Evaluation of *Burkholderia cepacia* Strains: Root Colonization of *Catharanthus roseus* and In-Vitro Inhibition of Selected Soil-borne Fungal Pathogens

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Three strains of *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) isolated from soil in Oklahoma were not pathogens on onion bulbs when compared to a known pathogenic strain. Inhibitory effects of antimicrobial compounds produced by each of the strains were evaluated *in vitro* against selected soilborne pathogenic fungi. Inhibition of radial growth was observed against *Fusarium oxysporum, Macrophomina phaseolina, Sclerotium rolfsii, Rhizoctonia solani,* and *Pythium ultimum.* Strain OK-2 colonized the rhizosphere of *Catharanthus roseus,* when applied to germinated seeds and population densities of 10^6-10^7 colony forming units (cfu)/cm were attained along the root. Densities were similar along the proximal, medial, and distal portions of the roots during a 17-d period.

INTRODUCTION

Although *Burkholderia cepacia* (ex. Burk) Yabuuchi (formerly *Pseudomonas cepacia*, Burk) was first recognized as a pathogen of onions, it is a successful biocontrol agent against foliar, soilborne, and post-harvest plant pathogens (*1-5*). Compounds potentially involved in biocontrol have been pyrrolnitrin and pyoluteolin (*6,7*), and the siderophore, cepabactin (*2,3,8*). Production of volatile ammonia also has been implicated as a possible mechanism to control soilborne pathogens (*9-12*). Certain strains of *B. cepacia*, isolated from the caryopses of grass plants, produce antifungal compounds suppressive to many phytopathogens (*13*).

Bedding plants, such as periwinkle *(Catharanthus roseus* L. 'Little Rosie'), are susceptible to many damping-off diseases. Although chemicals can effectively control these diseases, new forms of biological control should be evaluated and developed to provide more environmentally benign alternatives.

B. cepacia has been registered since 1992 as a microbial pesticide by the US Environmental Protection Agency. This agency recently issued a notice (*14*) to the research community about public health concerns regarding the registration and use of *B. cepacia* as a microbial pesticide (*15*). This concern is for opportunistic, serious infections in humans suffering from cystic fibrosis (*16*) where bacteria have been isolated and identified as strains of *B. cepacia.* The taxonomic classification of numerous strains identified as *B. cepacia* is uncertain and until the relationship between clinical and environmental strains is known further use of this microbe is being discouraged by the medical community. Biotypes have been separated and characterized (*17*) and clinical and environmental biotypes have been separated based on the production of bacteriocin and pectolytic enzymes produced by biotypes of plant origin. Because *B. cepacia* is such a diverse organism, we feel that further research should be done to understand its mode of action and spectrum of activity against soilborne, pathogens.

The ability to colonize and proliferate in the rhizosphere, i.e., rhizosphere competence, (*18,19*) and to produce antibiotics are desirable characteristics of bacterial organisms used for biological control (*18*). However, bacteria differ in their ability to colonize root systems (*20*), and thus vary in their degree of rhizosphere competence (*21-23*). In addition, densities of an introduced bacterium in the rhizosphere may not correlate with effects on the host, such as plant growth promotion (*20,22*) or suppression of pathogens (*24*). Some rhizosphere-competent bacteria may even be deleterious (*12,25,26*). Information about factors involved in rhizosphere competence is limited. Certain bacteria possess specific characteristics for successful root colonization. Pre-

diction of rhizosphere competence of bacteria along the root is important (*27*).

Parke (*19*) defined root colonization as the proliferation of microorganisms in, on, and around the growing root. The definition includes the dispersal of microorganisms from a source of inoculum to the actively growing root and multiplication or growth in the rhizosphere.

One of the major problems associated with biological control agents is variable efficacy in the field that limits agronomic application. Variability is due to any factor interfering with either the population size of a biocontrol agent or its expression of activity.

Key characteristics contributing to the success of a biocontrol organism, as well as physio-chemical factors that determine success or failure, are not well known (*19*). However, *B. cepacia* isolated from the rhizosphere of rice reduced the weight and the height of seedlings because of its accumulation of nitrite (*26*), thus implicating nitrite toxicity as one of the factors that interferes with seedling vigor.

