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Assessment of the diagnostic potential of Immmunocapture-PCR and Immuno-PCR for Citrus Variegated Chlorosis

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ABSTRACT

Xylella fastidiosa causes significant losses in many economically important crops. An efficient pathogen detection system is critical for epidemiology studies, particularly when large sample size is involved. In this study we report the development of immunomolecular assays like Immmunocapture-PCR and Immuno-PCR for direct detection of *X. fastidiosa* without DNA isolation. Whereas the reactivity of ELISA and PCR ranged from 10^6 to 10^4 bacterial cells, the IC-PCR sensitivity was up to 10^3 and the detection limit of I-PCR was up to 10^1 bacterial cells. These methods can use either plant sample extracts or cultivated media, and show no cross reaction for any other endophytic citrus-bacteria. Therefore, IC-PCR and I-PCR assays provide an alternative for quick and very sensitive methods to screening *X. fastidiosa*, with the advantage of not requiring any concentration or DNA purification steps while still allowing an accurate diagnosis of CVC.

1. Introduction

Xylella fastidiosa subsp. *pauca* (Schaad et al., 2004) is a xylem-limited Gram-negative bacterium (Hopkins, 1989) of a broad range of perennial plants and is responsible for significant losses in many economically important crops. In Brazil, this bacterium is associated with plum leaf scald (Rosseti et al., 1990), Citrus Variegated Chlorosis (CVC) (Chang et al., 1993) and coffee leaf scorch (Beretta et al., 1996). These different strains are transmitted during xylem sap feeding by insect vectors, such as common sharpshooters leafhoppers (Hemiptera, *Cicadelidae*) (Purcell and Hopkins, 1996), and currently, there are no adequate control measures for these diseases.

In citrus, *X. fastidiosa* infects mainly sweet orange trees (*Citrus sinensis* [L.] Osbeck cv. pera), and the chlorosis symptoms are initially restricted to leaf, but later progress to the fruits which make them useless economically, due to the drastic size reduction. The economic losses to São Paulo citriculture are estimated to range from US$ 286–322million annually (FUNDECITRUS).

At the present time, the diagnosis of *X. fastidiosa* is routinely carried out by standard polymerase chain reaction (PCR) using specific primer sets whereas its quantification is performed by agarose gel electrophoresis (Henson and French, 1993; Pooler and Hartung, 1995). The primer set usually used includes the RST31/33 (Minsavage et al., 1994), derived from the RNA polymerase genomic locus, 272-1-int and 272-2-int or those derived from the 16S rRNA gene (Chen et al., 2005). However all PCR procedures involve sample extraction to generate template DNA which can be obtained through petiole perfusion from fresh leaf petioles (Coletta-Filho et al., 1996) since the CVC bacterium forms a strong biofilm within the xylem vessels which render these extraction processes difficult to carry out, making these purification methods very tedious and labor intensive when conducted on a large scale. Furthermore, samples can also be obtained by grounding, but many times uncharacterized components of plant tissues may interfere and even inhibit the PCR (Wilson, 1997).

Therefore, to circumvent these purification problems and the drawback of the initial step of PCR that requires chloroform/phenol manipulation for the nucleic acid extraction, which is a potential environmental pollution risk, we established Immunocapture-PCR (IC-PCR) and Immuno-PCR (I-PCR) for CVC detection. These immunomolecular methods are highly sensitive and specific for detecting CVC, since they unite immunological and molecular detection technologies, which combine the specificity of enzyme-linked immunosorbent assay (ELISA) with the amplification power and sensitivity of the PCR (Sano et al., 1992; Niemeyer et al., 2005).

