Brazilian Journal of Microbiology (2006) 37:451-455
ISSN 1517-8382

CURTOBACTERIUM FLACCUMFACIENS PV. *FLACCUMFACIENS* DETECTION IN BEAN SEEDS USING A SEMI-SELECTIVE CULTURE MEDIUM

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Submitted: March 20, 2006; Returned to authors for corrections: May 15, 2006; Approved: October 06, 2006

ABSTRACT

The bacterial wilt caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* is currently considered one of the most important bacterial bean disease in Brazil. One of the most effective control methods against this disease is the use of healthy seeds. However, no methods are known that could be routinely used to detect this bacterium in bean seeds under Brazilian condition. The aim of this work was to evaluate qualitative and quantitative detection methods for *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in naturally-infected bean seeds, and the detection of this pathogen in thirty bean seed samples, by sowing onto a semi-selective culture medium the leachate obtained from soaked bean seeds. Both the qualitative and quantitative methods were effective for detecting the presence of the bacteria in the seeds samples analysed. The qualitative method proved more practical for rotine use; of the thirty bean seed samples analyzed by this method, fifty percent were infected with *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*.

Key words: bacterial wilt, isolation, seed pathology, *Phaeolus vulgaris*

INTRODUCTION

Bean bacterial wilt, caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cff), was first described in the USA in 1921 by Hedges (6), causing serious problems to the crop. This disease occurs in several European countries, as well as in Australia, Canada, Mexico, and Colombia (2). In Brazil, bacterial wilt has been verified in several regions resulting in loses in bean production (8,9,18).

Typical symptoms of the disease in bean plants are mainly wilting, vascular darkening, and death of the above-ground part of the plant (7). Under field conditions during mild-temperature seasons infected bean plants have developed that lack bacterial disease symptoms. This fact has been observed by Thomas and Graham (17), who isolated *Xanthomonas axonopodis* pv. *phaseoli* and Cff from bean plant stems without external symptoms. Other plants, in addition to beans, have been reported as Cff hosts including pea, soybean, *Phaseolus lunatus*, *Lupinus polyphyllus*, *Vigna cylindrica*, *V. sesquipedalis*, *Dolichos labllab*, *Phaseolus radiatus*, *P. lathyroides*, *P. calcaratus*, and *P. acutifolius* (7,15).

In bean plants, some control measures are employed against bacterial wilt include the use of healthy seeds, since Cff survives and is transmitted by seed (13,15). Burkholder (3) verified that Cff survived for 24 years in bean seeds stored under natural environmental conditions.

In practice, few methods are described in the literature for Cff detection in bean seeds. The European and Mediterranean Plant Protection Organization recommends a visual examination of seeds, direct or indirect isolation of the bacterium, and a serum test for Cff detection in bean seeds for quarantine purposes (4). In Japan, again for quarantine purposes Mizuno (11) and Mizuno and Kawai (12) recommended the isolation of Cff from bean seeds in semi-selective culture media, which use specific carbon sources and antimicrobial agents, together with serum tests but these media are very expensive for routine use in Brazil (10). Tegli *et al.* (16) developed specific primers for Cff that can be used for

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detection of this bacterium in naturally-infected bean seeds, by the PCR technique.

Considering the necessity for the development of a practical, effective, and low-cost method for the routine analysis of bean seeds in Brazil, the present work was carried out using Cff isolation in a semi-selective culture medium developed for this purpose (10).

