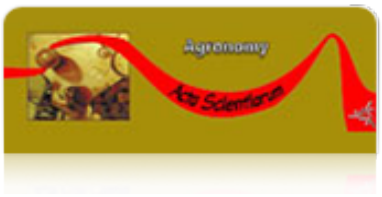

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Protocol for detecting *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in the leaves of infected bean plants (*Phaseolus vulgaris* L.)

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ABSTRACT. *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*), which is a gram-positive bacterium that causes wilting in several cultivated plants, is one of the main disease-causing bacteria in beans (*Phaseolus vulgaris* L.). Different techniques are employed to detect pathogens in bean plants; however, only a few slow and inefficient methodologies can be used to detect *Cff*. Hence, there is a need for rapid and efficient techniques to detect *Cff* for disease management. The objective of our study was to develop a technique to effectively detect *Cff* in the leaves of infected bean plants by optimizing a previously developed method. We modified the extraction method by reducing the amount of water used, replacing seeds with leaf tissues (obtained from the veins and petioles) and using *CffFOR2* and *CffREV4* primers specific for *Cff*. Our optimized method exploits a concentration gradient generated in the plant tissue through osmosis to disrupt the plant cell wall and efficiently isolate bacterial cells. DNA extracted from the isolated bacterial cells was amplified by polymerase chain reaction. This method allowed efficient detection of *Cff* in leaf tissues.

Keywords: *Curtobacterium* wilt; DNA extraction; leaf tissue; bacterium; common beans.

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Introduction

Curtobacterium wilt, which is caused by the bacterium *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*), is a major bacterial disease of bean crops (Hedges, 1922). It adversely affects bean cultivation by causing considerable yield loss (Herbes, Theodoro, Maringoni, dal Piva, & Abreu, 2008). Hedges (1926) reported that symptoms of *Cff* infection usually appear in the dead parts of a plant, but they can also be found in isolated living plants (Tegli, Sereni, & Surico 2002). The infection spreads via contaminated seeds or through injuries in plants (Leite Júnior, Meneguim, Behlau, Rodrigues, & Bianchini, 2001). Therefore, the chances of disease outbreaks in the field are high (Valentini, Guidolin, Cruz Baldissera, & Coimbra, 2010). Furthermore, this pathogen can survive for a long time in seeds, especially when seeds are stored under optimal conditions (Tegli et al., 2002). The bacterium colonizes the xylem vessels and thereby obstructs sap flow and affects the entire shoot, causing leaf wilting, sagging, and yellowing; vascular darkening; and stunted growth in plants (Wendland et al., 2008). The affected roots darken and exhibit soft rot under humid conditions (Behlau, Nunes, & Leite Junior, 2006). The most acute symptoms are observed under water and high temperature stress and in young plants (Harveson, Vidaver, & Schwartz, 2011).

Currently, there are a few different techniques to detect pathogens in bean plants. However, there are only a few methodologies that can be used to detect *Cff*, which are mainly used in infected seeds, that range from visual seed inspection to the use of molecular techniques to identify and quantify the bacterium. These involve seed culture in selective culture medium, plating of seed extract in selective or semiselective media, seed extract inoculation in susceptible plants, polymerase chain reaction (PCR) with *Cff*-specific primers, immunofluorescence, and the use of bacteriophages (Mafia, Alfenas, & Gonçalves, 2007; Badel, Arriel, Guimarães, & Ferraz, 2016). These methods vary in their cost, time, physical space, and equipment requirements, sensitivity, and practical application (Roth, Saettler, & Schaad, 1989). None of the methods use molecular techniques, such as real-time PCR, for rapid and efficient detection of *Cff* in bean leaves. Thus, the

objective of this study was to develop an efficient and practical method to extract and detect *Cff* in the leaf tissues of bean plants.

Material and methods

To obtain the necessary plant material, the bean cultivars IPR Tangará (*Cff* tolerant) and IAC Carioca (*Cff* susceptible) were grown in a greenhouse at the Agronomic Institute of Paraná (IAPAR), Londrina, Paraná State, Brazil. Both cultivars were inoculated with the *Cff* 14330 strain in the V3 phenological stage. The leaves and petioles that exhibited the symptoms of *Cff* infection were collected from the plants in the V4 phenological stage to extract bacterial cells.

The following three protocols were used to extract bacterial DNA from infected plant leaves: the method proposed by Doyle and Doyle (1987), the alkaline extraction method developed by Xin, Velten, Oliver, and Burke (2003), and a method proposed by Deuner, Souza, Zacaroni, Figueira, and Camera (2012), which we optimized and will thereafter denote as the protocol for detecting *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in the leaves of infected bean plants (*Phaseolus vulgaris* L.). The first two protocols were used to isolate bacterial DNA from plant tissues to detect bacterial cells (Moreira, Noschang, Neiva, Carvalho, & Vicente, 2010; Trindade, Marques, Lopes, & Ferreira, 2007). The protocol of Deuner et al. (2012) was developed for *Cff* cell extraction from infected seeds, and we modified it for bacterial cell extraction from the leaf tissues of bean plants as follows. We modified the extraction method by reducing the amount of water used, replacing seeds with leaf tissues (obtained from the veins and petioles), and using the *CffFOR2* and *CffREV4* primers specific for *Cff*.

