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First report of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* on soybean in Brazil

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ABSTRACT

Curtobacterium flaccumfaciens pv. *flaccumfaciens* (Cff) causes bacterial wilt on beans (*Phaseolus vulgaris*) and bacterial tan spot on soybeans (*Glycine max*). Cff was detected on beans in Brazil in 1995. Plants of commercial and experimental fields of soybean with typical symptoms of the disease were collected in the State of Paraná, Brazil, during the 2011/2012 growing season. The causal agent was identified as Cff by isolation from symptomatic leaves on CNS semi-selective medium, artificial inoculation test and re-isolation in soybean and bean, Gram staining test, solubility in KOH, and by PCR. This is the first report of Cff on soybean in Brazil.
Key words: *Glycine max,* bacterium, tan spot

Curtobacterium flaccumfaciens pv. *flaccumfaciens* (Hedges) Collins & Jones (Cff) was first described on beans (*Phaseolus vulgaris* L.) causing bacterial wilt in the state of South Dakota (USA) in 1920 (Hedges, 1922), and its presence has been recorded in diverse geographical areas in the world, as parts of East and South Europe (EPPO, 2011), Australia, Asia, North and South America, and Africa (Bradbury, 1986). In Brazil, Cff was first detected on beans in 1995 in the state of São Paulo (Maringoni & Rosa, 1997), and more recently has been observed in many beans producing areas, mainly in the South, Southeast and Central-West regions (Rava & Costa, 2001).

The first observation of Cff on soybean [*Glycine max* (L.) Merr.] was in the United States in 1975, and the disease was named bacterial tan spot (Dunleavy, 1983). Experiments showed maximum yield losses of 18.8% in tan spot susceptible soybean cultivars, with mean losses of 12.5% (Dunleavy, 1984).

The bacterium survives in the field in crop debris, seeds and soil for at least two winters and enters through seedlings during germination, spreading in vascular tissues (EPPO, 2011). Characteristics like long latency period, relatively slow growth on complex media, its endophytic nature and occurrence in low numbers have made disease diagnosis and pathogen detection difficult, especially in seed certification programs or quarantine inspection for importation (Guimarães et al., 2001).

Symptoms on beans start with wilt due to systemic infection, but chlorotic and necrotic leaf lesions can occur simultaneously. On soybeans, many chlorotic leaf spots appear after dry out and acquire a tan color (hence the name tan spot disease). Seedling death occurs in the case of an early infection. Older plants usually survive the attack, but growth and yield are significantly reduced. In a few cases, wilting symptoms are also observed on soybeans (Harveson & Vidaver, 2007).

The objective of this research was to identify the causal agent of a soybean disease suspected to be tan spot in the state of Paraná, Brazil.

Leaves of plants from commercial and experimental fields of soybean with typical symptoms of the disease were collected in Guarapuava (25°25'01" S, 51°34'35" O) and Londrina counties (23°11'35"S, 51°11'02"O) of the state of Paraná during the 2011/2012 growing season. To obtain the isolates, leaf pieces were disinfested in alcohol and sodium hypochlorite and used to make suspensions in sterile water which were plated in petri dishes with NSA medium. The isolates obtained were plated in modified CNS (*Clavibacter nebraskensis* Selective Medium), described by Behlau et al. (2006) as semi-selective to Cff, where the colony color and morphology were determined. Pathogenicity tests were done in soybean, cutting the first trifoliolate leaf with a scissor wetted in the bacterium suspension (Rava, 1984) and evaluating the presence of chlorosis around the cut. Inoculation in beans was done by the stem puncture method using a needle dipped in bacterial colonies grown in NSA medium for 96 h at 28 °C (Maringoni, 2002), and evaluating the presence of wilt in the plants. Soybeans and beans plants were grown in a greenhouse with natural lightening, temperature between 24–28 °C, in 2 liters pots with autoclaved substrate (soil:sand:manure, 1:1:1), and 2 plants per pot. Four-week-old plants were inoculated with the bacterial isolates and sterile water (control). The experimental design was randomized with four replications (four pots). Symptoms were evaluated 2 weeks later. Gram

type was determined by differential Gram staining and solubility in KOH (Halebian et al., 1981).

