PLANT-TO-SEED TRANSMISSION OF *CURTOBACTERIUM FLACCUMFACIENS* pv. *FLACCUMACIENS* IN A DRY BEAN CULTIVAR

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SUMMARY

Bacterial wilt is a disease that causes serious bean crop losses in Brazil, and its causal agent, the bacterium *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*), is seed transmitted. Recommendations for managing the disease in the field include the use of pathogen-free seed, crop rotation, and resistant cultivars of dry bean. Transmission of *Cff* from plant to seed was evaluated in three assays with six different dry bean cultivars (IAC Carioca, IAC Carioca Aruã, IAC Carioca Akytã, IAC Carioca Pyatã, IAC Carioca Tybatã, and Pérola). Plants of these cultivars were inoculated with a *Cff* isolate by stem puncture and the disease symptoms were evaluated using a scale. To assess bacterial transmission to seeds, three assays were made analysing in each 500 seeds of the cultivars IAC Carioca, IAC Carioca Aruã, IAC Carioca Akytã, IAC Carioca Pyatã, and IAC Carioca Tybatã respectively, whereas for the cv. Pérola 46, 155, and 87 seeds were analysed in the first, second, and third assay, respectively. These seeds were macerated individually in distilled and sterilized water, and soaked for 24 h at 5°C. The resulting suspension was streaked on Petri dishes containing semi-selective medium for *Cff*, and incubated at 28°C for 96 to 120 h. Typical colonies for *Cff* were purified on 7% NSA+NaCl medium, after which Gram staining, KOH, and pathogenicity tests were conducted. Isolates from the first and second assays were characterized by Microlog2™, and those of the third assay with PCR. Results showed that on cvs IAC Carioca Aruã, IAC Carioca Akytã, IAC Carioca Pyatã, and IAC Carioca Tybatã low levels of disease developed, indicating resistance to bacterial wilt, whereas cvs Pérola and IAC Carioca were highly susceptible in all the assays. With respect to transmission of *Cff* from plants to seeds, cvs IAC Carioca Akytã, IAC Carioca Pyatã, and IAC Carioca Tybatã showed no transmission, whereas IAC Carioca Aruã showed a 5.5-14.8% level of transmission. Cvs IAC Carioca and Pérola showed the highest levels of transmission, namely 10.4-70% and 32.61-74.2%, respectively.

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INTRODUCTION

Dry bean (*Phaseolus vulgaris* L.) is grown throughout Brazil and is the main source of protein in the Brazilian diet. The crop however is exposed to many diseases that can decrease production and quality (Sartorato, 2006). Various pathogens of this crop are known, including the bacterium *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) (Hall, 1991). Measures to control infection by this bacterium are based on using healthy seeds, crop rotation, and resistant cultivars (Maringoni, 2002; Maringoni and Camara, 2006; Maringoni *et al.*, 2006).

Studies on seed transmission of different cultivars are few. Knowledge regarding this is important, as the seeds are the source of initial inoculation in the field (Saettler, 1991). Under artificial inoculation, the transmission of *Cff* from bean plants to seeds is in the order of 51.4 to 52.5%, with additional negative effects on germination (Chavarro *et al.*, 1985). Schuster and Smith (1983) observed a rate of transmission from seeds to seedlings ranging from 83.2 to 98.2%, as well as a drastic effect on the emergence of seedlings (49-76% of non-emergence).

Since *Cff* is a vascular pathogen, differences in transmission capacity of the bacteria from diseased plants to seeds are likely to exist among dry bean cultivars with differing levels of resistance. This has been observed in some cultivars with different levels of resistance to common bacterial blight by *Xanthomonas axonopodis* pv. *phaseoli* (Maringoni *et al.*, 1993; Torres and Maringoni, 1997).

Hsieh *et al.* (2005) analyzed the reaction of 124 bean genotypes to bacterial wilt and found high levels of resistance in the strains L02E317, L02B662, and 9995-2 for yellow and orange isolates of *Cff*. In Brazil, good levels of resistance were found in cvs IAC Carioca Akytã, IAC Carioca Aruã, and IAC Carioca Pyatã (Maringoni, 2002; Souza *et al.*, 2006), and IAC Carioca Tybatã (Souza *et al.*, 2006). Always in Brazil, Theodoro *et al.* (2007) analyzed the reaction of 73 dry bean cultivars collected in State of Santa Catarina to bacterial wilt and found higher levels of resistance in the cvs Mouro Piratuba and Vagem Amarela. Cultivars with higher levels of resistance to bacterial wilt under artificial moculation were more vigorous than the more susceptible ones (Maringoni, 2003).

