

## Broad spectrum anti-biotic activity and disease suppression by the potential biocontrol agent *Burkholderia ambifaria* BC-F

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### Abstract

The potential biocontrol bacterium *Burkholderia ambifaria* isolate BC-F significantly suppressed damping-off of cucumber and soybean caused by *Pythium ultimum* when applied as a seed coating. Cucumber and soybean seed coated with a peat bond formulation of isolate BC-F had significantly greater plant stands than the non-treated pathogen controls in soil-less mix. Soybean seeds treated with isolate BC-F had significantly greater plant fresh weight per pot and plant height per pot than the pathogen control. Isolate BC-F effectively colonized the roots of corn, cucumber, soybean, and sunflower and was detected at  $\geq 10^5$  colony-forming-units per gram fresh weight of root after 4 weeks in two different natural soils. *Burkholderia ambifaria* BC-F grown on nutrient broth agar or potato dextrose agar released a diffusible metabolite(s) that inhibited hyphal growth of *P. ultimum*, *Rhizoctonia solani*, *Phytophthora capsici*, and *Fusarium oxysporum* f.sp. *lycopersici*. Culture filtrates from isolate BC-F grown on nutrient broth contained a metabolite(s) that inhibited egg hatch and mobility of second-stage juveniles of *Meloidogyne incognita*. These culture filtrates contained chitinase and protease activities. However, the <3 kDa fraction of these culture filtrates was responsible for the inhibition of *M. incognita*. The >8 kDa fraction contained no inhibitory activity, indicating that inhibition of egg hatch and mobility of second-stage juveniles of *M. incognita* was not directly due to chitinase and protease activities. The demonstrated ability of isolate BC-F to suppress disease caused by animal and fungal pathogens of many crop plants may be due to the ability of isolate BC-F to persist for long periods in association with roots of diverse crop plants in different soils and the production of a metabolite(s) with broad spectrum anti-biotic activity. Published by Elsevier Science Ltd.

**Keywords:** Bacteria; Fungi; Nematode; Rhizosphere

### 1. Introduction

The *Burkholderia cepacia* complex consists of a group of bacteria currently organized into seven genomovars (Coenye et al., 2001). Among the interesting features of the *B. cepacia* complex are the presence of multiple chromosomes with wide variation in genome size among isolates and the unusual nutritional versatility of these isolates (Lessie et al.,

1996). Isolates from this complex have been associated with clinical infections of immunocompromised patients (Nelson et al., 1994; Butler et al., 1995; Govan et al., 1996), bioremediation (Kilbane et al., 1982; Vega et al., 1988; Shields et al., 1991; Shields and Reagin, 1992), and suppression of plant diseases. There have also been a limited number of reports implicating certain isolates as plant pathogens (Lessie et al., 1996). Different isolates from the *B. cepacia* complex have been reported to be effective antagonists of several important soilborne fungal plant pathogens including *Sclerotium rolfsii*, *Rhizoctonia solani*, *Pythium* spp., *Fusarium* spp., and *Phytophthora capsici* (Parke et al., 1991; Hebbar

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et al., 1992; McLoughlin et al., 1992; Bowers and Parke, 1993; Mao et al., 1998), and to exhibit activity against a species of root-knot nematode (Meyer et al., 2000, in press). Certain isolates of the *B. cepacia* complex are commercially available for control of plant pathogenic fungi and nematodes (Deny™, Stine Microbial Products, USA).

Isolate BC-F has been placed into genomovar VII of the *B. cepacia* complex (P. Vandamme and E. Mahenthalingam, personal communication) and given the proposed name *Burkholderia ambifaria* (Coenye et al., 2001). This isolate suppressed damping-off of tomato caused by a mixture of the pathogens *S. rolfssii*, *R. solani*, *P. ultimum*, and *F. oxysporum* f.sp. *lycopersici*. Isolate BC-F also suppressed damping-off of pepper caused by the mixture of pathogens *S. rolfssii*, *R. solani*, *P. ultimum* and *P. capsici* (Mao et al., 1998). Pepper and tomato plants treated with BC-F had significantly greater plant stand, plant fresh weight, and fruit yield than the pathogen control in the field (Mao et al., 1998). In another study, isolate BC-F suppressed populations of the important plant-parasitic nematode *Meloidogyne incognita* on pepper (Meyer et al., in press). We extend our analysis of isolate BC-F here to include disease suppression of *P. ultimum* damping-off of cucumber and soybean and colonization of roots of a diverse group of important crop plants. In addition, we initiate studies directed at determining the mechanism of action of *B. ambifaria* isolate BC-F. We partially characterize culture filtrates and demonstrate *in vitro* inhibition of *M. incognita* and several important soilborne plant pathogenic fungi by this bacterium.