MATERIAL AND METHODS

Comparison of methods for *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* detection in bean seeds

Quantitative and qualitative Cff analysis were carried out in six 200 g samples of bean seeds (approximately 1,000 seeds), cultivar Campeão II, from commercial field where the occurence of bacterial wilt was verified. Each sample containing 200g of seeds was soaked in 600 mL distilled and sterilized water for 24h, under refrigeration (5ºC). Following soaking, the seeds were manually stirred in flasks and the leachate liquid was sampled. For the quantitative analyse, 100 µL of the leach liquid obtained from the seeds and their dilutions (10^{-1}, 10^{-2}, and 10^{-3}) were sown onto the surface of CFFSM culture medium (10) with the aid of a Drigalski spatula. Four Petri dishes were sown for each concentration. In the qualitative evaluation, however, the seed leachate (same of quantitative analysis) was sown by streaking with a loop onto the surface of the CFFSM semi-selective culture medium. Four Petri dishes were sown, in two halves per each plate, totaling eight halves.

The Petri dishes remained under incubation at 28-30ºC, for 96 to 120h, and colonies with cultural characteristics resembling Cff (colonies circular shape, with yellow to slighty orangish coloration; casein hydrolisis and slight fading of dye arround the colonies) were compared against the growth of a pure standard isolate (Feij-2634). Six bacterial isolates from each seed sample were selected for identification.

CFFSM semi-selective culture medium

This medium consisting of peptone - 5g, meat extract - 3g, sucrose - 5g, agar - 15g, skim milk powder* - 5g, Congo red* - 0.05g, chlorothalonil* - 0.01g, thiophanate methyl* - 0.01g, nalidixic acid* - 0.01g, nitrofurantoin* - 0.01g, oxacillin* 0.001g, sodium azide* - 0.001g, and distilled water q.s. 1L, *added after autoclaving the basal medium, according Maringoni *et al.* (10).

Curtobacterium flaccumfaciens pv. *flaccumfaciens* isolation in naturally infected bean seeds

Thirty seed samples from several regions of Brazil were analized for the presence of Cff. Twenty-two samples were analyzed twice, and eight samples were analyzed once. Five 200g subsamples were evaluated for each seed sample. Each subsample was transferred into a flask containing 600 mL distilled and sterilized water. The seeds were left in soaking for 24h, under refrigeration. After soaking, the seeds were manually stirred and the resulting suspension was plated by streaking on Petri dishes containing CFFSM culture medium. Four Petri dishes were sown for each 200g subsample, in two halves per each plate, totaling eight halves per subsample. The Petri dishes remained under incubation for a variable period between 96 and 120h, at 28 - 30ºC. After incubation, observations were made for bacterial colonies that showed cultural characteristics which resembled Cff when compared with a pure standard isolate (Feij-2634). A given seed sample was considered to carry Cff when Cff growth was observed in at least one of the eight sown fields. Two to four bacterial isolates from each positiv sample were selected to be submitted for identification.

Identification of bacterial isolates obtained from seeds

Cff-suspected bacterial colonies were initially purified in a nutrient sucrose-agar medium (NSA) containing sodium chloride at 7%, and evaluated using Gram-staining, KOH test, pathogenicity test in cultivar Pérola bean plants, and identification by the Biolog® method (Biolog, Hayward, USA) previous described by Maringoni *et al.* (10).

RESULTS AND DISCUSSION

The presence of Cff in the bean seeds analyzed was observed, regardless of the method used quantitative or qualitative (Table 1). Both methods were effective to isolate Cff-suspected colonies in the seeds. All isolates were identified as Cff by the Biolog® method, regardless of their pathogenicity (Table 1). Three Cff isolates were not pathogenic to bean plants.

The analysis of thirty bean seed samples from several localities in Brazil revealed that fifty percent of them were infected with Cff (Table 2). All suspected bacterial isolates were identificated as Cff, by the Biolog® method. Seven out of the ninety-seven bacterial isolates submitted to the pathogenicity test were non-pathogenic to Cultivar Pérola bean plants (Table 2). All suspected isolates showed cultural characteristics similar to Cff on the CFFSM culture medium (yellow colonies and the presence of casein hydrolysis and Congo red fading) Figure 1, and grew in NSA medium containing sodium chloride at 7%, were Gram-positive rods and did not form a string in the KOH test.