Water movement on roots colonized by bacterial strains (*28*, *29*) improved the downward movement of bacteria along the root. In the absence of downward water movement, Howie and coworkers (*29*) proposed that root colonization by bacteria introduced on wheat seeds occurs in two phases. In phase 1, bacteria are distributed by passive carriage downward with root extension through soil, accounting for progressively lower populations on the root at increasing distances from the seed. Phase 2 is the multiplication and survival phase and occurs during and after phase 1.

In our investigations, we (1) examined isolates of *B. cepacia* as potential biocontrol agents by evaluating their inhibitory effects against several soilborne pathogens, (2) characterized strains based on pathogenicity on onion, and (3) characterized strains for their root colonization potential. Associated objectives were (1) to determine if bacteria applied to small seeds, such as those of periwinkle would increase and obtain densities needed for disease suppression and (2) to compare the inhibitory capacity of each bacterial isolate against selected fungal pathogens.

Although Parke (*24*) and others (*29*) have determined that regardless of the initial bacterial densities on the seed, densities will increase over time to reach a maximum ìcarrying capacityî for the spermosphere and rhizosphere, most research has been conducted with large seeds; pea (*24*), soybean (*30*), wheat (*23*,*31*), and cotton (*29*).

MATERIALS and METHODS

Description of Strains: Five strains of *B. cepacia* were evaluated in selected portions of this study: OK-1 isolated from soil at the State Forest Nursery in Washington, OK; OK-2 was selected from OK-1 for resistance to 120 µg/mL nalidixic acid and 80 µg/mL chloramphenicol; OK-3 (*B. cepacia* 945), obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, England, isolated from onion tissue; OK-4, isolated from apple fruit by W. J. Janisiewicz; and OK-5 isolated from soil in Stillwater, OK. In addition, an isolate of *Pseudomonas aeruginosa* B-4, OK-B-4, from Peru was used in this study because it inhibited *Macrophomina phaseolina*, the causal agent of charcoal rot disease found in many economically important plants. All isolates were given an OK-isolate number for acquisition into our culture collection. Our isolate OK-1 was determined to be a soil biotype (*17*) by Dr. C. Gonzales, Department of Plant Pathology & Microbiology, Texas A&M University (personal communication).

Preparation of Bacterial Inoculum: Isolates of *B. cepacia* were streaked on King's B Medium (KB) (*33*) and incubated for 48 h at 28-30°C. Bacterial suspensions were prepared by removing the colonies from the media using a sterile solution of 0.85% sodium chloride. Bacterial suspensions were standardized to approximately 10^8 colony forming units(cfu)/mL using a Spectronic 20 at an absorbance value of 0.1 at 660 nm. Other concentrations (10^2-10^6 cfu/mL) were prepared by appropriate dilutions with a sterile solution of (0.85%) sodium chloride.

Pathogenicity of *B. cepacia* on Onion: Suspensions of 10^8 cfu/mL were prepared from fresh cultures of *B. cepacia* including OK-1, OK-2, OK-3, OK-4, and OK-5 in 250 mL sterile flasks. Several plastic, 12 oz cottage cheese containers (diameter: 11 cm and depth 5.5 cm) were used as moist chambers. Containers and lids were soaked in 95% ethanol and 0.525% sodium hypochlorite solution (50/50%, v/v) for 1 h and washed with sterile water. Either sterile, clean tissue or filter paper was placed inside each container and moistened with 5 mL sterile distilled water.

Two inoculation techniques were used for each bacterial isolate. In both techniques, three yellow onion bulbs approximately 5-7 cm in diameter were inoculated. The extent of tissue maceration was used as an indication of pathogenicity. In the first method, the outer three scales of each onion and the newly exposed scale was surface sterilized by wiping it with 95% ethanol. A suspension of 0.5 mL of bacteria containing 10^8 cfu/mL was injected into three locations on two onion bulbs at d depths of 1, 2, and 3 cm. One of the covering scales was placed back into its original position to protect the injected area from drying and to prevent contamination by other microorganisms. For the control, the third onion was injected with 0.5 mL sterile distilled water.