The leaf samples of the two cultivars were surface-sterilized by immersing them for 1 min. each in 70% alcohol and 1% hypochlorite. They were then rinsed with distilled water five times. After sterilizing the leaves, the limbs were discarded, and the veins and petioles were cut into 1-cm-long fragments using a scalpel. Five such fragments were packed into individual graduated tubes, and then sterile distilled water was added to the tubes until the final volume was 10 mL; these tubes were incubated overnight at 4°C. The obtained supernatant was filtered through gauze to obtain the crude extract, which was divided into two aliquots of 750 µL each. The aliquots were centrifuged at 8,000 rpm for 15 min. to obtain an aqueous medium with a high cell concentration. Thereafter, the supernatant was discarded to obtain a concentrated cell suspension of 200 µL. This suspension was incubated overnight at -20°C, and 1 µL of the aliquot was directly used for *Cff* detection by PCR.

All three methods were used to obtain the extracts of both cultivars, and thus six extracts were obtained. PCR was performed in duplicate for each of these extracts, which were labeled A1 and A2 for IPR Tangará and A3 and A4 for IAC Carioca, and the results were compared with those for the positive (sample containing *Cff* DNA) and negative (sample containing only water) controls. The primers used in the reaction were specific for *Cff*: *Cff*FOR2 (5′-GTTATGAACTTCACTCC-3′) and *Cff*REV4 (5′-GATGTTCCCGGTGTTCAG-3′) (Tegli et al., 2002); the primers amplify a 306-bp fragment. The reaction mixture consisted of 0.5 µM of each primer, 0.1 mM of each of the four deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, and dTTP), 1.5 mM of $MgCl_2$, 1 U of Taq DNA Polymerase, and 1 µL of DNA sample, and the final volume of the mixture was brought to 25 µL with 1× reaction buffer. The PCRs were carried out in a Veriti® 96-Well Thermal Cycler (Model 9902; Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with the following program: initial denaturation at 94°C for 3 min., followed by 30 cycles of denaturation at 94°C for 1 min., annealing of the primers at 60°C for 45 seconds, and extension at 72°C for 30 min., and a final extension at 72°C for 5 min.

The quality of the PCR products was determined by electrophoresis in a 1% (w/v) agarose gel with 1× SB buffer and a voltage of 90 V. The gel was visualized under ultraviolet light (UV) and photographed using a UV transilluminator (L. PIX; Loccus Biotechnology). The sizes of the fragments were determined using a 1-Kb plus DNA ladder.

Results and discussion

Among the three methods, only the modified extraction protocol used for detecting *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in the leaves of infected bean plants (*Phaseolus vulgaris* L.) was efficient in extracting *Cff* DNA from bean leaf samples, and this was further confirmed by amplification of the target DNA fragments by PCR. By using this method, we successfully detected a *Cff* DNA fragment of approximately 300 bp (Figure 1) for both bean cultivars as well as the positive control. For PCR, we used the primers described

by Tegli et al. (2002), which were confirmed to be highly specific for *Cff* by Souza, Maringoni, and Krause-Sakate (2004) after testing different primers. Owing to their high sensitivity, these primers avoided the amplification of DNA fragments from the bean cultivars, which are genetically close to those of the pathogen.

The optimized methodology proposed herein leads to the rupture of the conducting vessels of the plant and the cell wall of bacteria as they become saturated with water, thereby extracting the bacterial cell content, which is then precipitated by centrifugation. Thus, the efficiency of this methodology depends on the concentration gradient generated by osmosis. We were unable to detect *Cff* in the leaf tissues by using the extraction methods proposed by Doyle and Doyle (1987) and Xin et al. (2003) (Figure 1). These methods use detergents to solubilize the cell wall membranes, which form a complex with the DNA and subsequently cause differential precipitation of the DNA. However, we did not observe *Cff* DNA fragments on the agarose gel for any of the samples obtained using these two extraction methods.