Dry bean cultivars with high resistance to bacterial wilt when inoculated by puncture of the cotyledonary node (Schuster *et al.*, 1964) or by high-pressure spraying of the leaflets (Halluka *et al.*, 1978) presented a lower amount of bacteria in the tissues compared to susceptible cultivars.

Since it is likely that a difference in *Cff* transmission from plant to seed exists in dry bean with different levels of resistance, a study was carried out to investigate this aspect in six dry bean cultivars with differing levels of resistance, in three assays in three different periods under greenhouse conditions.

MATERIAL AND METHODS

Assays. Three assays were conducted under greenhouse conditions in 2004, 2005, and 2006. In the first, the bean cultivars used were IAC Carioca, IAC Carioca Aruã, IAC Carioca Akytã, IAC Carioca Pyatã, and Pérola. In the second and third assays all these cultivars were tested plus IAC Carioca Tybatã. Seeds from the different cultivars were germinated in Germ test paper until radicle emergence and were transplanted in 8 liter pots (three plants per pot) containing autoclaved substrate with fertilizer. They were inoculated nine days after emergence with an aggressive *Cff* isolate (Feij 2634, FCA/UNESP, culture collection), by the stem puncture method using a needle dipped in bacterial colonies grown in NSA medium for 96 h at 28°C (Maringoni, 2002). Each experimental plot was represented by one pot randomly placed in the arrangement of blocks, with five repetitions. The plants were watered daily and sprayed weekly with pesticides recommended to control mites and insects.

Disease symptoms were evaluated 37 days post-inoculation using an arbitrary scale (Maringoni, 2002), as follows: 0 = no symptoms; 1 = mosaic on the leaves; 3 = some wilted leaves (1 to 3 leaves, less than 10% of the leaves); 5 = approximately 25% of the leaves wilting or turning yellow; 7 = approximately 50% of the leaves wilted, yellow, and with necrosis of the leaflets, plants stunted; 9 = approximately 75% or more of the leaves wilted or necrotic, premature loss of leaves, severe stunting and/or death of the plant. The plants were maintained until seeds were produced. These were collected, dried, and stored under refrigeration (5-8ºC) for detection of *Cff*.

Detection of *Cff* in the seeds. In the first assay, 500 seeds of each of the cultivars IAC Carioca, IAC Carioca Aruã, IAC Carioca Akytã, and IAC Carioca Pyatã were macerated individually in 5 ml of distilled and sterilized water, and soaked for 24 h at 5ºC. For cv. Pérola 46 seeds were analyzed. In the second and third assays, 500 seeds of each of the cvs IAC Carioca, IAC Carioca Aruã, IAC Carioca Akytã, IAC Carioca Pyatã, and IAC Carioca Tybatã were analyzed, whereas for the cultivar Pérola, 155 and 87 seeds were analyzed in the second and third year, respectively. A loopful of the suspensions from macerated seeds was streaked on the surface of the semi-selective medium MSCFF, composed of 5 g peptone (Difco Bacto, USA), 3 g meat extract (Difco), 5 g sucrose (Difco), 15 g agar (Oxoid, UK) in 1 litre of distilled water, with the following added after autoclaving in basal medium, 5 g skim milk power (Difco, USA), 0.05 g Congo red (Difco, USA), 0.01 g chlorothalonil, 0.01 g thiophanate-methyl, 0.01 g nalidixic acid (Difco, USA), 0.01 g nitrofurantoin (Dfico, USA), 0.001 g oxacillin, 0.001 g sodium azide (Difco, USA) (Maringoni *et al.*, 2006). Plates were then incubated at 28-30°C for 96 to 120 h, colonies with characteristics similar to *Cff* were purified in 7% NSA+NaCl medium (15 g agar, 5 g sucrose, 5 g peptone, 3 g meat extract, 70 g sodium chloride), then submitted to differential Gram staining, KOH test, and pathogenicity tests on the cv. Pérola