The second method involved the removal of small triangular pieces of the onion flesh. A technique modified from that of Gonzalez and Vidaver (*17*) was used for this test. After removing the outer scale layers of three yellow onions and surface sterilizing the exposed scale, a triangular section, 1 cm on each side was cut into each bulb to a depth of three layers. Dissected bulbs were placed in a sterile container lined with two layers of sterile Whatman No.1 filter paper moistened with sterile water (*17*) and covered with plastic wrap. Each strain of *B. cepacia* to be tested was cultured overnight on KB medium. Cells of *B. cepacia* from colonies of each strain were transferred in mass into the depth of each triangular cut. Control onions were cut in a similar manner but no bacteria were applied. For both experiments onions were kept in closed containers and incubated at 27°C for 1 wk. The width and length of water soaking (cellular damage) were measured for each inoculation point. The experiment was conducted twice. The pathogenic strain OK-3 was the positive control.

Comparison of Inhibition by Strains of *B. cepacia* and *Pseudomonas aeruginosa* B-4 against Selected Pathogenic Fungi: Agar blocks (2 mm diameter) from fresh cultures of *Fusarium oxysporum, Macrophomina phaseolina, Sclerotium rolfsii, Rhizoctonia solani,* and *Pythium ultimum* were separately tested in a dual culture on potato-dextrose agar (PDA) and KB media against *B. cepacia* (OK-1, OK-2, OK-3, OK-4, and OK-5) and *P. aeruginosa* (B-4) for inhibition of growth. To standardize our technique, a template was made from cardboard in the shape of an isosceles right triangle with the sides 5.8 cm in length and a height of 4.5 cm from the angle to the base. The outline of the template was marked with a permanent marker on the bottom of all petri dishes. All bacteria tested were streaked onto PDA media and after 48 hr an individual bacterial colony was transferred by loop and inoculated along the hypotenuse of the triangle. Fungal inoculum was placed in the right angle so that the plug was a maximum of 4.5 cm distance from the bacteria streak. Similar procedures were conducted for six bacterial strains versus five fungal pathogens on PDA and KB media. All cultures were randomly placed and incubated at 27°C, with a 12 h alternating light/dark cycle. The zone of inhibition between the bacteria and the leading edge of the fungal colony was measured with a metric ruler after 4 d for fast growing and 10 d for slow growing fungi. The experiment was performed twice.

Root Colonization by *B. cepacia*: Plastic centrifuge tubes (40 cc) were cut longitudinally into two halves but not separated, soaked in 10% Clorox and soap for 1 h, washed, dried, microwaved for 90 s, and wrapped with parafilm. One hundred tubes were filled with 25 g of Redi-Earth (RE) (W.R. Grace, Inc., Tifton, GA) and moistened to 15% water holding capacity RE was microwaved in the tubes (Kenmore, SEARS, Chicago, IL Model 564.8878310, 1400W) for 90 s to kill contaminating fungi (*34*) and to reduce fungal over-growth in dilution plates. Plastic caps were left on the tubes after microwave treatment to maintain water content until ready to use. For uniformity and to avoid damage to the radicle, germinated periwinkle seeds were selected with radicles ≤1 mm in length. Each seed was placed 1 cm below the surface of the RE and to prevent dessication, 0.1 mL of polysurf C gel (modified hydroxyethyl cellulose, Hercules, Inc., Wilmington, DE) was placed on top of the seed. Seeds were covered by additional RE. Sets of 20 germinated seeds each were submerged in either 10^2, 10^4 or 10^6 cfu/mL suspensions of *B. cepacia* OK-2 for 30 min, and dried for 1 h before they were placed them in RE and covered with gel in each tube. Tubes with seeds were incubated for 17 d in a growth chamber adjusted to 27°C day/30°C night, 12/12 h cycles. No additional water was added during the experiment.

Shoots emerged after 3 d, and 3 tubes per concentration level were split and the soil gently removed. After the 17-d growth period, the average length of roots was 8 cm. Smaller roots (1-2 cm) were ignored. Roots (>4 cm) with rhizo-

sphere soil were weighed, and sectioned into 3 equal parts. A 1 cm segment was cut from the lower portion of each root section and separately ground in 1 mL distilled water with a sterilized mortar and pestle. A 10-fold dilution series was prepared with 1% PDA in Petri dishes. Cultures were incubated at 27°C for 48 h. The number of colonies was counted and densities (cfu/mL) were determined from each 1 cm root segment. The agar medium for dilutions was later modified because of contaminating microorganisms, by amending the Pseudomonas F agar (Difco, Detroit, MI) with 120 µg nalidixic acid and 80 µg chloramphenicol.

