one plant was left in each pot.

All experiments were repeated twice. Each assay was performed in a greenhouse where the temperature and humidity are not controlled. The tests were carried out at favorable temperature: Cff 37,2 °C and 35,2 °C, Fop 32,6 °C, Mp 35,1 °C, Pg 26,7 °C in the first and second assays respectively. The humidity was maintained using wet plastic bags over each pot, 24 h before and after the pathogens inoculations.

Table 1
Identity and the ecological niche of the biocontrol bacteria used in the study.

Isolate	Identity*	Ecological Niche
DFs093	*Bacillus cereus* Frankland & Frankland	Soil
DFs348	*Bacillus* sp. Cohn	Onion leaf
DFs513	*Pseudomonas veronii* Elomari	Onion scales
DFs769	*B. cereus* Frankland & Frankland	Snap beans
DFs831	*P. fluorescens* Migula	Bean Rhizosphere
DFs842	*P. fluorescens* Migula	Bean leaf
DFs843	*Rhodococcus fascians* Goodfellow ex. Tilford	Bean leaf
DFs912	*R. fascians* Goodfellow ex. Tilford	Bean leaf

* Determined by sequencing of gene 16S rDNA (unpublished data).

2.3. Spectrum of biocontrol agents for disease control

Four species of plant pathogens listed in Table 2 were used to evaluate the spectrum of biocontrol. All they were prior tested for pathogenicity.

2.3.1. Fusarium wilt (F. oxysporum sp. phaseoli – Fop)

Two isolates were grown on potato dextrose agar (PDA) in culture plates (Dhingra and Sinclair, 1995) and incubated for 7 days under constant light at 25 ± 2 °C. The aqueous suspension containing micro and macro conidia was prepared according to Nascimento et al. (1995). Bean seedlings, 10-days after emergence, were exposed to each isolate, separately, by pouring 5 mL of the in conidial suspension in each of two holes made in the soil around the plants (Cavalcanti et al., 2002). After 15 days the disease severity was evaluated according to Nascimento et al. (1995).

2.3.2. Charcoal rot (Macrophomina phaseolina-Mp)

The inoculum of the fungus was prepared according to Abawi and Pastor Corrales (1990) and the soil was infested with 2 g of this inoculum before seed planting. The disease severity was evaluated 15, 20 and 25 days after seeding according to Abawi and Pastor-Corrales (1990), and the disease progress was calculated.

2.3.3. Angular leaf spot (P. griseola - Pg)

The fungus was cultivated on tomato paste-$CaCO_3$ agar (Dhingra and Sinclair, 1995) and the conidia were washed off with plates the sterile saline solution. The plants were inoculated 10 days after emergence by spraying the conidial suspension (2×10^4/mL), and then placed in a moist chamber for 24 h before transferring to greenhouse bench. The disease severity was quantified 10, 12, 14 and 16 days after inoculation according to Godoy et al. (1997), and the disease progress curve was calculated.

2.3.4. Bacterial Wilt (Curtobacterium flacumfaciens pv. flacumfaciens – Cff)

Each five isolates of Cff were cultivated for 48 h on medium 523 (Dhingra and Sinclair, 1995) at 28 °C. The cell suspension was prepared in the sterile saline solution not washed or centrifuged and the cell concentration was adjusted to absorbance of $A_{540} = 0.20$ (10^2 cfumL^{-1}).

The seedlings were inoculated 15 days after emergence, by puncturing the hypocotyls at two points between the cotyledonary and the primary leaves, followed by depositing 10 μL of the suspension. The wilt disease symptoms were evaluated after 15 days according to Maringoni (2002).

2.4. Experimental design and statistical analysis

All experiments were performed under greenhouse conditions at the Universidade Federal de Pelotas (UFPel), in Pelotas, Rio Grande do Sul, Brazil (level 13, 24 m, 31° 52′ 00″ S and 52° 21′ 24″ W). The experimental design was completely randomized.

Charcoal rot and angular leaf spot diseases were evaluated in more than one time in each experiment (Mp e Pg) had their area under disease progress curve (AUDPC) calculated by using GwBasic

Table 2
Origin and source of the bean pathogens used in this study.