For DNA extraction, the bacterial strains were grown in Nutrient Broth medium for 48 h at 28 °C and harvested by centrifugation. DNA extraction was carried out according to the method previously described (Li & De Böer, 1995). The bacteria cells were washed once in sterile distilled water, and the pellet was frozen at -20 °C for 1 h and thawed at room temperature. After being treated with 100 µL of cold acetone (-20 °C) for 10 min, the pellet was suspended in 500 µL of TE (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0) buffer, followed by addition of 50 µL of 500 mM EDTA (pH 8.0), 50 µL of 14% sodium dodecyl sulfate, and 10 µL of 0.1% proteinase K and incubation for 1 h at 55 °C, at 37 °C. An equal volume of 7.5 M ammonium acetate was then added to separate the DNA in solution from most cell debris, which precipitated, and was removed by centrifugation at 17,310 *g* for 20 min. DNA in the supernatant was precipitated using isopropanol at -20°C for 30 min. The DNA was pelleted, washed with 70% ethanol, and vacuum-dried before dissolving in 100 µL of sterile distilled water.

For specific detection of Cff by PCR, the primer pair *Cff*FOR2 5'-GTTATGACTGAACTTCACTCC-3' and *Cff*REV4 5'-GATGTTCCCGGTGTTCGA-3' (EMBL accession numbers AJ318036 and AJ318037, respectively) (Tegli et al., 2002) was used to amplify bacterial DNA from selected isolates. For analysis, 50 ng of DNA were used as template in a 25 µL reaction containing buffer (100 mM Tris-HCl, 500 mM KCl), 1.5 mM $MgCl_2$, 100 µM of each dNTP, 0.5 µM of each primer, and 1 U of Taq DNA Polymerase. The PCR conditions were an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min; annealing at 62°C for 45 s, extension at 72°C for 30 s and a final extension at 72°C for 5 min. The amplified product were separated by electrophoresis in 1.2% agarose gel in TAE buffer, stained with ethidium bromide and visualized under UV light.

The Cff isolates Feij-2500 and Feij-2912 were provided by Dr. Antonio Carlos Maringoni (Faculdade de Ciências Agronômicas, UNESP, Botucatu, SP, Brazil) and used as the PCR positive controls, while a DNA sample from *Xanthomonas axonopodis* pv. *glycines* was used as the negative one. Each sample was extracted and amplified in triplicate by PCR.

Seven bacterial isolates were obtained in NSA medium and plated in modified CNS. Three isolates grew in modified CNS and were selected for the other tests. Those isolates inoculated into plants at greenhouse condition, caused the symptoms of the disease on soybean (Figure 1)