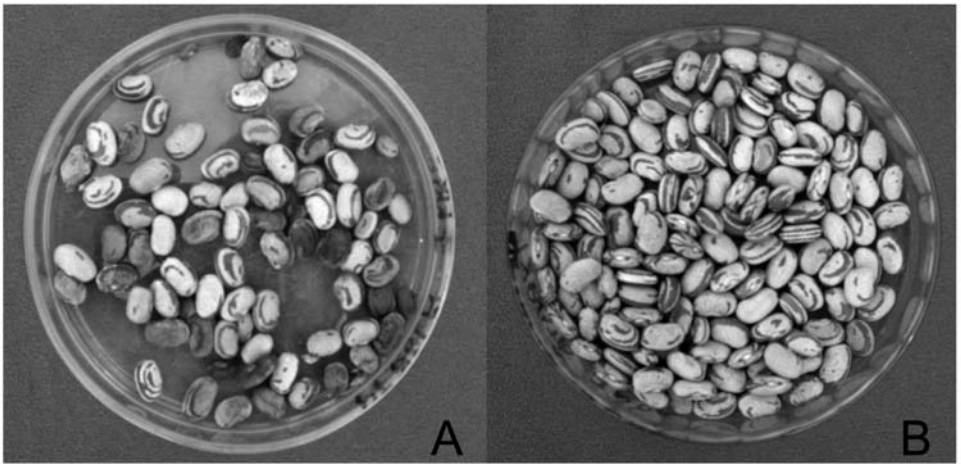

Fig. 1. Aspect of the seeds of dry bean cultivars IAC Carioca (A) and IAC Carioca Tybatã (B) from plants inoculated with *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*.

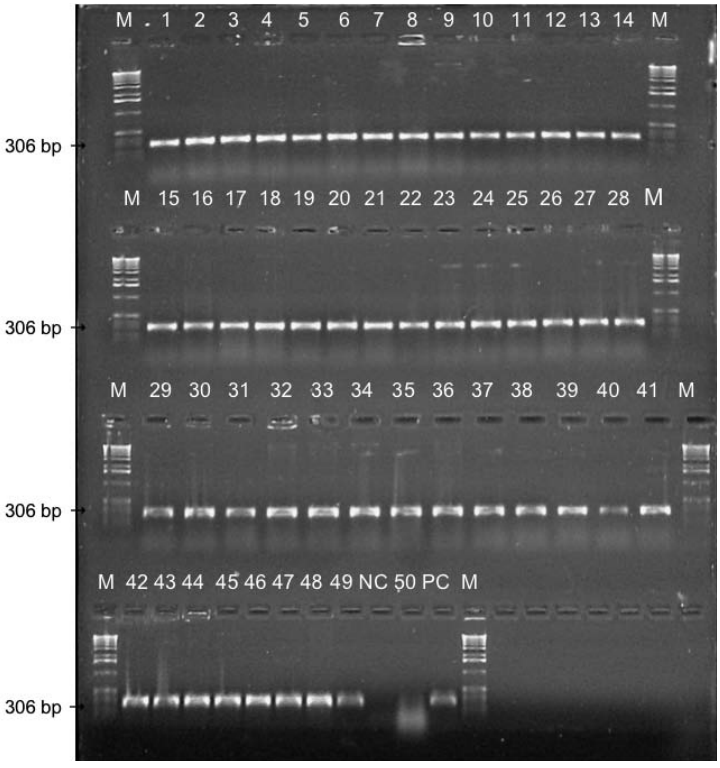

Fig. 2. Agarose gel eletrophoresis of PCR products obtained with primers CffFOR2-CffREV4 specific to *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*. Lanes 1 to 49, isolates obtained from seeds; lane 50, *Pseudomonas fluorescens*; lane PC, positive control; lane NC, negative control; M, molecular marker.

(Maringoni and Camara, 2006; Maringoni *et al.*, 2006) to determine the percentage of *Cff* transmission

Characterization of bacterial isolates obtained from seeds. *Microlog2*^{TM}. From the first and second assay, 56 and 27 isolates with culture characteristics typical of *Cff*, were further characterized. The isolates were cultured in NSA medium for 48 h, then cultured twice in Biolog Universal Growth (BUG^{TM}) Agar for 24 h at 30 °C. Bacterial colonies were transferred and homogenized in inoculant fluid, and the resulting suspension was standardized by colorimetry to 20% transmittance at 600 nm wavelength. One hundred and fifty microliters of bacterial suspension were deposited in each of the microplate wells (GP2 MicroPlate^{TM}) containing 95 different carbon sources. The material was incubated in a moist chamber at 30ºC for 24 h. Following incubation, readings were made, plotted and analyzed using the MicroLog2^{TM} System. Results were expressed as an index of similarity.