Although a specific standard method for Cff detection in bean seeds does not exist in Brazil, especially for routine analysis, the methodology herein employed was effective to isolate this bacterium from naturally-infected bean seeds. The method of plating on a semi-selective culture medium is used in the detection of a number of phytopathogenic bacteria in seeds of several crops, and usually the seed leachate dilutions, either concentrated or not by centrifugation, are sown onto specific culture media as, for example, *Pseudomonas savastanoi* pv. *phaseolicola* in bean seeds (19), *Xanthomonas translucens* pv. *translucens* in wheat seeds (14), and *Clavibacter*

Table 1. Recovery of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* from bean seeds by two methods.

Sample	Quantitative method	Qualitative method	Pathogenicity (Nº. pathogenic isolates /Nº inoculated isolates)	Similarity index value (Biolog®)[a]
	Cff-suspected colonies ($CFU.mL^{-1}$)	Nº fields containing Cff-suspected colonies/Nº fields sown		
A	$1.87 \times 10^2 \pm 0{,}41$	1/8	6/6	0.710-0.844
B	$1.39 \times 10^4 \pm 0.28$	7/8	5/6	0.844
C	$7.48 \times 10^4 \pm 0.26$	8/8	6/6	0.730-0.865
D	$3.54 \times 10^4 \pm 0.25$	8/8	6/6	0.844
E	$6.10 \times 10^3 \pm 2.46$	7/8	6/6	0.860
F	$8.90 \times 10^4 \pm 0.81$	8/8	4/6	0.775-0.810

[a]Range of the similarity index value of isolates compared against *C. flaccumfaciens* database.

Table 2. Recovery of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* from naturally-infected bean seeds collected from several localities in Brazil.

Sample	Locality (State)	Cff-suspected isolates[a]	Pathogenicity (Path/inoc)[b]	Similarity index value (Biolog®)[c]
F-1	São Paulo			
A[d]		+ (1)	3/3	0.780-0.978
B		+ (1)	3/3	0.821-0.876
F-3	São Paulo			
A		+ (1)	2/2	0.876-0.926
B		+ (2)	2/2	0.844-0.943
F-11	São Paulo			
A		+ (4)	4/4	0.713-0.860
B		+ (5)	3/4	0.775-0.978
F-12	São Paulo			
A		+ (3)	4/4	0.756-0.876
B		+ (2)	4/4	0.735-0.926
F-13	São Paulo			
A		+ (3)	3/3	0.750-0.775
B		+ (4)	4/4	0.775-0.926
F-15	Paraná			
A		+ (3)	3/3	0.860-0.926
B		+ (3)	3/3	0.775-0.779
F-19	Paraná			
A		+ (5)	5/5	0.741-0.802
B		+ (5)	5/5	0.741-0.860
F-20	São Paulo			
A		+ (3)	4/4	0.659-0.741
B		+ (2)	3/3	0.730-0.738
F-21	Paraná			
A		+ (2)	3/4	0.730-0.736
B		+ (5)	2/4	0.720-0.730

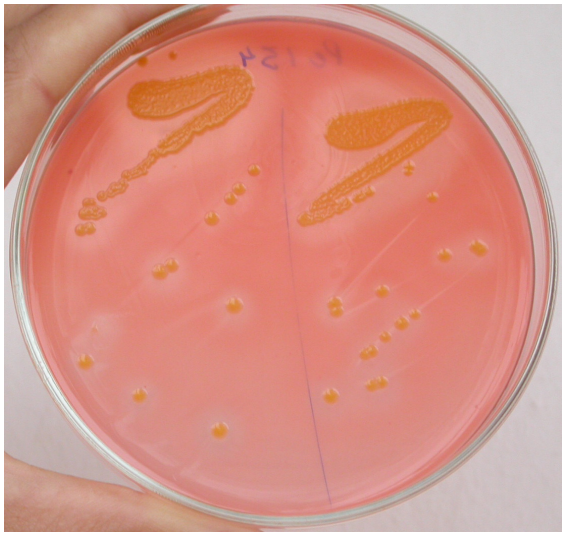

Figure 1. Cultural characteristics of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* isolated from bean seeds on the CFFSM culture medium.

michiganensis subsp. *michiganensis* in tomato seeds (5). Although the literature does not contain references about the direct sowing of seed leachate suspensions by streaking onto the surface of the culture medium (qualitative analysis) in order to isolate phytobacteria in seeds, this procedure proved as viable, since the isolation of Cff from the seed samples analyzed was consistent.