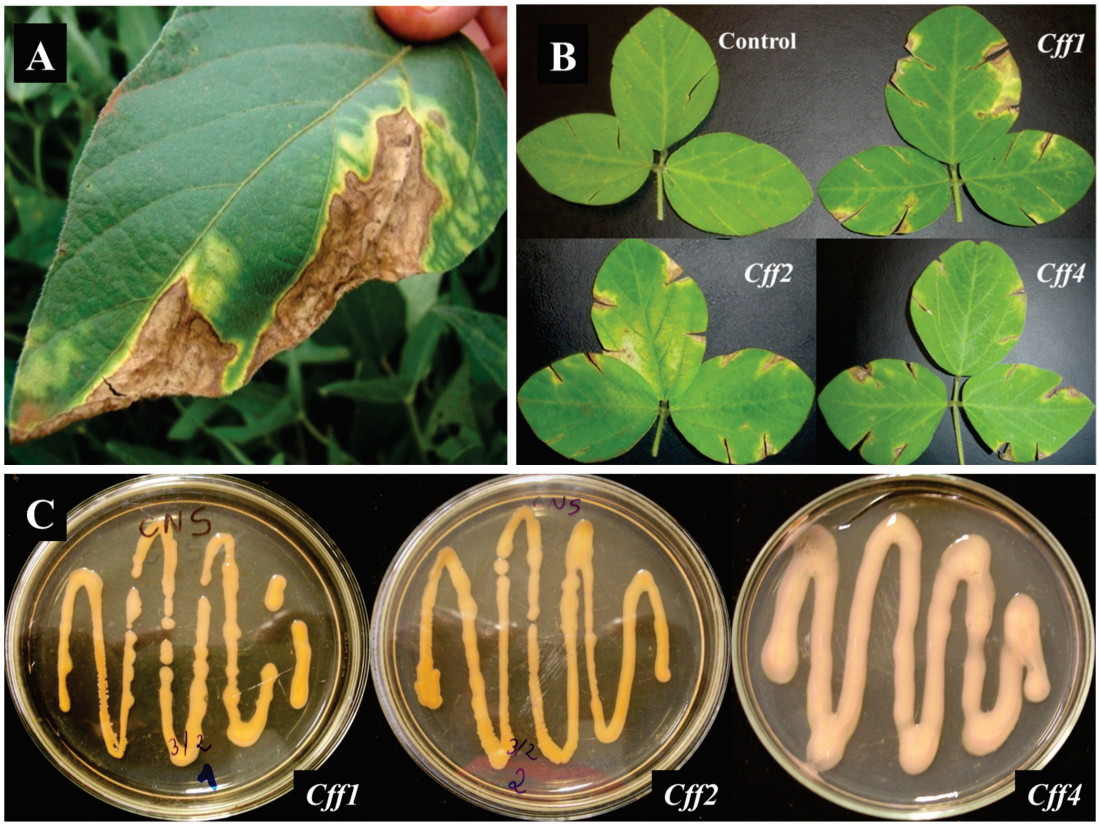

FIGURE 1 - A. Chlorotic and dried leaf tissue caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cff). **B.** Symptoms of scissor inoculation with three isolates of Cff. **C.** colonies of three isolates of Cff in CNS medium.

and bean plants. They were re-isolated from soybean, stored and cataloged as *Cff*1, *Cff*2 and *Cff*4 at Embrapa Soybean Bacteria Collection.

The characteristics showed by the three isolates were: Gram positive; not soluble in KOH 3%; rod-shape cells; pigmentation in NSA medium, yellow for isolates *Cff*1 and *Cff*2, pink for isolate *Cff*4, and pigmentation in CNS medium, orange for *Cff*1 and *Cff*2, pink for *Cff*4 (Figure 1).

PCR analysis using the primers *Cff*FOR2-*Cff*REV4 confirmed the results based on morphological symptoms previously observed. An expected single DNA band of 306 bp was specifically obtained in all Cff strains tested (positive controls: *Cff* Feij-2500 and Feij-2912) and in the isolates collected in Brazilian fields (*Cff*1, *Cff*2 and *Cff*4). No amplification was detected when DNA sample from *X. axonopodis* pv. *glycineswas* was tested (Figure 2).

Based on the results obtained, we conclude that the disease observed in soybean fields was caused by Cff. This is the first report of Cff on field-grown soybean plants in Brazil.

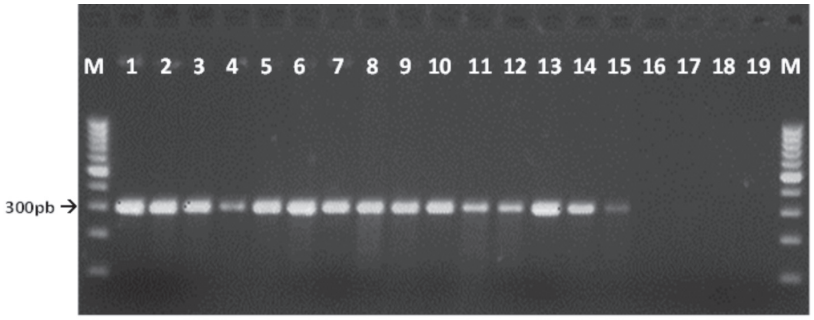

FIGURE 2 - Agarose gel electrophoresis of the PCR products obtained with the primers *Cff*FOR2 and *Cff*REV4. **M.** 100bp DNA ladder. **1-6.** Positive controls *Cff* Feij-2500 and Feij-2912 isolates. **7-15.** Three candidates samples *Cff*1, *Cff*2 and *Cff*4 respectively. **16-18.** Negative controls. **19.** No template control (sterile distilled water).

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