RESULTS

Pathogenicity Test of *B. cepacia* on Onion: Strain OK-3 produced the largest macerated area, an average of, 3.8 mm wide and 8.2 mm long on the onion bulbs. The other isolates, OK-1, OK-2, OK-4, and OK-5, caused sunken dried lesions, 0.8-1.9 mm in diameter, around their points of inoculation similar to the control. Both inoculation techniques produced similar results.

TABLE 1. Comparison of inhibition (cm) caused by five strains of *Burkholderia cepacia,* and a strain B-4 of *Pseudomonas aeruginosa* against selected pathogenic fungi on King's B Medium (KB[1] and Potato-Dextrose Agar (PDA)[1].

BACTERIAL STRAIN [2]	Mean[3] (cm) PDA	KB
OK-1	1.26 [a]	2.01 [a]
OK-2	1.26 [a]	2.07 [a]
OK-3	0.90 [b]	1.35 [c]
OK-4	0.47 [c]	0.34 [d]
OK-5	1.35 [d]	1.67 [b]
B -4	0.88 [a]	1.31 [c]
FUNGAL STRAIN [4]	**Mean[3] (cm) PDA**	**KB**
Fusarium oxysporum [5]	0.62 [d]	0.68 [d]
Sclerotium rolfsii [6]	0.00 [e]	1.57 [b]
Macrophomina phaseolina [6]	1.22 [b]	1.51 [b]
Rhizoctonia solani [5]	2.31 [a]	2.40 [a]
Pythium ultimum [6]	0.95 [c]	1.12 [c]
Main effects		
Bacteria	** [7]	**
Fungi	**	**
Media	**	**
Interaction		
Bacteria X media	**	**
Bacteria X Fungi	**	**
Bacteria X Fungi X Media	**	**

[1] PDA: Difco; KB: (33).
[2] Streak of 72 -h bacterial cultures were used against selected fungi on both media. Means are pooled data from all fungi for each bacterium.
[3] Experiment was conducted based on three -way completely randomized block. Numbers followed by different superscript letters are significantly different according to analysis of variance ANOVA and the student-Newman-Keuls multiple range test ($P \leq 0.05$). Means are five replications with the total of 300 observations.
[4] Indicates mean values of inhibition (cm) between each fungus and all bacterial strains at 27°C. Distance between the block of each fungus to the bacterial streak line on all petri dishes was 4.2 cm.
[5] Data were collected after 10 da among slow growing fungi.
[6] Data were collected after 4 da among fast growing fungi.
[7] ** indicates significant difference ($P \leq 0.05$) among bacteria, fungi, media, and their interactions.

Comparison of Inhibition by Strains of *B. cepacia* and *P. aeruginosa* against Selected Pathogenic Fungi: Inhibition zones were observed against selected fungi on KB and PDA (Table 1). There were significant interactions ($P \leq 0.05$) between bacteria X fungi X media, with all bacterial strains except OK-4 being more inhibitory on KB medium compared to PDA. There was also a significant interaction ($P \leq 0.05$) between bacteria X fungi with strains OK-1 and OK-2 more inhibitory to all fungi compared to the other strains. *Sclerotium rolfsii, F. oxysporum* and *P. ultimum* were the least inhibited of the fungi tested and *R. solani* was the most inhibited.

On KB media, *B. cepacia* strain OK-5 had the greatest inhibition against all fungal pathogens. Only strain OK-4 had greater inhibition on PDA compared to KB medium, whereas it had the least inhibition compared to the other bacteria. There was slight difference in inhibition between OK-3 and OK-2, but OK-1 and OK-2 had similar inhibition. On the other hand, *R. solani* on KB medium was the most inhibited fungus whereas *F. oxysporum* was the least inhibited. There was a significant ($P \leq 0.05$) bacteria X media interaction within fungi. Most selected fungi were more inhibited by strains of *B. cepacia* and *P. aeruginosa* on KB compared to those of on PDA medium except *F. oxysporum* which showed similar inhibition on both media.