## 2. Materials and methods

### 2.1. Bacteria, fungi and nematode isolates

*B. ambifaria* BC-F was isolated by W. Mao from roots of corn plants grown in Beltsville, MD soils and identified as a member of the *B. cepacia* complex using Biolog and FAME analysis (Mao et al., 1998; Buyer and Roberts, unpublished). A spontaneous, rifampicin-resistant mutant of BC-F was isolated by standard methods (Miller, 1972). This rifampicin-resistant mutant, designated BCFR8, was similar in growth rate in nutrient broth and on seeds, in colony morphology, and had the same antibiotic resistance profile as BC-F. The fungi *P. ultimum*, *R. solani*, *P. capsici*, and *F. oxysporum* f.sp. *lycopersici* were from the Sustainable Agricultural Systems Laboratory and the Alternative Crops and Systems Laboratory (formerly Biocontrol of Plant Diseases Laboratory). The nematode *M. incognita* was from the Nematology Laboratory.

### 2.2. Seed treatments

Cultures of BC-F were grown overnight in Tryptic Soy Broth (Difco), washed in sterile distilled water (SDW), and incorporated into treatments of cucumber (*Cucumis sativum* cv Marketmore 76), corn (*Zea mays*), soybean (*Glycine max* cv Chesapeake), and sunflower (*Helianthus giganteus*) seeds with Pelgel (LiphaTech Inc., Milwaukee WI) and peat as described by Roberts et al. (1997). Seeds coated with Pelgel, peat, and SDW but without bacteria, were used as controls. Bacterial cell densities in seed treatments were determined by dilution plating.

### 2.3. Biocontrol assays

*P. ultimum* was grown at 25°C for 3 d, flooded with soil extract (Ayers and Lumsden, 1975), and incubated at 25°C for 7–28 d. Sporangia from these plates were washed and incorporated into soil-less mix. Soil-less mix, soil-less mix amended with sporangia of *P. ultimum* or with SDW, treated cucumber or soybean seeds, and soil-less mix amended with sporangia of *P. ultimum* or SDW, were added as sequential layers to small cups as described by Roberts et al. (1997). For each seed type, eight replicate cups were sown with five seeds each and incubated in a growth chamber at 24°C for 8 d for cucumber and 11 d for soybean with a 12 h photoperiod. Plant stand per cup for cucumber and plant stand per cup, plant weight per cup, and plant height per cup for soybean were determined. Analysis of variance (ANOVA) was carried out and differences among means estimated using a protected least significant difference (LSD) test (SAS, Cary NC). The experiment was performed at least four times with each seed type. Repeated experiments were not combined prior to analysis.

### 2.4. Root colonization assays

Cucumber, corn, soybean, and sunflower seeds, treated with *B. ambifaria* BCFR8, were sown in a natural Galestown gravelly loamy sand soil (pH 5.8) and in a natural Rumford sandy loam soil (pH 6.4) in 6.5 cm diameter × 25 cm deep pots and incubated in a constant temperature room in a greenhouse at 22°C. Plants were removed after 28 d and the root system sampled by cutting the root just below the seed coat. The root and attached soil were placed in SDW, sonicated, and colony-forming units (cfu) per root system determined as described by Roberts et al. (1997). Root system fresh weight was determined for each sample. The experiment was performed twice with each soil type with eight replicates per treatment. Repeated experiments were not combined prior to analysis. Log<sub>10</sub> cfu per gram fresh weight root tissue was determined, analyzed by ANOVA, and differences among means estimated by LSD.

