Epiphytic Bacteria from Various Bean Genotypes and their Potential for Biocontrol of *Xanthomonas axonopodis* pv. *phaseoli*

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Abstract

Naturally occurring epiphytic non-pathogenic bacteria were isolated from reproductive tissue of various bean genotypes grown in the field, and screened for both **in vitro** *and* **in vivo** *antagonism to* ***Xanthomonas axonopodis*** *pv.* ***phaseoli*** *(formely X. campestris* pv *phaseoli). Of the 22 potential bacterial antagonists screened in vitro (at 27 + 10C) only 3 isolates exhibited antagonism to* ***X. a.*** **pv.** ***phaseoli.*** *Two of the 3 isolates were identified as* **Bacillus** spp. *and the third as* ***Pseudomonas fluorescens.*** *When screened in vivo in the green-house, all three bacterial antagonists delayed the development of common bacterial blight symptoms for 2-3 days, when spray-inoculated prior to* ***X. a.*** *pv.* ***phaseoli.*** *The rate of common bacterial blight disease development was significantly reduced. Bean plants treated with bacterial antagonists had smaller disease lesions than the phosphate buffer treated controls. These results suggest that phylloplane microflora from beans influence the development of common bacterial blight on the bean crop. These antagonists are promising potential biocontrol agents for bean common bacterial blight disease.*

Key words: Epiphytic bacteria, biocontrol, *Phaseolus vulgaris*, *Xanthomonas axonopodis* pv. *phaseoli*

Introduction

Common bacterial blight of beans caused by *Xanthomonas axonopodis* pv. *phaseoli*, Vauterin *et al*, formerly *Xanthomonas campestris* pv. *phaseoli* (E.F. Smith) Dawson is one of the most serious bean diseases in many bean growing areas of the world (Wilson and Lindow, 1993; Zaumeyer and Thomas, 1957). The bacteria usually infect leaves causing leaf blight, but may go systemic and invade the vascular tissue of the plant resulting in infection of the stems, pods and seeds (Wilson and Lindow, 1993). The worldwide distribution of this pathogen is in part associated with its ability to infect seed. Control of common bacterial blight is difficult because of the seed-borne nature of the pathogen. Short term control measures include crop rotation, the use of pathogen free seed produced in dry areas and use of resistant varieties (Wilson and Lindow, 1993; Zaumeyer and Thomas, 1957).

However, even resistant varieties may produce both infested and infected seed. Cafati and Saettler (1980) and Schuster *et al*. (1979) reported that seeds of resistant bean genotypes can become infected with *X. a.* pv. *phaseoli* to the same level as seeds from susceptible genotypes. In addition, differential reaction of tepary bean to *X. a.* pv. *phaseoli* has been reported (Zaiter *et al., 1989)*.

Aerial surfaces of plants are very selective habitats for microbial growth. Ample evidence for the occurrence of epiphytic microflora have been given (Blakeman and Brodie, 1976; Kaneda, 1986; Leben, 1965; Leben *et al.*, 1965). Some of these micro-organisms are deposits of airflora or result from the activities of insects or animals. The in-depth exploration of epiphytic microflora on various crops have stimulated an interest in managing microflora for disease control. There is an extensive literature on antagonistic interactions between epiphytic microflora and plant pathogens, leading to the need for exploring the use of such antagonistic interactions in biocontrol of plant pathogens (Elad and Kirshner, 1993; Leben,

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1964; Pandey *et al.*, 1993; Punjar, 1997; Reinecke, 1981; Wilson and Lindow, 1993). Moreover, biological control of plant disease is currently receiving increased research efforts (Balanger, *et al.*, 1994; Hussain *et al*, 1994; Laha *et al.*, , 1992; Pandey *et al.*, *1993, Utkhede and Smith, 1993) to reduce the use of chemical pesticides in agricultural systems (Johnson, 1994) and the environment in general. Biological control has become an important component of the integrated pest management systems (Agnihtri, 1992; Punja, 1997).*

Many pathogens, including *X. a.* pv. *phaseoli* grow in a non-pathogenic resident phase as epiphytes on the phylloplane of host and non-host plants (Leben, 1965; Parashar, 1993). Bacterial antagonists isolated from resident microflora populations would seem to be good candidates for controlling foliar diseases. However, interactions between *X. a.* pv. *phaseoli* and resident bacterial microflora on bean plants have not received much attention. Thus, a clear understanding of such interactions is needed to determine the potential of epiphytic bacterial flora for control of common bacterial blight.