PCR. Of the bacterial isolates obtained in the third assay, 49 were selected, cultured on NSA culture medium, and following growth on the plate, were transferred to 5 ml of nutrient broth medium (NB), and kept at 30°C for 72 h (approximately 10^9 CFU/ml). Two milliliters of bacterial suspension were centrifuged at 8,000 *g* for 2 min. The sediment obtained was resuspended and washed in two successive centrifugations (8,000 *g* for 5 min) in 1 ml of sterile distilled water. Following the second wash, pellets were resuspended in 50 µl TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) and the tubes containing the suspensions were autoclaved for 1 min at 120°C. The autoclaved suspension was centrifuged (12,000 *g* for 5 min) and the supernatant collected and stored at -20ºC for later use as a DNA template for PCR (Simmon *et al.*, 2004).

The primers used were CffFOR2 5' GTTATGACTGAACTTCACTCC 3' and CffREV4 5' GATGTTCCCGGTGTTCAG 3' (Tegli *et al.*, 2002). DNA of Feij 2634 *Cff* isolate was used as the positive control and sterile distilled water and DNA isolated from *Pseudomonas fluorescens* as negative controls. PCR conditions were according to Tegli *et al.* (2002) with 25 µl of reaction being added to 2.5 µl of DNA, 0.5 µM of each primer, 1 U of Taq DNA polymerase, and 25 mM dNTP. PCR consisted of an initial cycle at 94°C for 3 min; 30 cycles denaturation at 94°C for 1 min, annealing at 60ºC for 45 sec, and extension at 72°C for 30 sec, with a final extension cycle at 72°C for 5 min. The final product amplified by PCR was visualized in a 1% agarose gel in 0.5% TBE with ethidium bromide, using 1Kb DNA ladder (Invitrogen, Brazil) as the marker.

RESULTS AND DISCUSSION

Cultivars IAC Carioca Aruã, IAC Carioca Akytã, IAC Carioca Pyatã (assays 1, 2, and 3) and IAC Carioca Tybatã (assays 2 and 3) were highly resistant to bacterial wilt, whereas cvs Pérola and IAC Carioca were highly susceptible in all assays (Tables 1, 2 and 3). The resistant reaction observed in IAC Carioca Aruã, IAC Carioca Akytã, IAC Carioca Pyatã, and IAC Carioca Tybatã, as well as the susceptibility of Pérola and IAC Carioca, agree with other findings (Maringoni, 2002; Souza *et al.*, 2006; Theodoro and Maringoni, 2006).

Plant-to-seed transmission of *Cff* was found to vary with the level of resistance of the plant. Transmission of the bacteria from plant to seed was not observed in IAC Carioca Akytã, IAC Carioca Pyatã (Tables 1 and 3) and IAC Carioca Tybatã (Tables 2 and 3). It is probable that some type of post-infection resistance developed, which blocked bacterial colonization in the xylem vessels, preventing infection of the seeds. Souza *et al.* (2006), after *Cff* inoculation of dry bean cultivars with high levels of resistance, observed in xylem vessels agglutinations of bacterial cells surrounded by a structure that apparently blocked further colonization and, consequently, the advance of the disease.

IAC Carioca Aruã, despite the good resistance to bacterial wilt, showed 5.5 to 14.8% plant-to-seed trans-

Table 1. Plant-to-seed transmission of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in different dry bean cultivars. First assay, 2004.

Cultivar	Disease severity	No. of seeds analyzed/ No. seeds with *Cff*	*Cff* transmission (%)	No. of isolates tested for pathogenicity/No. pathogenic isolates	Range of similarity índex values (Microlog2™)
IAC Carioca Aruã	1.68	500/74	14.80	20/19	0.74 – 0.84
IAC Carioca Akytã	0.74	500/0	0	-	-
IAC Carioca Pyatã	0.33	500/0	0	-	-
Pérola	9.00	46/15	32.61	11/11	0.76 – 0.86
IAC Carioca	8.87	500/221	44.20	25/25	0.66 – 0.80

Table 2. Plant-to-seed transmission of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in different dry bean cultivars. Second assay, 2005.

