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A nested-PCR assay for detection of *Xylella fastidiosa* in citrus plants and sharpshooter leafhoppers

L.P. Ciapina, L.M. Carareto Alves and E.G.M. Lemos

Laboratório de Bioquímica de Microrganismos e Plantas, Departamento de Tecnologia, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal-SP, Brazil

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ABSTRACT

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Aims: Detection of *Xylella fastidiosa* in citrus plants and insect vectors.

Methods and Results: Chelex 100 resin matrix was successfully standardized allowing a fast DNA extraction of *X. fastidiosa*. An amplicon of 500 bp was observed in samples of citrus leaf and citrus xylem extract, with and without symptoms of citrus variegated chlorosis, using PCR with a specific primer set indicating the presence of *X. fastidiosa*. The addition of insoluble acid-washed polyvinylpyrrolidone (PVPP) prior to DNA extraction of insect samples using Chelex 100 resin together with nested-PCR permitted the detection of *X. fastidiosa* within sharpshooter heads with great sensitivity. It was possible to detect up to two bacteria per reaction. From 250 sharpshooter samples comprising four species (*Dilobopterus costalimai, Oncometopia facialis, Bucephalogonia xanthopis* and *Acrogonia* sp.), 87 individuals showed positive results for *X. fastidiosa* in a nested-PCR assay.

Conclusions: The use of Chelex 100 resin allowed a fast and efficient DNA extraction to be used in the detection of *X. fastidiosa* in citrus plants and insect vectors by PCR and nested-PCR assays, respectively.

Significance and Impact of the study: The employment of efficient and sensitive methods to detect *X. fastidiosa* in citrus plants and insect vectors will greatly assist epidemiological studies.

Keywords: Chelex 100, citrus variegated chlorosis, DNA extraction, nested-PCR, sharpshooter, *Xylella fastidiosa*.

INTRODUCTION

Xylella fastidiosa (Wells *et al.* 1987), the causal agent of citrus variegated chlorosis disease (CVC) in Brazil (Chang *et al.* 1993), is a gram-negative bacterium limited to the xylem system of plants. CVC has caused great economic losses within Brazilian citriculture. Various strains of *X. fastidiosa* cause numerous other plant diseases such as Pierce's disease (Davis *et al.* 1978), alfalfa dwarf (Goheen *et al.* 1973), phony peach (Wells *et al.* 1983), and others (Hopkins 1989).

Xylella fastidiosa is transmitted by leafhoppers. Members of the Cicadellidae (Cicadellinae subfamily, named sharpshooters) and Cercopidae (spittlebugs) families are the most common vectors (Purcell 1990). *X. fastidiosa* is present in the xylem of host plants and in the foregut and alimentary canal of its vectors (Raven 1984). Hill and Purcell (1995) estimated that less than 100 live bacterial cells within an insect vector were sufficient for the transmission of *X. fastidiosa* to plant tissues. This level is below that for routine detection of *X. fastidiosa* by '*in vitro*' culturing. The detection of *X. fastidiosa* in insects and asymptomatic plants has been limited because of the low concentration of the bacterium and the low sensitivity of serological techniques such as ELISA (Raju *et al.* 1983; Yonce and Chang 1987). The limit of detection of *X. fastidiosa* using ELISA is in the order of more than 10^4 cells ml^{-1} (Nomé *et al.* 1980; Minsavage *et al.* 1994). This limit is two orders of magnitude greater than the number of bacteria in the vector's head that is sufficient for transmission to plants (Hill and Purcell 1995).

Correspondence to: E.G.M. Lemos, Via de Acesso Prof. Paulo Donato Castellane, s/n, km 05, Jaboticabal, SP, Brazil (e-mail: egerle@fcav.unesp.br).

Molecular methods based on DNA amplification by polymerase chain reaction (PCR) are important to detect *X. fastidiosa* in asymptomatic plants and in insect vector samples. This is because the direct isolation by culturing of this slow-growing bacterium is complicated. The use of PCR to detect *X. fastidiosa* in samples of infected plant tissues, based on specific primers, theoretically permits the detection of 10 to 100 bacteria per PCR reaction (Pooler and Hartung 1995). However, in most cases the DNA extraction methodology is not efficient and can release agents that inhibit the PCR reaction generating a false-negative result. In order to detect *X. fastidiosa* in plant and insect samples, it is, therefore, important to choose an adequate DNA extraction procedure and to use a suitable sensitive DNA amplification technique.