Therefore, the objectives of this study were to isolate epiphytic bacteria from seven bean genotypes and to evaluate their potential for biocontrol of common bacterial blight of beans.

Materials and Methods

Epiphytic bacteria

Epiphytic bacteria were obtained from reproductive tissues of seven bean genotypes (Valley, I-84100, Pinto UI-114, Cranberry Taylor Hort, Charlevoix, C-20 and Black Magic) grown in the field at Sokoine University of Agriculture, Morogoro, Tanzania using a completely randomized design with four replications. Flower buds, blossoms, flat and bumpy pods were assayed for presence of non-pathogenic epiphytic bacteria. At flower bud formation, samples of 25 flower buds were taken at random from each replication using steam sterilized forceps and placed in sterile glass test tubes. Similar sampling procedures were used for blossoms and pods. Samples were kept cold at 8^0C in the refrigerator until processed within 24 hours.

Flower buds, blossoms, flat and bumpy (half grown) pods were shaken for 30 minutes in 40 ml of 0.01M phosphate buffer containing 0.01 percent Tween 20 on a horizontal shaker set at a speed of 75 x 1.5 - inch strokes per minute. The resulting washates were decimally diluted in the same buffer and 0.1 ml aliquots from each dilution were plated in triplicate on a non-sective yeast extract carbonate agar (YCA) (g/L distilled water: yeast extract (DIFCO) 10.0; calcium carbonate 2.5; bacto-agar (DIFCO) 15.0). Inoculated plates were incubated at 27 + 1^0C for up to 5 days. Single bacterial colonies of presumed bacterial epiphytes, based on colony morphology and color, were further purified by a series of transfers on YCA. Purified epiphytic bacteria were maintained in phosphate buffer (0.01M, pH 7.2)/glycerol (40%)) mixture at 5 - 8^0C. Prior to use, epiphytic bacterial isolates were streaked on Nutrient Broth Glucose Agar (NBGA) (g/L distilled water: Nutrient broth (DIFCO) 8.0; glucose 5.0, bacto-agar (DIFCO) 15.0) and incubated at 27 $\pm$ 1^0C for 5 days.

Pathogenicity tests

All epiphytic bacteria isolated were tested for pathogenicity on varieties Canadian Wonder and Charlevoix. Bean seedlings of the test varieties were grown in the greenhouse and inoculated when possessing two trifoliolate leaves (14-20 days old). Epiphytic bacterial suspensions were prepared from 24-48 hr-old NBGA cultures using 0.01M phosphate buffer, pH 7.2. The suspensions were adjusted using a Spectronic 20 colorimeter (Bausch and Lomb Company, NY.) to an optical density of 0.1 at 620, nm ((ca. 1.7-3.9 x 10^7 colony forming units (CFU)/ml). Bean seedlings were inoculated with each isolated epiphytic bacteria by infiltrating the cell suspensions abaxially by pressing the end of a 3cc disposable pre-sterilized hypodermic syringe against the forefinger-supported leaflets and slowly introducing in suspensions of epiphytic bacteria. Pathogenicity tests were done twice using four bean plants for each bacterial epiphyte. Positive and negative control bean plants were inoculated with *X. a.* pv. *phaseoli* and sterile phos-

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phate buffer, respectively. Inoculated plants were maintained in the glasshouse with temperature ranging from 25 to 30°C, and observed daily for symptom development up to 16 days.