## 2.5. Preparation of culture filtrates

Strain BC-F was grown for 2 d at 37°C by shaking at 250 rpm in nutrient broth (NB), or Berka and Lessie minimal medium (BL) (Berka and Lessie, 1984) plus 0.2% glycerol, 0.2% carboxymethylcellulose (CMC), chitin, or laminarin, or BL plus 0.2% glycerol plus 0.2% CMC, chitin, or laminarin. Cultures were centrifuged at  $6000 \times g$  for 10 min and passed through a  $0.2 \mu$  filter. Culture filtrates were stored at  $-20^\circ\text{C}$  until used.

Culture filtrates were size-fractionated by centrifugation in a centriprep 3 (molecular weight cut off = 3 kDa) apparatus (Amicon Corp., Lexington, MA) essentially as described by Meyer et al. (2000). The filtrate (<3 kDa fraction) was frozen until used. The retentate was dialyzed (molecular weight cut-off = 8 kDa) against 50 mM potassium phosphate pH 7.0, and restored to the original volume with sterile NB. The non-fractionated culture filtrate and the dialyzed preparation (>8 kDa fraction) were also frozen until used. Sterile, non-inoculated NB was size-fractionated as above and used as controls.

## 2.6. Enzyme assays

Culture supernatant was assayed for carboxymethylcellulase (CMCase), chitinase, laminarinase, and protease activities. For CMCase activity, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 5.0 was mixed with 0.2% CMC and incubated at 37°C. For laminarinase activity, 50 mM MES buffer, pH 5.0, was mixed with 0.2% laminarin and incubated at 37°C. Reducing sugars liberated due to CMCase or laminarinase activities were determined by the method of Nelson (1944) with a glucose standard. One unit of CMCase or laminarinase activity was defined as the amount of enzyme that released 1 mM glucose reducing equivalent  $\text{min}^{-1} \text{ml}^{-1}$  culture. Chitinase activity was determined by incubation of culture supernatant with 50 mM citrate buffer, pH 5.0, and 0.2% chitin at 37°C. Liberated *N*-acetylglucosamine equivalents were determined by the method of Reissing et al. (1955). One unit of chitinase activity was the amount of enzyme that released 1 mM *N*-acetylglucosamine equivalent  $\text{min}^{-1} \text{ml}^{-1}$  culture. Protease activity was determined by incubating culture filtrate in 100 mM potassium phosphate buffer, pH 7.0, plus 0.4% azocoll at 37°C (Chavira et al., 1984). One unit of protease activity was the amount of enzyme that increased absorbance at 520 nm  $1 \text{ unit hr}^{-1} \text{ml}^{-1}$  culture filtrate. Lower limits of detection were 2.3 U for CMCase and laminarinase activities, 0.3 U for chitinase activity, and 0.001 U for protease activity.

## 2.7. In vitro inhibition of *M. incognita*

Culture filtrates and size-fractionated culture filtrates were tested in 24-well tissue culture plates for effects on

*M. incognita* egg hatch and mobility of hatched second-stage juveniles (J2) as described by Nitao et al. (1999). A suspension of eggs in 0.1 ml sterile distilled water was placed into each well in combination with 0.9 ml of a treatment or a control. Counts were made of total J2 and of mobile J2 in each well after 3 d and again after 14 d. Each treatment was replicated six times. Experiments for the non-size-fractionated culture filtrates contained 158 and 187 eggs per well. Experiments with size-fractionated preparations contained 170 and 120 eggs per well. Percent hatch, active J2, and inactive J2 were determined, analyzed by ANOVA, and differences among means estimated by LSD. Repeated experiments were not combined prior to analysis.

## 2.8. In vitro inhibition assays for soilborne fungal plant pathogens

Isolate BC-F or *Escherichia coli* DH5 $\alpha$  were spotted onto NB agar or potato dextrose agar plates twice at opposite sides near the edge and incubated 48 h at 37°C. A single plug (4 mm diameter) of *P. ultimum*, *R. solani*, *P. capsici*, or *F. oxysporum* f.sp. *lycopersici* was subsequently placed at the center of each plate and the plates incubated at 20°C for 3, 5, 7, and 7 d, respectively, prior to measuring the zone of inhibition of hyphal growth. The experiment was performed once with five replicates for each fungal isolate on nutrient broth agar and once on potato dextrose agar.