Root Colonization Ability by *B. cepacia*: Regardless of the initial concentrations of *B. cepacia* on periwinkle seeds, the population densities usually increased rapidly for the first 3 d and were maintained near 10^6-10^7 cfu/cm for the 17-d period (Fig. 1). High initial concentrations of bacteria on seeds declined for the first 3 d at the top and tip of the root, but increased to 10^7 cfu/cm by the fifth day.

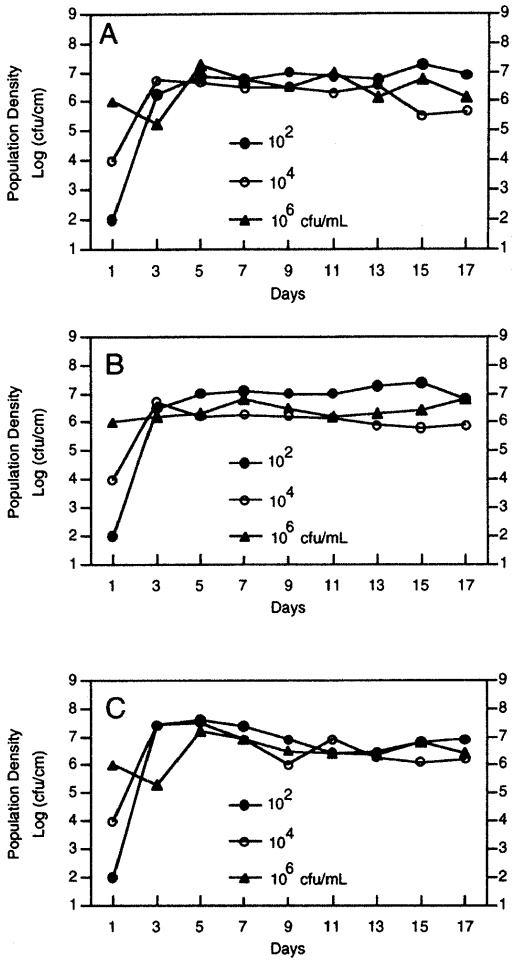

Figure 1. Population densities (colony forming units (cfu)/ cm) of *Burkholderia cepacia* OK-2 used as a seed treatment on three sections of periwinkle root for a 17- d period. (A) proximal third. (B) medial third. (C) distal third of root. Initial densities on germinated seeds were 10^2, 10^4, or 10^6 cfu/seed.

DISCUSSION

Only *B. cepacia* OK-3 was a pathogen to onion and it produced the greatest amount of macerated tissue in each test. Limited maceration of onion by the other strains suggested they were not pathogenic. *B. cepacia* could not be reisolated from macerated tissue. In previous investigations (*35*), inoculation of onion bulbs with *B. cepacia* resulted in the death of 80% of bacterial cells within 3 d and an increase in phenolic concentrations in onion scales up to 5 d after inoculation. A similar phenomenon may have occurred here, preventing the isolation of our isolates from onion tissue.

B. cepacia and *P. aeruginosa* produce antibiotics and siderophores (*8*,*36*,*38*). Strains B-4, and OK-1 through OK-5 were tested for such activities. Although these products were not identified in this investigation, inhibition zones on PDA and KB media demonstrated the possible presence of such compounds. The mechanism of such inhibition in our experiment is unknown. However, inhibitory compounds are produced by strains of *B. cepacia*, including pyrrolnitrin and

pyoluteolin (*6*,*28*), ammonia (*9*), and siderophore (*8*). The variability of inhibition among strains of *B. cepacia* against selected pathogens on the two different media, PDA and KMB, illustrates the importance of nutrition on the efficacy of biological control microorganisms.

We did not attempt to identify or quantify the inhibitory factor, although isolate OK-4 obtained from Dr. W. Janisiewicz (USDA/ARS, Kearneysville, WV) was a known producer of pyrrolnitrin (*6*) and reacted differently when compared to the other strains on the two agar media indicating a different mode of action than the other strains.