In vitro screening for antagonism

Epiphytic bacteria isolated from bean reproductive parts were screened *in vitro* for antagonistic activity to *X. a.* pv. *phaseoli* and the brown pigment producing variant *X. a.* pv. *phaseoli* var *fuscans* from the laboratory stocks, using the overlay method (Pointius, 1983). NBGA was used for both the upper soft layer and the lower layer. Inoculum for overlays was prepared by washing 24 to 48 hr-old NBGA cultures grown at 27 ± 1°C, with phosphate buffer. Suspensions were adjusted turbidimetrically to an optical density of 0.25 at 620nm. Decimal milliliter portions of suspensions of *X. a.* pv. *phaseoli* were mixed with 4.0 ml of soft NBGA at 44°C and vortexed for 10 seconds at medium speed to obtain a uniform distribution of the pathogen, and immediately poured onto a 10 ml base layer in sterile petri plates. Inoculated plates were left in the laminar flow transfer chamber for 30 minutes to dry. After drying, 4 droplets of 2 l volume of each epiphytic bacteria were spotted onto *X. a.* pv. *phaseoli* - inoculated plates, incubated at 27 ± 1°C and observed for three days for presence of inhibition zones. Rating was done following the procedures of Pierson and Weller (Pierson and Waller, 1994). The size of the inhibition zones around each epiphytic bacteria tested was used as a measure of the ability of that strain to inhibit *X. a.* pv. *phaseoli* and the *fuscans* variant, and was scored as - = negative, no inhibition zone and the pathogen overgrew the epiphytic bacteria strain; + = a distant inhibition zone with the pathogen growth inhibited less than 6 mm from the point where epiphytic bacteria were spotted; ++ = a distant inhibition zone with pathogen growth inhibited 6-10 mm from point where epiphytic bacteria were spotted. The control plates were spotted with sterile phosphate buffer. For each epiphytic bacterial isolate four experiments were conducted, each replicated three times.

Characterization of epiphytic bacterial antagonists

Three epiphytic bacterial antagonists were subjected to physiological and biochemical tests for identification following procedures of Schaad (1998) and tobacco hypersensitivity to confirm pathogenicity tests (Klement *et al.*, *1964)*.

In vivo screening for antagonism

Epiphytic bacterial isolate which inhibited growth of *X. a.* pv. *phaseoli in vitro* were tested for ability to reduce common blight disease severity in the greenhouse. Bean plants, variety Charlevoix, were grown in the greenhouse in 10 cm diameter clay pots (one plant per pot) in a 3:1 mixture of soil: vermiculite, respectively. Inoculum was prepared from 48 hr-old cultures grown on NBGA medium as earlier described and adjusted to contain ca. 2 x 10^7 CFU/ml as estimated by plate dilution counts. Test plants were spray-inoculated with epiphytic bacteria to run-off without water-soaking, at 2-day intervals starting from when plants had 2 trifoliolate leaves. Control plants were spray-inoculated with sterile phosphate buffer. Four treatments, each replicated 5 times were used as follows: treatments 1 = control, plants sprayed with phosphate buffer; 2 = plants sprayed once with antagonists individually 2 days before inoculation with *X. a.* pv. *phaseoli*; 3 = plants sprayed twice with antagonists at 2-day intervals (2 and 4 days before inoculation); 4= plants sprayed three times at 2-day intervals (6,4 and 2 days before inoculation). After the last treatment with antagonists, test plants were challenge spray-inoculated with *X. a.* pv. *phaseoli* to run-off without water-soaking. Inoculated plants were maintained at a temperature range of 24-30°C and observed for common blight symptom development on daily basis. Disease assessment was done at 10, 14 and 18 days after inoculation using the CIAT (1987) scale of 0-9 where 0=immune, no symptoms; 9= plants dead or nearly so and 50 percent or more of the leaf area covered with lesions. The experiment was repeated twice.

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Data analysis

Data analysis was done using MSTAT-C. Data from the three experiments were pooled for analysis of variance (P = 0.05) and Duncan's new multiple range test was used to separate differences between means.

Results

Pathogenicity tests

A total of 22 epiphytic bacteria were isolated from reproductive parts of 7 bean genotypes and tested for pathogenicity on bean varieties Canadian Wonder and Charlevoix. All 22 isolates were non-pathogenic on the tested bean plants (Table 1).