Pathogen	Code	Origin	Donor	Institution
Curtobacterium flacumfaciens pv. *flacumfaciens*	Cff GO	Goiás	A.C. Maringoni	Unesp
	Cff SP	São Paulo	A.C. Maringoni	Unesp
	Cff MG	Minas Gerais	A.C. Maringoni	Unesp
	Cff DF	Distrito Federal	A.C. Maringoni	Unesp
	Cff SC	Santa Catarina	A.C. Maringoni	Unesp
Pseudocercospora griseola	Pg	São Paulo	M.F. Ito	IAC
Macrophomina phaseolina	Mp	São Paulo		USP/Esalq
Fusarium sp.	Fop CA	Rio Grande do Sul		UFPel
	Fop SC	Santa Catarina		UFPel

Institutions- Unesp (Universidade Estadual Paulista), IAC (Instituto Agronomico de Campinas), USP (Universidade de São Paulo), UFPel (Universidade Federal de Pelotas).

program (Maffia, 1995). AUDPC data were submitted to one-way variance analysis. The severity of the common bacterial wilt and Fusarium wilt diseases (evaluated only once and for multiple isolates, Cff and Fop) were submitted to two-way variance analysis.

The data of AUDPC and severity were submitted for significant means Duncan's multiple range tests ($P = 0.05$) that were applied by using the SASM-Agri (Canteri et al., 2001).

3. Results

In general, there was some control of different diseases by all bacterial biocontrol agents, individually or in combination, in both experiments. However, the extent of control differed significantly among the isolates, combinations and disease.

There was no interaction between the biocontrol agents and two *F. oxysporum* f. sp. *phaseoli* isolates, therefore the results were analyzed separately. The control of wilt caused by the isolate Fop CA varied between 14.3 and 43% and between 10 and 50% in the first and second experiment, respectively while the control of the disease caused by the isolate Fop SC ranged between 6.2 and 31.2 and 0 and 28.6%, respectively (Table 3). The disease severity caused by the isolate Fop CA was signicantly reduced by eight and nine biocontrol treatments in the first and second experiment, respectively, with mean reduction of 32.2%. In the first experiment, the maximum severity reduction was shown by the biocontrol isolate DFs093 (43%), while in the second experiment, the treatment with the combination C01 resulted in maximum disease severity reduction (50%). Averaging the data from the two experiments revealed that the combination C01 had the greatest reduction of disease severity (43.6%). However, decline of disease severity caused by the isolate Fop SC was lower (19.1%), even though the disease severity in the control plants was also lower than that caused by the isolate Fop CA. In this case, only four microbiolization treatments significantly differed from the control in both experiments, and the combination C01 was most stable (mean 28.6%).

The control of charcoal rot averaged 22.8% (Table 3), with eight and six treatments being significantly effective in the first and the second experiments, respectively. The symptom severity in control plants was higher in the first experiment but the extent of control and the number of treatments providing significant control was also higher. In both experiments the maximum disease control was imparted by the biocontrol isolate DFs843 (mean 42.6%).

Although the percent reduction of AUDPC of angular leaf spot was high in some treatments in the first experiment (Table 3), the degree of disease control was higher in the second experiment, with the mean control of 64.8% imparted by the isolate DFs769 and the combinations C01 and C02 which was significant only in experiment 2.

In general the combination C01 was most effective and stable in controlling the fungal diseases. The control was significant in seven out of eight opportunities evaluated (four diseases in two experiments).

Table 3
Effect of microbiolization of bean seeds on the severity of fusarium wilt (Fop) and on the area under the disease progress curve of charcoal rot (MP) angular leaf spot (Pg) bean plants in two experiments.

Treatments[I]	Fop CA[II] Experiment		Fop SC[II] Experiment		Mp[II] Experiment		Pg[II] Experiment	
	1	2	1	2	1	2	1	2
DFs093	2.0c[IV]	2.0bc	2.5ab	2.0b	61.2c	52.5cd	7.5cde	8.3b
DFs513	2.2bc	2.0bc	2.5ab	1.7b	45.0e	50.0cd	4.1def	5.6bc
DFs769	2.2bc	2.0bc	2.7ab	2.0b	85.0a	72.5a	2.5ef	2.6d
DFs831	2.2bc	2.0bc	2.2b	2.2ab	52.5d	55.0bc	5.7cdef	4.0cd
DFs842	2.5bc	2.0bc	2.2b	2.2ab	35.0f	50.0cd	10.3bc	7.4b
DFs843	3.0ab	2.7a	2.7ab	2.2ab	35.0f	45.0d	8.4cd	6.1bc
DFs912	2.2bc	1.7bc	2.2b	2.7a	85.0a	73.5a	14.7ab	14.2a
C01	2.2bc	1.5c	2.2b	2.0b	52.5d	52.5c	2.9ef	2.4d
C02	2.5bc	2.2b	3.0ab	2.7a	68.7b	55.0bc	1.7f	2.4d
C03	3.0ab	2.0bc	3.0ab	2.7a	50.0d	52.5c	16.3a	7.0bc
Control	3.5a	3.0a	3.2a	2.7a	85.0a	61.2b	6.6cdef	7.0bc
CV (%)[III]	17.9	14.8	17.9	18.3	4.9	7.8	49.6	31.7