Several DNA extraction procedures, using a variety of biological sources, include the use of phenol–chloroform for protein extraction with further nucleic acid ethanol precipitation (Sambrook *et al.* 1989), high salt concentration (Dykes 1988) and treatment with enzymes (Grimberg *et al.* 1989). These methods normally demand several manipulations for salt removal or involve elements that might inhibit the PCR amplifications. Pure DNA samples are considered highly desirable (Wilson 1997) and so consequently these steps in DNA extraction from bacterium present in plant or insect tissues are considered laborious and inefficient producing preparations containing low concentrations of DNA. For application in PCR reactions, alternative methods using special matrices frequently allowed faster and easier DNA extraction because of the elimination of phenol–chloroform treatments. These matrices comprise ion exchange resins that, together with adequate buffer solutions, allow the resin to exhibit the correct electric charges required for the adsorption of compounds that could inhibit PCR reactions.

To increase the efficacy and sensitivity of PCR assays and avoid inhibitors of DNA amplification, the immunocapture and immunomagnetic separation techniques have been widely used to concentrate and purify bacteria present in food (Hedrum *et al.* 1992; Kapperud *et al.* 1993), in clinical samples (Muir *et al.* 1993; Seesod *et al.* 1993; Wolfhagen *et al.* 1994), in water (Morgan *et al.* 1991; Christensen *et al.* 1992; Kapperud *et al.* 1993), in plants (Hartung *et al.* 1996; Llop *et al.* 2000) and in insects (Pooler *et al.* 1997). The nested-PCR technique has also improved and sometimes even permitted the fast detection of pathogens and has improved the sensitivity of such analysis (McManus and Jones 1995; Hartung *et al.* 1996; Llop *et al.* 2000). Nested-PCR is an effective method for detecting organisms or their products in environmental samples where the presence of low concentrations of the DNA targets and high concentrations of contaminants could inhibit DNA amplification. The detection of *X. fastidiosa* in insect samples is difficult as the number of bacterial cells present in insects is less than that present in samples of plant tissues, so the use of nested-PCR could be an efficient alternative assay.

The aim of this work was to optimize bacterial DNA extraction by the use of ionic exchange resin to detect *X. fastidiosa* in plant materials through PCR assays and to provide a fast and efficient procedure for the detection of this bacterium within sharpshooter tissue samples. DNA extracted by this procedure combined with nested-PCR assays allowed the detection of very low number of cells of *X. fastidiosa* in sharpshooter tissue samples. Such low levels would be expected to occur within many infectious sharpshooters.

MATERIALS AND METHODS

Bacterial cells

Cells from *X. fastidiosa* strain 9a5c were grown in Glutamate Yeast Extract (GYE) broth medium (Campanharo *et al.* 2003) and DNA was directly extracted from an aliquot of 100 μl of cultured cells.

Plant tissues

Plant leaf extracts for PCR were obtained from 100 μg of liquid nitrogen macerated citrus leaves and citrus xylem extracts were obtained from branches by passing 1 ml of sterile water through the xylem vessels of the branches using a vacuum system. Citrus xylem samples were centrifuged (12 000 *g*, 20 min, at room temperature). The supernatant was discarded and 100 μl of sterile ultrapure water was added to the samples before extraction.

Insect samples

The four species of sharpshooters described as *X. fastidiosa* vectors: *Dilobopterus costalimai, Oncometopia facialis, Bucephalgonalia xanthopis* and *Acrogonia* sp., were kindly provided by FUNDECITRUS (Foundation for Citriculture Protection) from orange orchards. For *X. fastidiosa* acquisition the insects were maintained from 1·5 to 48 h on sweet orange infected plants with CVC symptoms, then sealed in cryovials and stored at −20°C. Controls were used in the experiment to evaluate the efficiency of the DNA extraction procedure and also the specificity and sensitivity of the DNA amplifications. The controls consisted of: (i) 100 μl of cultured bacteria strain 9a5c; (ii) 100 μl of cultured bacteria strain 9a5c mixed with the macerated heads of healthy sharpshooters to evaluate the presence of agents released by the insect samples that might inhibit the amplification of the bacterial DNA; (iii) samples of

sharpshooters raised under laboratory controlled conditions which were never exposed to CVC-diseased plants (these sharpshooters were kindly provided by João R. S. Lopes, Departamento de Entomologia, ESALQ, USP, Piracicaba, S.P); (iv) PCR control mixes without bacteria or sharpshooter samples.