In vitro screening for antagonism

Of the 22 bacterial epiphytes isolated and tested individually for *in vitro* inhibition of *X. a.* pv. *phaseoli* strain MI-7 and *X. a. phaseoli* var. *fuscans* strain MI-5, only 3 isolates were positive (Table 2). Inhibition zones were observed around colonies of the epiphytic bacterial isolates 4, 8 and 13, with inhibition zones of 2.1, 6.5 and 6.3 mm, respectively (Figure 1A & B). These strains were isolated from flower buds, blossoms and flat pods. Isolate No. 8 provided larger inhibition zones than the other two isolates that were positive for antagonism. All three bacterial epiphytes equally suppressed growth of *X. a.* pv. *phaseoli* strain MI-17 and *X. a.* pv. var. *fuscans* strain MI-5 (Table 2). These observations were consistent over all four experiments conducted. On the basis of these results, strain No. 8 performed the best of all the three bacterial epiphytes tested. Effective epiphytic bacterial isolates were used in further experiments in the glasshouse.

Characterization and identity of epiphytic bacterial antagonists

The 3 epiphytic bacterial antagonists were subjected to physiological and biochemical tests for identification. Strain No. 4 and 13 were identified as *Bacillus spp*. based on positive Gram reaction, facultatively anaerobic, spore forming rod-shaped cells, non-pathogenic to bean and produced no hypersentive reaction on tobacco. Strain No. 8 was identified as *Pseudomonas fluorescens*. This strain was Gram negative rod, aerobic, produced fluorescent pigment

Table 1: Origin and pathogenicity tests of epiphytic bacteria used in the current study.

Isolate Number	Source (genotypes)	Isolated From	Pathogenicity on bean[Y]
1	Black Magic	Blossoms	[z]
2	Black Magic	Blossoms	
3	Black Magic	Flower buds	
4	Black Magic	Blossoms	
5	C - 20	Flower buds	
6	C - 20	Flower buds	
7	Cranberry Taylor Horn	Bumpy pods	
8	Cranberry Taylor Horn	Flat pods	
9	Pinto UI-114	Flower buds	
10	Charlevoix	Blossoms	
11	I-84100	Bumpy pods	
12	I-84100	Flat pods	
13	Valley	Flower buds	
14	Valley	Blossoms	
15	I-84100	Blossoms	
16	I-84100	Blossoms	
17	I-84100	Flower buds	
18	I-84100	Blossoms	
19	I-84100	Flat pods	
20	C - 20	Flat pods	
21	Pinto UI-114	Bumpy pods	
22	Black Magic	Bumpy pods	

[Y]pathogenicity was tested by infiltrating leaflest of bean plants of varieties Canandian Wonder and Charlevoic with bacterial suspencions adjusted to an potical density of 0.1 at 620nm (ca. 1.7 - 3.9 x 107 CFU/ml). [Z]=Non-pathogenic, negative host reac-

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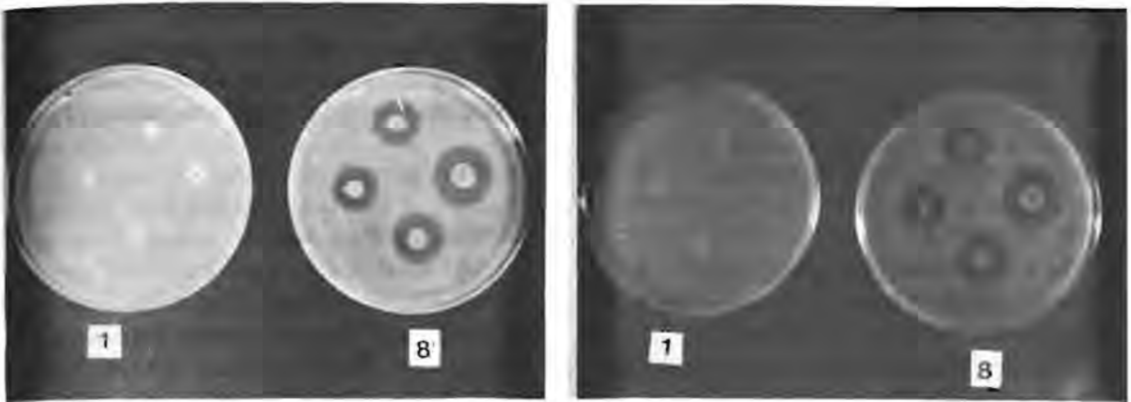

Figure 1: Inhibition zones of *Xanthomonas axonopodis* pv. *phaseoli* (A) and *X. a.* pv. *phaseoli* var. *fuscans* (B) around colonies of bacterial epiphyte No. 8., bacterial epiphyte No. 1 in the same lawn is without inhibition zones.