Cultivar	Disease severity	Nº. of seeds analyzed/ No. of seeds with *Cff*	*Cff* transmission (%)	No. isolates tested for pathogenicity/No. of pathogenic isolates	Range of similarity índex values (Microlog2™)
IAC Carioca Aruã	1.0	500/29	5.5	10/10	0.53 – 0.73
IAC Carioca Akytã	0.5	500/0	0	-	-
IAC Carioca Pyatã	0.5	500/0	0	-	-
IAC Carioca Tybatã	0	500/0	0	-	-
Pérola	8.6	155/121	74.2	8/8	0.51 – 0.74
IAC Carioca	6.6	500/350	70	9/9	0.59 – 0.79

Table 3. Plant-to-seed transmission of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in different dry bean cultivars. Third assay, 2006.

Cultivar	Disease severity	Nº. of seeds analyzed/ No. of seeds with *Cff*	*Cff* transmission (%)	No. of isolates tested for pathogenicity/ No. of pathogenic isolates	Amplification of 306 pb band with primers CffFOR2-CffREV4
IAC Carioca Aruã	1.00	500/0	0	-	-
IAC Carioca Akytã	1.00	500/0	0	-	-
IAC Carioca Pyatã	0.90	500/0	0	-	-
IAC Carioca Tybatã	0.90	500/0	0	-	-
Pérola	8.50	87/32	36.78	32/32	+
IAC Carioca	7.70	500/52	10.40	17/17	+

mission (Tables 1 and 2), but the susceptible cvs IAC Carioca and Pérola had higher transmission percentages, IAC Carioca from 10.4 to 70%, and Pérola from 32.61 to 74.2 % (Tables 1, 2 and 3). Infected seeds from cvs IAC Carioca and IAC Carioca Tybatã (third assay) are shown in Fig. 1. These findings regarding plant-to-seed transmission of *Cff* in susceptible bean plants partially agree with those of Chavarro *et al.* (1985), who found a transmission rate in the order of 52.5% for the PI 204600, and 42.5% for the cv. Porrillo Sintetico. According to Chavarro *et al.* (1985), transmission of *Cff* in seeds of *Zornia glabra* was 88.4%, significantly higher than that observed in bean plants.

Resistance as well as susceptibility of the beans to *Cff* can vary with climatic conditions and aggressiveness of the bacteria. Bean genotype PI 136677, for example, which is considered resistant to *Cff* isolates from the USA, proved to be susceptible to Colombian isolates of *Cff* originating from *Z. glabra* (Chavarro *et al.*, 1985). Such observations may explain the variation observed in *Cff* transmission in the assays carried out in our study with IAC Carioca Aruã, IAC Carioca, and Pérola (Tables 1, 2, and 3), especially if one considers the variations in the environment inside the greenhouse over a three-year period.

Differences in plant-to-seed transmission of *Xanthomonas axonopoidis* pv. p*haseoli* on bean genotypes with diverse levels of resistance to this bacterium have been reported (Maringoni *et al.*, 1993; Torres and Maringoni, 1997). *Cff* showed a similar behaviour in the present study for higher percentages of transmission occurred in susceptible genotypes compared to those with higher levels of resistance.

Bacterial isolates obtained from seeds were consistently identified as *Cff*, by the Microbiolog2^{TM} method in the first and second assays (Tables 1 and 2), with similarity indices ranging from 51 to 86%, or by PCR in the third assay (Table 3), using *Cff*-specific primers (Fig. 2), yielding a specific band of 306 bp. The Microbiolog2^{TM} method had been employed successfully before to identify *Cff* from naturally infected bean seeds (Maringoni and Camara, 2006; Maringoni *et al.*, 2006) and cultures from international collections (Harris-Baldin and Gudmestad, 1996). Of the *Cff* isolates identified, only one was not pathogenic to cv. Pérola (Table 1), as previously observed by Maringoni and Camara (2006) and Maringoni *et al.* (2006) while identifying *Cff* in naturally infected bean seeds.

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