These methodologies are claimed to be several orders of magnitude more sensitive than conventional ELISA or PCR, thereby enhancing dramatically sensitivity and enabling for a broad range of applications in diagnostics. Recently, IC-PCR and I-PCR have been used to detect a variety of very low antigen concentrations, such as tumor markers (Zhou et al., 1993), microorganisms and toxins (Liang et al., 2003; Chao et al., 2004; Allen et al., 2006; Lubelli et al., 2006; Mason

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et al., 2006; Fischer et al., 2007), cytokines and hormones (Sanna et al., 1995; Sims et al., 2000; Kamatsu et al., 2001), and human and plant pathogens (Luo et al., 2002; Chye et al., 2004; Adler, 2005).

2. Materials and methods

2.1. Bacterial strain and culture

The 9a5c bacterial strain of *X. fastidiosa* (Schaad et al., 2004) was used in this study. This strain was the same one used for genome sequencing and obtained from the Institut National de La Recherche Agronomique (INRA, Bordeuax, France). Bacterial cells were grown for 9days either in solid or liquid periwinkle wilt (PW) media (Davis et al., 1981), at 29 °C and with or without an orbital shaker at 130 to 150rpm. The cells were harvested from liquid and solid medium cultures, pelleted by centrifugation. The suspension of bacteria was standardized to an optical density of 0.25 (10^7 to 10^8CFU/mL) at A_{600}nm with a spectrophotometer (Minsavage et al., 1994).

2.2. Plant samples

The leaves were obtained from healthy, CVC-symptomatic and -asymptomatic citrus sweet orange trees (*C. sinensis* cv. pera), from the crop of the Centro APTA 'Sylvio Moreira', Instituto Agronômico, Cordeirópolis, SP, Brazil. Samples were obtained by grinding 0.5g petioles in 5mL of grinding buffer (2% polyvinylpyrrolidone 40 in PBS-T (136 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 8 mM Na_2HPO_4, 0.05% Tween 20, pH 7.4), using a manual bead grinder. One milliliter of plant extract was transferred to microfuge tubes and clarified by centrifugation at room temperature for 2 min at 21,000×*g*.

2.3. Viability of X. fastidiosa in infected plants

The CVC-infected plant sterilized samples (150 mg/mL) previously milled in sterile PBS, were diluted to 10-fold serial dilutions in sterile PBS (10^{-1} to 10^{-9}). So, 100µL of each solution was placed on three sterile plates with PW medium (0.4% w/v phytone peptone, 0.1% w/v trypticase peptone, 7.35 mM KH2PO4, 6.89 mM K2HPO4, 1.62 mM MgSO4, 0.001% w/v hemin chloride, 0.002% phenol red, 0.6% w/v BSA, 0.4% w/v glutamine, pH 6.8) and one sterile plate with BCYE medium (1% w/w yeast extract, 0.04% w/v, L-cysteine-HCl, 0.025% w/v ferric pyrophosphate, 30 mM KOH, and 54.9 mM ACES; pH 6.85). The growth profile was estimated by counting the colony forming units (CFU) after growing at 25 °C for 10–12days. Finally, the CFU per mg of infected tissue was calculated and all assays (ELISA, IC-PCR and I-PCR) were carried out with 100mg of infected tissue.

2.4. Endophytic bacterial strains

The endophytic bacterial strains were obtained from the collection of the Laboratório de Genética de Microrganismos (Departamento de Genética, ESALQ/USP, Piracicaba, Brazil). The growth conditions were as recommended in the Handbook of Microbiological Media. The bacteria were cultured under aerobic conditions and harvested at the end of the exponential growth phase, centrifuged, washed with PBS and stored at 4 °C.

2.5. Polyclonal antiserum

Female New Zealand white rabbits weighing 2kg were endovenously inoculated with standardized bacterial suspension at 1×10^6 *X. fastidiosa* cells/mL in PBS, and this procedure was repeated three times at 14day intervals. Ten days after the last injection, the blood was collected through cardiac puncture and the serum titer and specificity were determined by indirect enzyme-linked immunosorbent assay (ELISA) (Clark et al., 1986) and preimmune serum was used as a negative control.