## 3. Results

### 3.1. Biocontrol

*B. ambifaria* BC-F was effective in suppressing *P. ultimum* damping-off of cucumber and soybean in growth chamber studies using soil-less mix (Table 1). Cucumber and soybean seed treatments with isolate BC-F resulted in significant ( $P \leq 0.05$ ) increases in mean

Table 1  
Suppression of damping-off of cucumber and soybean caused by *Pythium ultimum* with *Burkholderia ambifaria* BC-F<sup>a</sup>

Treatment	Cucumber		Soybean	
	Stand	Weight	Stand	Height
Healthy check	4.88 A	8.36 A	5.00 A	6.44 A
BC-F + <i>P. ultimum</i>	4.25 A	6.32 B	4.30 A	4.47 B
Pathogen check	0.00 B	2.92 C	2.10 B	1.83 C
LSD	1.07	1.13	0.72	1.15

<sup>a</sup> Means followed by the same letter in each column are not significantly different ( $P \leq 0.05$ ). Stand, mean stand per pot; weight, mean fresh weight per pot; height, mean height per pot. Isolate BC-F was demonstrated not to be phytotoxic to soybean and cucumber in separate experiments. LSD = least significant difference ( $P \leq 0.05$ ).

stand per pot compared with the pathogen check. Cucumber and soybean seed treatments with BC-F resulted in a mean stand per pot that was similar to that of the healthy check. Soybean seed treatment with isolate BC-F resulted in mean plant weight and mean plant height per pot that were significantly greater ( $P \leq 0.05$ ) than the pathogen check but lesser than the healthy check. Similar results were obtained with all experiments. There was no evidence of phytotoxicity to cucumber or soybean due to seed treatment with BC-F.

### 3.2. Root colonization

*B. ambifaria* BCFR8 effectively colonized the roots of corn, cucumber, soybean, and sunflower plants when introduced into two different natural soils as a seed treatment (Table 2). Populations of BCFR8 were significantly greater ( $P \leq 0.05$ ) per gram of fresh root on corn than on the other three crops tested in both natural

Table 2  
Populations of *Burkholderia ambifaria* BCFR8 on roots of 4-week-old crop plants<sup>a</sup>

Crop plant	Mean log <sub>10</sub> colony-forming unit per gram fresh weight of root	
	Rumford soil	Galestown soil
Corn	7.69 A	6.61 A
Cucumber	6.24 B	5.63 BC
Soybean	6.22 BC	5.34 C
Sunflower	5.54 C	5.92 B
LSD <sup>b</sup>	0.69	0.54

<sup>a</sup> Means followed by the same letter are not significantly different ( $P \leq 0.05$ ) for that soil type.

<sup>b</sup> LSD = least significant difference ( $P \leq 0.05$ ).

Table 3  
In vitro inhibition of *Meloidogyne incognita* with culture filtrates from *Burkholderia ambifaria* BC-F<sup>a</sup>

Treatment <sup>b</sup>	Percent egg hatch		Active J2s <sup>c</sup>		Inactive J2s <sup>c</sup>	
	3 d	14 d	3 d	14 d	3 d	14 d
NB	25.8 A	42.6 A	39.9 A	34.8A	4.0 A	37.8 BCD
<3 KDa NB	20.1 B	36.6 B	30.3 BC	6.3 D	3.9 A	55.9 A
>8 KDa NB	19.9 B	35.9 B	29.8 BC	30.5 AB	3.9 A	30.5 D
Sterile water	21.7 B	37.5 AB	35.1 AB	19.0 C	1.8 BC	44.8 B
dialysis buffer	18.3 B	39.3 AB	27.9 C	30.9 AB	3.3 AB	35.9 CD
BC-F	1.3 C	2.0 C	0.3 D	0.0 D	2.0 ABC	3.4 E
<3 KDaBC-F	0.5 C	1.5 C	0.0 D	0.1 D	1.0 C	2.5 E
>8 KDaBC-F	20.5 B	38.0 AB	32.4 BC	24.3 BC	2.5 ABC	40.4 BC
LSD <sup>d</sup>	3.5	5.8	5.5	6.7	2.1	8.3

<sup>a</sup> Means followed by the same letter in a column are not significantly different ( $P \leq 0.05$ ).