[I] Bacterial treatments DFs093 (*Bacillus cereus*), DFs513 (*Pseudomonas veronii*), DFs769 (*Bacillus cereus*), DFs831 (*Pseudomonas fluorescens*), DFs842 (*Pseudomonas fluorescens*), DFs843 (*Rhodococcus fascians*) and DFs912 (*Rhodococcus fascians*), C01 = DFs093 + DFs769 + DFs831; C02 = DFs093 + DFs769 + DFs842; C03 = DFs348 + DFs769 + DFs831. Control: seeds treated with saline solution (NaCl 0.85%).

[II] Phytopathogen Fop CA (*Fusarium oxysporum phaseoli* isolate Cascata), Fop SC (*F. oxysporum phaseoli* isolate Santa Catarina), Mp (*Macrophomina phaseolina*) and Pg (*Phaeoisariopsis griseola*). Severity of fusarium wilt (Fop CA and SC) was assessed once, 15 days after inoculation. Severity of charcol rot (Mp) and angular leaf spot (Pg) were evaluated respectively 15, 20, 25 days and 10, 12, 14, 16 days after inoculation and AUDPCs were calculated.

[III] CV: Coefficient of variation.

[IV] Different letters within the column indicate a statistically significant difference (Duncan's test $p = 0.05$).

There were different responses of five *Curtobacterium* wilt isolates to the microbiolization treatments (biocontrol agent vs pathogen isolate interaction) (Table 4). The disease severity was lowest in the first experiment with Cff GO and Cff SP wilt isolates, in control plants as well as in plants originating from microbiolized seeds. In the second experiment, in control plants, the disease severity incited by the isolate Cff GO was the highest one and by the isolate CFF SP, the lowest. The maximum disease control in the first (48.4%) and the second (59.4%) experiment was obtained when the plants were inoculated with the isolate Cff GO. On the other hand, microbiolization with the combinations was most effective, and stable, since the disease was controlled in both experiments independent of the pathogen isolate except the Cff SP which was not controlling in experiment 1. Mean control achieved by the combinations C01 and C02 was 48.7% and 38.9%, respectively, in contrast to the mean control of 26.6% by the individual biocontrol agents.

Table 4
Severity of bacterial wilt incited by five isolates of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* on bean plants originating from microbiolized seeds.

Treatments[I]	Experiment 1					Experiment 2				
	Cff GO[II]	Cff SP[II]	Cff MG[II]	Cff DF[II]	Cff SC[II]	Cff GO[II]	Cff SP[II]	Cff MG[II]	Cff DF[II]	Cff SC[II]
DFs093	1.0bB[IV]	1.0aB	5.0cdA	5.0dA	5.0bA	2.0dC	5.0bcB	4.5cdeB	7.0abA	4.5cdeB
DFs513	1.2bC	2.0aC	8.0aA	5.0dB	5.0bB	4.5bcB	3.5cdB	7.0bA	7.0abA	4.0cdeB
DFs769	2.0abC	1.5aC	4.5dB	5.0dB	7.0aA	5.0bB	6.0abB	5.0cdB	5.5bcB	8.0aA
DFs831	1.0bC	2.0aC	5.5cdB	5.5dB	7.0aA	5.0bA	2.0dB	4.5cdeA	5.0cA	5.5bcA
DFs842	1.5bC	1.7aC	8.5aA	9.0aA	5.5bB	3.0cdC	5.0bcB	6.0bcB	8.5aA	5.0cdB
DFs843	1.0bC	1.0aC	2.5eB	5.0dA	5.0bA	3.0cdC	6.0abAB	5.0cdB	5.0cB	7.0abA
DFs912	1.2bC	1.7aC	6.0cB	8.0bA	6.0abB	3.0cdD	7.0aB	9.0aA	7.0abB	5.0cdC
C01	1.2bC	2.0aC	3.0eAB	3.0dAB	3.5cA	4.5bcA	2.0dB	2.7eAB	4.0cA	3.0eAB
C02	1.0bC	2.0aC	5.0cdA	5.0dA	3.5cB	3.0cdBC	2.0dC	5.0cdA	5.0cA	4.5cde AB
C03	2.0abBC	1.2aC	3.0eB	5.0dA	3.0cB	3.5bcdA	1.7dB	4.0deA	5.0cA	3.5deA
Control	2.5aB	2.0aB	7.0bA	7.0cA	6.0abA	9.0aA	4.2bcC	7.5abAB	7.5aAB	7.0abB
[III]CV			18.5					23.4		

[I] Bacterial treatments DFs093 (*Bacillus cereus*), DFs513 (*Pseudomonas veronii*), DFs769 (*Bacillus cereus*), DFs831 (*Pseudomonas fluorescens*), DFs842 (*Pseudomonas fluorescens*), DFs843 (*Rhodococcus fascians*) and DFs912 (*Rhodococcus fascians*), C01 = DFs093 + DFs769 + DFs831; C02 = DFs093 + DFs769 + DFs842; C03 = DFs348 + DFs769 + DFs831. Control: seeds treated with saline solution (NaCl 0.85%).