2.6. Biotinylation of antibody

The immunoglobulin G (IgG) fraction previously purified from the antisera by protein G affinity column chromatography (Sigma Chemical, Saint Louis, MO, USA), according to manufacturer's instructions using low-salt conditions and eluting with 0.1M glycine, pH 3.0, was biotinylated using the photobiotin method according to Heggeness and Ash (1977). Briefly, the purified antibodies (1 mg/mL) were dialyzed in 0.2M borate buffer, pH 8.5 and mixed with a fresh solution of biotinyl-*n*-hydroxy-succinimide ester (Sigma) in DMSO (1 mg/mL), at the molar ratio of 1:20 in a final volume of 50µL. The conjugation was developed at room temperature for 4 h, dialyzed overnight at 4 °C against PBS pH 7.2, and finally mixed with glycerin 1:1 (v/v) and stored at − 20 °C.

2.7. Synthesis of biotinylated-reporter DNA

The biotinylated-reporter DNA was prepared following the method described by Liang et al. (2003). The *X. fastidiosa usp*A1 (XF1516) gene that encodes a surface-exposed outer membrane protein was previously cloned into vector pGEM-T (Promega Corporation, Madison, WI, USA). This construction was used as the template to produce the reporter 1.122kb DNA sequence. One microgram of the uspA1-R-Biotin primer was used as reporter. The labeling was done using 1× terminal transferase buffer, 25 nmol of Biotin-16 ddUTP (Boehringer Mannheim, Germany) and 60units of terminal transferase (Promega). The reaction was carried out for 30 min at 37 °C. The labeling was confirmed by dot blot using 200ng of uspA1 gene from *X. fastidiosa* as template and labeled primer as probe.

PCR amplifications were performed using the following primers: forward, uspA1-F: 5′5′-AACTCGAGAGCAGGCCGCCGGTGATAG-CAGTA-3′ and reverse, uspA1-R-Biotin: 5′-GACGCTCGAGCCCCCGCCG-CAAGAT-3′. Each 25 µL PCR reaction contained Milli-Q water, 10× buffer (Invitrogen Corporation, Carlsbad, CA, USA), 1.25 mM $MgCl_2$, 0.25 mM dNTP, 0.1 mM of each primer, 0.25U *Taq* DNA polymerase (Invitrogen) and approximately 1ng/µL template plasmid. PCR amplifications were performed at an initial denaturation step at 96 °C for 30 s, followed by an annealing step at 55 °C for 30s and extension step at 72 °C for 1 min and 30s for 35 cycles. The final extension was at 72 °C for 10 min. The PCR products were quantified using gel electrophoresis with a 1.0% agarose gel in 1× TAE (45 mM Tris–acetate, 1 mM EDTA, pH 8.0) followed by ethidium bromide (1 mg/mL) staining and visualization under UV light.

2.8. Enzyme linked immunosorbent assay

Polystyrene microtiter plates (Pierce Biotechnology, Rockford, IL, USA) were coated with 100 µL/well of standardized *X. fastidiosa* cells or plant extract samples diluted serially (10-fold) with carbonate coating buffer (16 mM sodium carbonate, 34 mM sodium bicarbonate, pH 9.6), and incubated overnight at 4 °C. The plates were washed three times with 200 µL of washing buffer (PBS-T), and blocked with PBS containing 2% BSA for 1 h at 37 °C. After washing, the plates were incubated for 3 h at 37 °C with 100 µL/well of anti-*X. fastidiosa* diluted to 1:1000 in PBS. For detection of the antigen–antibody reaction, 100 µL/well of goat anti-rabbit IgG conjugated with alkaline

Table 1
Viability of *X. fastidiosa* in infected plant tissue

Dilution	Mean of colonies	CFU/mg
10^{-4}	40	1.33×10^3
10^{-5}	10	3.33×10^3
10^{-6}	3.3	1.11×10^4
CFU/mg		5.25×10^3

After growing at 25 °C for 10–12 days, the colony forming units (CFU) were counted in three different dilutions and the CFU/mg was calculated.