<sup>b</sup> NB, sterile non-fractionated nutrient broth; <3 kDa NB, <3 kDa non-inoculated nutrient broth fraction; >8 kDa, >8 kDa non-inoculated nutrient broth fraction; dialysis buffer, 50 mM potassium phosphate, pH 7.0; BC-F, sterile BC-F culture filtrate; <3 kDa BCF, <3 kDa BC-F culture filtrate fraction; >8 kDa BC-F, >8 kDa BC-F culture filtrate fraction.

<sup>c</sup> J2s, second-stage *M. incognita* juveniles.

<sup>d</sup> LSD = least significant difference ( $P \leq 0.05$ ).

soils. However, BCFR8 was present at substantial levels after 28 d on the roots of all crop plants tested in both soil types. Data from both experiments with each soil type were similar.

### 3.3. Enzyme assays

Culture supernatants contained enzyme activities potentially capable of degrading cell walls of *P. ultimum* and life cycle stages of *M. incognita*. These supernatants contained 13.9 and 2.5 U laminarinase activity when strain BC-F was grown in BL plus 0.2% laminarin and BL plus 0.2% glycerol and 0.2% laminarin, respectively. Laminarinase activity was not detected in BC-F cultures grown on NB. Culture supernatants also contained 3.9 and 0.7 U chitinase activity when BC-F was grown in NB and BL plus 0.2% chitin, respectively. Chitinase activity was not detected in culture filtrates from BC-F grown on BL plus glycerol or BL plus glycerol plus chitin. 0.39 and 0.19 U protease were detected in culture filtrates from BC-F grown on BL plus glycerol and NB, respectively. CMCase activity was not detected in cultures from BC-F grown on any medium. Enzyme activities in a particular culture filtrate were not determined unless mentioned above.

### 3.4. In vitro inhibition of *Meloidogyne incognita*

Non-size-fractionated culture filtrates from BC-F grown in NB contained a metabolite(s) that resulted in the inhibition of egg hatch and of mobility of *M. incognita* J2s (Table 3). After 3 d, 21.7%, 18.3%, and 25.8% of *M. incognita* eggs hatched when treated with the SDW, 50 mM phosphate dialysis buffer, or non-size-fractionated NB controls, respectively. In contrast, only

1.3% of *M. incognita* eggs hatched when treated with non-size-fractionated culture filtrates from BC-F (significantly lower than all controls at  $P < 0.05$ ). Essentially all the J2s that developed in the BC-F treatment were inactive while most of the J2s treated with the SDW, phosphate buffer, and non-size-fractionated NB controls were active. After 14 d, 37.5%, 39.3%, and 42.6% of *M. incognita* eggs hatched when treated with the SDW, phosphate buffer, or NB controls, respectively. In contrast, only 2.0% of *M. incognita* eggs hatched when treated with non-size-fractionated culture filtrates from BC-F (significantly lower at  $P < 0.05$ ). As with the 3 d results, all of the J2s that developed in the BC-F treatment were inactive. Similar results were obtained from other experiments.

The inhibitory metabolite(s) that resulted in the inhibition of egg hatch and of mobility of *M. incognita* J2s was of low molecular weight (Table 3). Only 0.5% and 1.5% of *M. incognita* eggs hatched after 3 and 14 d, respectively, when treated with the <3 kDa preparation from culture filtrates of BC-F. This percentage of egg hatch was statistically similar ( $P \geq 0.05$ ) at 3 and 14 d to that of the non-size-fractionated BC-F culture filtrate preparation and statistically different from the SDW, phosphate buffer, or NB controls. In contrast, the >8 kDa preparation from BC-F culture filtrate was not inhibitory to egg hatch or activity of J2s and was similar to the controls (Table 3). Similar results were obtained from a second experiment. Results from preliminary experiments suggest that an inhibitory metabolite(s) is volatile (data not shown).