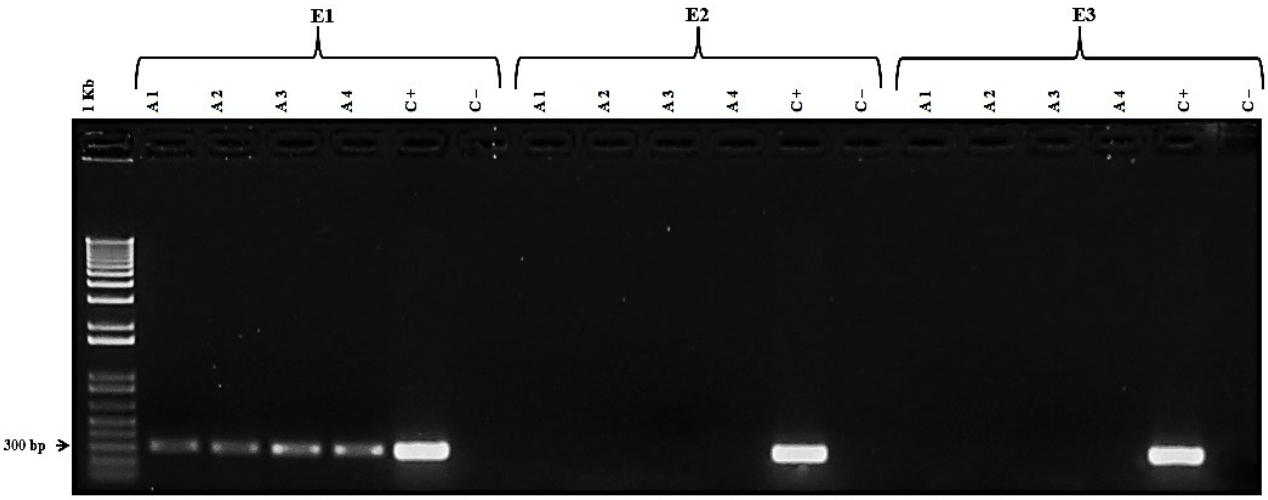

Figure 1. Agarose gel electrophoresis of the PCR products obtained with the primers *Cff*FOR2 and *Cff*REV4. *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) DNA was obtained using the extraction method proposed by Deuner et al. (2012) and our modified method (E1); Doyle and Doyle (1987) (E2); and Xin et al. (2003) (E3). Bean leaf samples from cultivars IPR Tangará (A1 and A2) and IAC Carioca (A3 and A4) were inoculated with the *Cff* 14330 strain: positive control (C+), negative control (C-), and molecular ladder (1 Kb plus).

Although several studies have successfully extracted genomic DNA using the methods proposed by Doyle and Doyle (1987) and Xin et al. (2003), these methods were found to be ineffective in extracting *Cff* DNA from bean leaf tissues. Similarly, Moreira et al. (2010) were also unsuccessful in extracting genomic DNA from several species of *Streptococcus*, which are gram-positive bacteria, using the method of Doyle and Doyle (1987). This is because the cell wall of gram-positive bacteria has a thick peptidoglycan layer (Junqueira & Carneiro, 2012). Thus, the extraction of DNA from gram-positive bacteria requires reagents that can lyse the cell wall of both plants and bacteria (Badel et al., 2016), which cannot be performed with CTAB detergent at 65°C or the alkaline solutions used in the methods proposed by Doyle and Doyle (1987) and Xin et al. (2003), respectively. Thus, no pathogen DNA was detected in the samples by PCR.

Most bacterial detection methods are designed for gram-negative bacteria, as most phytobacteria belong to this category (Salton, 1953), while protocols for isolating DNA of gram-positive bacteria from diseased plant tissues are scarce (Trzewik, Nowak, & Orlikowska, 2016). Hence, there is a need for methodologies that can be used to extract DNA from gram-positive bacteria to enable their rapid detection.

Studies have shown that hydrolase enzymes, such as lysozyme, can lyse the cell wall of gram-positive bacteria and thereby help release bacterial DNA (Nogueira, Momesso, Rossit, Almeida, & Rossit, 2004). However, these enzymes are expensive and therefore cannot be applied to the development of a cost-effective method of DNA extraction. Likewise, although it speeds up the processing of samples and generates a high yield, the use of commercial DNA extraction kits increases the cost per sample (Junqueira & Carneiro, 2012). Moreover, such kits do not always produce a satisfactory quantity of DNA or high-quality DNA, which is an essential requirement for PCR analysis (Trzewik et al., 2016).

There is a need for methods to detect *Cff* in the leaves of infected plants, as detecting *Cff* in the early stages of *Curtobacterium* wilt, which is when the plants just begin to show symptoms of infection, will allow more informed decision-making regarding disease control. In addition, most methods of bacterial detection are

designed for gram-negative bacteria, while protocols to isolate DNA from gram-positive bacteria from diseased plant tissues are scarce, indicating a need for methodologies to extract DNA from gram-positive bacteria to allow for rapid detection and reporting of results to the farmer. In this scenario, the PCR technique is a tool for the accurate identification of bacterial isolates.

Conclusion

Here, a protocol for detecting *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in the leaves of infected bean plants (*Phaseolus vulgaris* L.) established an efficient method for the extraction and detection of bacterial DNA in bean leaf samples infected with *Cff*. The present protocol does not require expensive reagents and provides results in less time. The protocol developed in this study to detect *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in leaves of infected bean plants (*Phaseolus vulgaris* L.) is efficient and demonstrates speed and safety in the diagnosis of *Curtobacterium flaccumfaciens pv. flaccumfaciens* infection in leaves of bean plants.

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