Table 2: In vitro screening of epiphytic bacteria from seven bean genotypes for ability to inhibit growth of *Xanthomonas axonopodis* pv. *phaseoli* (strain MI-17) and X.a. Pv. *phaseoli* var. *fuscans* (strain MI-5).

Isolate Number	Inhibition X zone	Size of Inhibition zone (mm)YZ	
		Strain MI-17	Strain MI - 5
1	-	-	
2		-	
3	-		
4	+	2.1	2 3
5		-	-
6	-		-
7		-	-
8	+ +	6 5	6.4
9	-	-	-
10	-		-
11			-
12			-
13	+ +	6.3	6.1
14	-	-	
15		-	
16	-	-	
17		-	
18		-	-
19			-
20			-
21			
22			-

[X] = No inhibition zone observed; + inhibition zone present but less than 6 mm; ++ = inhibition zone ranging between 6 - 10mm; [Y]Strain MI-17 = *Xanthomonas axonopodis pv. phaseoli*, and strain MI-5 = X.a.pv. *phaseoli var. fuscans;* [Z]means of four experiments each replicated three times.

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on Kings *et al.*, (1954) medium B (KB), was not pathogenic on bean and produced no hypersensitive reaction on tobacco. It was oxidase and arginine dihydrolase positive, hydrolized gelatin, but not starch. The compounds B-alamine, DL-arginine, glucose, m-inositol, trehalose and L-valine were also utilized as carbon sources by strain No. 8.

***In vivo* screening for antagonism.** In order to verify results from *in vitro* studies on the effect of the three epiphytic bacterial antagonists on *X. a.* pv. *phaseoli*, greenhouse experiments were conducted. Results from greenhouse experiments are shown in Table 3. On the basis of these experiments, bean plants treated four times with antagonists before challenge inoculation with *X. a.* pv. *phaseoli* had significantly reduced common blight disease severity (P = 0.05) for all the three epiphytic bacterial antagonists used (Table 3). Compared to the phosphate buffer - treated controls, bean plants treated with epiphytic bacterial antagonists had smaller lesions initially, but the lesions enlarged slowly with time. Development of symptoms on bean plants treated with antagonists was delayed for an average of 2-3 days as compared to control plants. Control plants which received phosphate buffer were affected by the disease much more rapidly and more severely than plants treated once with bacterial antagonists (Table 3).

Discussion

Common bacterial blight of beans is generally managed through preventive measures such as the use of pathogen-free seed and tolerant varieties (Cafati and Saettler, 1980). Interactions between *X. a.* pv. *phaseoli* and resident bacterial microflora on bean plants have not received attention. Based on results from the current investigation, it is hypothesized here that epiphytic bacterial on bean plants may play a role in suppression of common bacterial blight disease. The study has demonstrated that part of the epiphytic microflora that occur on reproductive tissues (flower buds, blossoms and pods) have the potential for biocontrol activity. Out of 22 bacterial epiphytes isolated from seven bean genotypes and screened for antimicrobial activ-

Table 3: The effect of Bacillus sp. (4,13) and Pseudomonas Fluorescens (8) on the development of common bacterial blight on beans in the greenhouse.

Antagonist identity number	Treatment Number[X]	Disease severity rating/Days after inoculation[YZ]		
		10	14	18
4	1	4.1a	7.1a	8.5a
	2	4.7a	6.9a	7.7ab
	3	3.3a	4.3b	6.7bc
	4	1.8b	3.1b	6.5c
8	1	5.2a	7.2a	8.3a
	2	4.1a	7.9a	8.5a
	3	3.7a	7.0a	7.9a
	4	1.0b	3.6b	4.7b
13	1	6.7a	8.4a	8.9a
	2	4.7ab	7.3ab	8.5ab
	3	5.5ab	7.8ab	8.8a
	4	2.9b	6.5b	7.9b