It was verified the presence of Cff in fifty percent of the thirty seed samples analyzed from several regions of Brazil (Table 2). A lower number of seed samples infected by Cff was expected, but the results here obtained confirmed the widespread dissemination of this pathogen in several bean-producing regions in Brazil (8,9,18).

F-22	Goiás			
A		+ (4)	3/4	0.720
B		+ (3)	3/3	0.720-0.802
F-24	São Paulo			
A		+ (2)	4/4	0.698-0.735
B		+ (3)	4/4	0.720-0.736
F-25	Rio Grande do Sul			
A		+ (2)	2/4	0.735-0.736
B		+ (1)	4/4	0.720-0.736
F-28	São Paulo			
A		+(4)	4/4	0.720
F-29	Paraná			
A		+(1)	2/2	0.720-0.735
F-30	Goiás			
A		+(4)	4/4	0.775-0.912
F-6, F-7, F-8, F-9, and F-10 (A)	Goiás, Paraná, Rio Grande do Sul, and São Paulo	-		
F-2, F-4, F-5, F-14, F-16, F-17,F-18, F-23,F-26, and F-27 (A and B)	Goiás and São Paulo	-		

[a]Number of subsamples containing Cff-suspected isolates; [b]Number of pathogenic isolates/number of inoculated isolates; [c]Range of the similarity index value of isolates compared against *C. flaccumfaciens* database; [d]One (A) or two (A and B) analysis.

The bacterial isolates submitted to the Biolog ® test were identified as Cff. However, 7.5% of the Cff isolates were non-pathogenic in the pathogenicity test. The non-pathogenicity of those isolates may be due to the mixture of both pathogenic and non-pathogenic Cff in the diseased bean plants, which would transmit them into their seeds, with the non-pathogenic strains having an endophytic status, similarly as *Curtobacterium flaccumfaciens* in citrus plants (1).

ACKNOWLEDGEMENTS

The authors express thanks for the financial support received from Fundação ao Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and FUNDUNESP in the conduction of the present research.

RESUMO

Detecção de *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* em sementes de feijoeiro utilizando meio de cultura semi-seletivo

Atualmente, a murcha-de-curtobacterium, causada por *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, é considerada uma das principais doenças baterianas da cultura do feijoeiro, no Brasil. Um dos métodos eficazes de controle desta doença é o emprego de sementes sadias. Entretanto, não se tem conhecimento de métodos que possam ser utilizados em rotina para o isolamento desta bactéria em sementes de feijoeiro. O presente trabalho teve por objetivo avaliar os métodos qualitativos e quantitativos de isolamento de *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* em sementes de feijoeiro naturalmente infectadas e a detecção deste patógeno em trinta amostras de sementes de feijoeiro, pela semeadura do líquido de maceração das sementes em meio de cultura semi-seletivo. Tanto o método quantitativo quanto o quantitativo foram eficazes em detectar a presença da bactéria nas amostras de sementes analisadas. O método qualitativo mostrou-se mais prático para o uso em rotina e das trinta amostras de sementes de feijoeiro analisadas por este método cinqüenta porcento delas estavam infectadas com *Curtobacterium flaccumfaciens* pv. *flaccumfaciens.*

Palavras-chave: murcha-de-curtobacterium, isolamento, patologia de sementes, *Phaeolus vulgaris*

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