To determine the sensitivity of detection, *X. fastidiosa* was cultured on GYE media for 4 days and then diluted in a 10-fold series from 2×10^9 to 2 CFU ml^{-1} in sterile ultrapure water. One hundred microlitres of each serially diluted bacterial suspension were then added to tubes, with and without the macerated sharpshooter heads, for DNA extraction. A further 100 μl from the dilutions were plated on GYE plates to obtain direct estimates of the number of viable bacteria added to each PCR reaction.

Activation of the Chelex 100 resin

A suspension of 5 g of the Chelex 100 resin in 50 ml of sterile ultrapure water was incubated for 12–14 h at 4°C. The suspension was filtered in a Buchner funnel with fritted disc (Corning, cat. no. 36060 – 150 ml), then re-suspended in 100 ml of 1N HCl and incubated with gentle agitation for 30 min at room temperature. The suspension was washed by filtration several times in sterile ultrapure water, until the filtered water reached pH 7·0. The resin was then re-suspended in 100 ml of 1N NaOH and incubated with gentle agitation for 30 min at room temperature. The material was then washed three times by filtration in 100 ml of Tris-HCl 500 mmol l^{-1}, pH 8·0 buffer, with incubation periods of 30 min and gentle agitation. Following the last wash, the resin was re-suspended in 250 ml of Tris-HCl 500 mmol l^{-1}, pH 8·0 buffer and stored at 4°C until use.

DNA extraction from cultured bacteria and plant material

Samples of cultured bacteria, citrus xylem sap and citrus leaf tissue were mixed with 100 μl of styrene divinylbenzene resin (Chelex 100; Bio-Rad, Hercules, CA, USA) that had been processed, as described above, to have its binding-sites activated. The suspensions were vortexed for 10 s; centrifuged briefly and incubated at 56°C in a water bath for 30 min; vortexed again for 10 s; centrifuged briefly; boiled for 8 min and centrifuged (12 000 g, 3 min). The supernatant was collected and transferred to new tubes for storage at −20°C until use. Bacterial DNA was also obtained from citrus leaf samples with CVC symptoms and bacterial suspensions through traditional extraction methodologies using phenol–chloroform (Shillito and Saul 1988) and the InstaGene Matrix (Bio-Rad), respectively. These DNA samples were used as controls.

Xylella fastidiosa DNA extraction from sharpshooter tissues

To extract bacterial DNA present in sharpshooters, their heads were removed using flame-sterilized forceps. Each head was then finely homogenized with a glass stick in 200 μl of a 0·25% solution of insoluble polyvinylpolypyrrolidone (PVPP), that had previously been washed with acid, as described by Holben *et al.* (1988). The samples were centrifuged (12 000 *g*, 20 min, at room temperature) and the supernatant was discarded. One hundred microlitres of the Chelex 100 resin, prepared as described above, was added to the samples and bacterial DNA was extracted as described for cultured bacteria and plant material.

PCR analyses

The conditions for DNA amplification were 8 μl of extracted DNA, 1X PCR Buffer (Invitrogen, Carlsbad, CA, USA); 2 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} of each dNTP; 1·0 unit of *Taq* polymerase (Invitrogen) and 30 ng of each primer: CVC-1/272-2 int or 272-1/272-2 (Pooler and Hartung 1995). The final reaction mixture volume was 20 μl. The first primer set was used for plant and bacterial samples and the second was used for insect samples in the first round of the nested-PCR. The PCR cycling conditions were: 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 90 s. After a final extension at 72°C for 5 min, reactions were held at 4°C. For the nested-PCR, 3 μl of the first PCR reaction was used as a template using the CVC-1/272-2 int primer set and it was amplified using the conditions for DNA amplification and PCR cycling programme described above.