[X]Treatments: 1=Control, plants sprayed with phosphate buffer; 2= plants sprayed once with antagonists two days before inoculation with X.a.pv. Phaseoli; 3 = plants sprayed twice with antagonists at 2-day intervals two and four days before inoculation; 4 = plants sprayed three times at 2-days interval (2,4 and 6 days before inoculation).
[Y]Disease was rated on a 0-9 scale: 0 =immune, no symptoms, 9= plants dead or nearly so, 50 percent or more of leaf area covered with lesions. Data are means of three experiments.
[Z]for each bacterial antagonist, means within the same column followed by the same letter are not significantly different (P = 0.05) according to Duncan's new multiple range test.

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ity against *X. a. phaseoli*, only 3 (isolates) were positive for such activity. These were identified as *Pseudomonas fluorescens* and *Bacillus* spp. Thus, the potential use of these bacteria for biological control of common bacterial blight of beans deserves attention.

Strains of *Pseudomonas fluorescens* have frequently been suggested and used as important natural antagonist of plant pathogens. These bacteria have been considered very useful, particularly to microbial suppression of the take-all fungus *Gaeumannomyces graminis* var. *tritici* following wheat monoculture and in various experiments with other pathogens (Hussain *et al.*, 1994; Pierson and Waller, 1994; Schippers, 1993; Wilson and Lindow, 1993). The bacteria is commonly found in the soil and also can compose a significant part of the bacterial microflora of plant rhizosphere. This work has revealed that *P. fluorescens* and *Bacillus spp.* can be a significant part of the phylloplane microflora of the bean plants, specifically on flower buds, blossoms and pods.

In greenhouse studies, significantly greater ($P = 0.05$) biological control activity was observed in plants treated three times at 2-day intervals with *P. fluorescens* and *Bacillus spp.* individually than in plants treated once. This repeated spraying significantly reduced the amount of disease. Several reasons may account for such results. The ability of biocontrol agents to survive and establish an active population in the phylloplane has been reported to be affected by phyllosphere inhabitants, nutrient availability and microclimatic conditions (Schippers, 1993). Therefore, repeated treatment increased the active population of these biocontrol agents on the phylloplane as compared to single application. Such findings have been observed by other workers who described the correlation between fungal antagonists inoculum dosage and percentage of disease reduction (Tosi and Zezzerini, 1994). The current results seem to suggest that the ability of epiphytic bacterial antagonists to control common bacterial blight infection may be related to the production of antibiotic substances. This work has also revealed that the higher the population of the biocontrol agent on the phylloplane, the better the control of disease (Table 3). However, further detailed population dynamic studies of *P. fluorescens* and *Bacillus* sp. will be needed to better understand the actual behavior of these biocontrol agents on phylloplane of bean plants.

Antagonism of micro-organisms to pathogens has been observed to involve a variety of ways (Elad *et al.*, 1994). Gueldner *et al.*, (1988) found that *Bacillus subtilis* expressed its antagonism to *Monilinia fructicola* by the production of turin antibiotic. These workers found that *Pseudomonas fluorescens* strain 2-79 produced antibiotic phenazins, anthranilic acid and siderophores inhibitory to many plant pathogens. Siderophores are well known competitors for iron with pathogens to an extent which depends on the environment (Tosi and Zazzerini, 1994). In the current investigation, results from laboratory studies indicate that antibiosis is one of the mechanisms involved in the suppression of *X. a.* pv. *phaseoli* by both *Bacillus spp* and *Pseudomonas fluorescens* (Figure 1).

Understanding the actual mechanisms involved in the biocontrol of *X. a.* pv. *phaseoli* by the current biocontrol agents is crucial for developing and improving application methods and strategies. Therefore, further studies are needed to determine precisely the actual mechanisms involved, to screen more effective isolates and to develop better formulations for introducing bioprotectants on bean plants to enhance their survival. It is hoped that this work will be an added stimulant to plant pathologists to consider such investigations. The systemic nature of *X. a.* pv. *phaseoli* in beans also calls for a search for endophytic antagonists that would complement epiphytic antagonists in biocontrol of common bacterial blight of beans.

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