[II] Five isolates of phytopathogenic bacterium *C. flaccumfaciens* pv. *flaccumfaciens* were used: Cff Go (Goiás isolate); Cff SP (São Paulo isolate), Cff MG (Minas Gerais isolate), Cff DF (Distrito Federal isolate), Cff SC (Santa Catarina isolate). Evaluation was performed 15 days after inoculation assigning scores for severity.

[III] CV: Coefficient of variation.

[IV] Means followed by the same letter in columns and capital in rows do not differ by Duncan's test ($p = 0.05$). Severity were submitted for significant means Duncan's multiple range tests ($p = 0.05$).

4. Discussion

Only the combination C01 imparted control of all diseases evaluated in this study, although considering the diseases individually, other treatments were effective. The isolate DFs831 was most effective individual treatment, except for the control of angular leaf spot. Earlier studies showed that the combination C01 and the isolates DFs831 also were effective in controlling *X. axonopodis* pv. *phaseoli* (Corrêa, 2007) and *C. lindemuthianum* (Corrêa et al., 2008).

The data from this study, together with those published earlier, show that the isolate DFs831 and the combination C01 possess mechanisms that act on different diseases of bean, and also on the different isolates of the same pathogen. The combinations appear to have the synergistic effect (DFs093, DFs769 and DFs831). The isolate DFs831 alone and the combination C01 controlled the diseases in two experiments, independent of the pathogen isolate, exemplified by the results against two isolates of *F. oxysporum* f. sp. *phaseoli* and five of *C. flacumfaciens* pv. *flaccumfaciens*. So these treatments demonstrate biocontrol behavior stability. It corroborates earlier findings showing the effectiveness of the same treatments in controlling common bacterial blight incited by the 16 isolates of *X. axonopodis* pv. *phaseoli* or when used for seed microbiolization on 23 different bean cultivars (Corrêa, 2007).

The synergistic effect between different biocontrol agents in combination in general is due to different components expressing different protection mechanisms (Jetiyanon and Kloepper, 2002; Boer et al., 2003). The association of different biocontrol agents has been shown not only to intensify the effects as reported for the combination of *Pichia guilliermondii* Wick and *Bacillus mycoides* Flügge on strawberry leaves affected by *Botrytis cinerea* (Fr.) Pers., where the mechanism of protection, parasitism and production of toxic compounds act together (Guetsky et al., 2001); but also increase the spectrum of diseases controlled, as seen in the combination of *Bacillus pumilus* Meyer & Gottheil, *C. flaccumfaciens* (Hedges) Collins & Jones and *B. subtilis* Cohn inducer of resistance and producers of antibiotics, to control *Pseudomonas syringae* pv. *lachrymans* (Smith & Bryan)Young, Dye & Wilkie, *Erwinia tracheiphila* Smith and *Colletotrichum orbiculare* (Berk. & Mont.) Arx in cucurbits (Raupach and Kloepper, 1998).

In this study the combination C01 is composed of two isolates of *Bacillus cereus* (DFs093 and DFs769) and one isolate of *Pseudomonas fluorescens* (DFs831), and both species are known as biocontrol agents, and already have marketable formulations for different crops and pathogens (Bettiol et al., 2012). These isolates have been reported to have lipolytic and proteolytic activity and also produce ammonia, and the isolate DFs769 also has chitinolytic activity (Corrêa et al., 2006) and produces antibiotics against bean pathogens (Silva et al., 2008). The possibility of resistance induction should not be discarded, because these isolates show some characteristics generally associated with this mechanism of action, such as the time interval between application and manifestation of disease control, spatial separation between the biocontrol agent and the pathogen and non-specificity of protection (Steiner and Shonbeck, 1995).

It is well established that biocontrol should not be the only control measure to be used to manage bean diseases. But it is important to select a bio-treatment that has wide spectrum of action, which allows for greater effect under diverse conditions, thus imparting stability to the control measure.

5. Conclusion

The biocontrol bacterial isolates studied possess the potential of controlling the fungal and bacterial leaf, vascular and root diseases of bean. The combination of different isolates intensified the effect of disease control compared.

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