Six traits, including growth rate, extent of growth on root exudates, chemotaxis to root exudates, and tolerance of low osmotic potentials, were thought to be essential for successful root colonization (*18*). *B. cepacia* OK-2 strain inoculated on periwinkle seed provided increased population densities and colonized roots. Bacterial populations distributed among three sections of root (top, middle, and tip) indicated that there were similar densities of *B. cepacia* OK-2. Eventually populations stabilized and were maintained near 106 to 107 cfu/cm of the root for a 17-d period. We may conclude that root exudates of periwinkle can serve as the nutrient for initial population of the biocontrol organism. We have shown that *B. cepacia* can survive in the spermosphere of small seeds and colonize roots. Colonization of periwinkle roots would best be described by phase 2 colonization as defined by Howie and coworkers (*28*).

In other investigations (*34*), *B. cepacia* was able to maintain population densities on cotton roots ranging from 10^6-10^9 cfu/g. In addition, Parke (*24*) established that densities of bacteria on pea seed reached a carrying capacity of approximately $10^{8.4}$ cfu/seed at 48 hr. The lower densities for periwinkle reported here may be a function of a smaller seeds or a smaller root system compared to cotton and pea. Cartwright and Benson (*37*) also describe a carrying capacity for isolates of *B. cepacia*.

According to Homma and Suzui (*2*), bacterization with *B. cepacia* of the planting materials, such as seeds or roots, of the host plants successfully restricted soilborne pathogens. When radish seeds were submerged in 3 different concentrations of *B. cepacia*, root diseases were controlled by rapid colonization of the bacteria in the rhizosphere of the host plant. Colonization of the rhizosphere changed the quantity and quality of the rhizosphere microflora, and suppressed the pathogen in the infection court on the seed and the surface of young roots by producing antibiotics or siderophores (*38*).

Short and Lacy (*39*) determined that pea seeds exuded from 185 to 7,119 µg glucose equivalents per seed during germination. These exudates had a direct relationship to disease incidences and would most likely be a source of energy for bacteria introduced on seeds. Interestingly, Parke (*24*) questioned whether the increase in density of *B. cepacia* on pea seed would be great enough to provide biological control of *Pythium ultimum*, as higher initial densities on seeds were more effective than lower densities. Even though doubling times were shorter for lower densities compared to higher densities, lower densities did not reach levels quickly enough to suppress infection by *P. ultimum*.

In previous experiments (*1*), *B. cepacia* reduced the stand of periwinkle seedlings compared to the control in non-pathogen-amended soil. However, under severe disease pressure from *R. solani* (>90% reduction in the control), the stand of periwinkle was increased when *B. cepacia* was applied to the seeds using a carboxymethycelluluose (CMC) sticker (*11*,*12*). Improved formulations and methods of applying this bacterium to seeds in soil may improve its efficacy as a biological control for damping-off diseases. For instance, a formulation using a mixture of *Trichoderma harzianum* and *B. cepacia* incorporated into a granule and/or powder using sodium alginate, zeolite, or diatomaceous earth was used to control blight of red pepper caused by *Phytophthora capsici* (*40*). An amendment of rice in the alginate formulation increased the viability of *B. cepacia*. Suppression of the disease on red pepper was significant when *P. cepacia* was applied to the soil as a pellet when compared to direct drenching into the soil (*40*). Formulation may be the key for successful biocontrol agents because these organisms must be handled carefully to maintain viability through processing, storage, and application (*41*). In addition, lyophilized formulations of *B. cepacia* also were successfully used for control of foliar diseases (*4*). A commercialized preparation of *B. cepacia* was introduced in 1996 by CCT Corporation, 5115 Avenida Encinas, Ste. A, Carlsbad, CA (*30*). Perhaps a combination of genetic manipulation and formulation technology will allow this bacterium

to be used as a successful biocontrol agent (*42*). In fact, inhibition of *R. solani* by all strains of *B. cepacia* tested in our experiments would indicate that suppression of diseases caused by *R. solani* is a possibility. This was validated by Cartwright and Benson (*37*) using our OK-2 isolate to suppress *R. solani* stem rot of pointsettia.

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