### 3.5. *In vitro* inhibition of soilborne fungal plant pathogens

Cultures of *B. ambifaria* BC-F grown on NB agar produced a metabolite(s) that was released into the agar and inhibited hyphal growth of all four fungi tested. Zones of inhibition due to BC-F were  $6.6 \pm 0.7$ ,  $15.5 \pm 0.9$ ,  $11.0 \pm 0.7$ , and  $8.8 \pm 0.2$  mm for *P. ultimum*, *R. solani*, *P. capsici*, and *F. oxysporum* f.sp. *lycopersici*, respectively. There were no zones of inhibition around *E. coli* DH5 $\alpha$  colonies on NB agar plates. Results from a second experiment performed on potato dextrose agar were similar.

## 4. Discussion

*B. ambifaria* BC-F is an extremely versatile biocontrol agent capable of suppressing disease caused by a number of soilborne plant-pathogens on pepper and tomato (Mao et al., 1998) and, as reported here, on cucumber and soybean. Isolate BC-F also suppressed populations of the plant-parasitic nematode *M. incognita* on pepper (Meyer et al., in press). Prior to intensive

field studies, interactions between *B. ambifaria* BC-F, plants, and pathogens should be investigated. Through an understanding of these interactions limitations for biocontrol can be determined and strategies to optimize biocontrol efficacy established (Larkin et al., 1998). We show here that BC-F is capable of colonizing and persisting on the roots of a diverse collection of crops when introduced into natural soil as a seed treatment. We also show that BC-F produces a metabolite(s) with broad spectrum anti-biotic activity. This combination of traits makes BC-F an excellent candidate for field applications as a seed treatment for control of damping-off and root-infecting pathogens. Isolate BC-F was shown to control several root-infecting pathogens in limited field trials (Mao et al., 1998).

Clarification of the taxonomy of the *B. cepacia* complex and the pathogenic potential of environmental isolates, such as *B. ambifaria* BC-F, is critical prior to intensive field studies directed at commercial development. The pathogenic potential of BC-F has not been determined with regard to humans. However, BC-F is not a member of genomovar III of the *B. cepacia* complex (P. Vandamme and E. Mahenthalingam, personal communication) which contains the highly epidemic strains isolated from patients with cystic fibrosis (Mahenthalingam et al., 2000). In addition, isolate BC-F is negative for the BCESM genetic marker and positive for the *recA* type C PCR primer (E. Mahenthalingam, personal communication). The BCESM genetic marker is a novel DNA marker associated with epidemic strains of the *B. cepacia* complex which have spread among patients with cystic fibrosis (Mahenthalingam et al., 1997). The *recA* PCR type C positive strains have all been isolated from the rhizosphere of corn. No strains of clinical origin are *recA* PCR type C positive (E. Mahenthalingam, personal communication).

The inhibitory metabolite(s) produced by BC-F was active against diverse organisms, including the plant-parasitic nematode *M. incognita* and several important soilborne plant-pathogenic fungi. Various bacterial isolates from the *B. cepacia* complex produce inhibitory metabolites including pyrrolnitrin and other phenylpyrroles (Mahoney and Roitman, 1990; Roitman et al., 1990a,b; McLoughlin et al., 1992; Burkhead et al., 1994), cepacin A, cepacin B (Parker et al., 1984), altericidin A, altericidin B, altericidin C (Kirinuki et al., 1977, 1984), bacteriocins (Gonzalez and Vidaver, 1979), siderophores (Smirnov et al., 1990) and a novel lipopeptide (Kang et al., 1998). To our knowledge, none of these compounds have been shown to be active against both nematodes and fungi. A second *Burkholderia* strain has been isolated, that inhibits growth of bacteria, fungi, and protozoa. However, there is no indication of inhibition of nematodes by this isolate (Cain et al., 2000).

*B. ambifaria* BC-F remains of interest despite some concern with environmental application of this organism. This strain is unusual due to its ability to: (1) suppress a range of pathogens on several important crop plants, (2) colonize and persist on the roots of a diverse collection of crops, and (3) produce a metabolite(s) with broad spectrum anti-biotic activity. Even if *B. ambifaria* BC-F is never used in commercial environmental applications, knowledge of the genes and pathways responsible for these unique traits will help screen for other bacteria with similar biocontrol capabilities.

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