The PCR products were separated by 1·5% Ethidium Bromide Agarose Gel Electrophoresis (EB-AGE) at 100 V, in 1X TBE buffer (Sambrook *et al.* 1989) containing ethidium bromide. The fragments were visualized with UV illumination and documented with a Gel Doc 1000 system (Bio-Rad). The images were saved as inverted data.

RESULTS

Xylella fastidiosa DNA extraction from plants

The Chelex 100 matrix was equally as efficient for DNA extraction of *X. fastidiosa* in the different tissues used (cultured cells, samples of leaves and xylem extracts of citrus plants) as extraction using phenol–chloroform or Instagene matrix methods. An amplicon of 500 bp (Pooler and Hartung 1995) was observed in all DNA samples used in PCR with the CVC1/272-2 int primer set (Fig. 1), currently recommended for the diagnosis of CVC. Cultivated bacteria and symptomatic citrus plant samples (Fig. 1 - lanes 2, 4

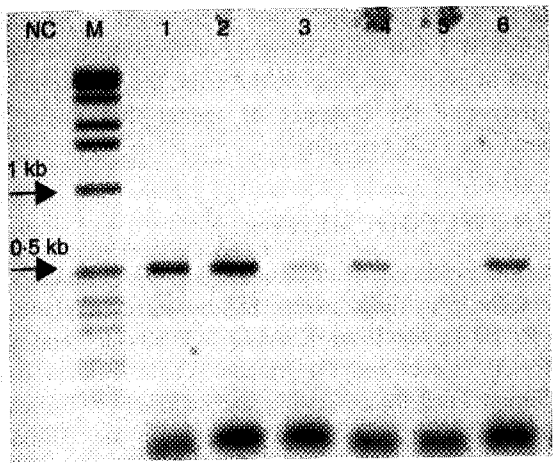

Fig. 1 EB-AGE of the amplification product using the CVC-1/272-2 int primer. Lanes: NC, Negative control; M, Ladder 1 kb DNA molecular size (Invitrogen); 1, DNA from cultured *Xylella fastidiosa* and extracted with InstaGene matrix (Bio-Rad); 2, DNA from cultivated *X. fastidiosa* cells; 3, DNA extraction from citrus xylem extracts without symptoms of CVC; 4, DNA extraction from citrus xylem extracts with symptoms of CVC; 5, DNA from citrus leaves without symptoms of CVC; 6, DNA from citrus leaves with symptoms of CVC. Lanes 2 to 6 DNA extracted using Chelex 100 resin

and 6) presented this fragment. It was still possible to observe the presence of a weak band in lane 3 (Fig. 1) with the material originating from the xylem extract of a citrus plant without symptoms. This result could be an indication of the presence of *X. fastidiosa* at a stage where the plants are yet to exhibit the disease symptoms.

Xylella fastidiosa DNA extraction from sharpshooters by Chelex 100

The presence of PVPP in the macerated insect samples and the extraction of DNA using Chelex 100, combined with the nested-PCR approach, allowed the detection of *X. fastidiosa* cells when in very low numbers. To determine the detection limit of this procedure, 2×10^8 to 2 bacteria were added to the macerated sharpshooter heads. From the extracted DNA, 8 μl were used in the first round of PCR with the 272-1/272-2 primer set generating an amplicon of 700 bp as expected (Pooler and Hartung 1995). It was possible to detect up to 2×10^2 and 2×10^5 bacteria in the reactions without and with the macerated sharpshooter head, respectively. In the nested-PCR assay, using the CVC-1/272-2 int primer set, an amplicon of 500 bp (Pooler and Hartung 1995) was obtained in all reactions. It was possible to detect two bacteria per sample using the Chelex extraction methodology.

Because of the probable low concentration of bacteria present in the heads of sharpshooters fed on infected plants, no bacterial DNA was detected in insects by the standard PCR without the further additional nested-PCR amplifications. An amplicon of the expected size (500 bp) was obtained in the second step of the nested-PCR for some of the sampled insects, DNA extracted from cultured cells and suspensions of cells added to the macerated insect samples (Fig. 2).

In this study, 250 sharpshooters were tested from which 87 (34·8%) showed positive results for the presence of *X. fastidiosa* with nested-PCR. Table 1 summarizes the results of the nested-PCR sharpshooter tests. About 40% of

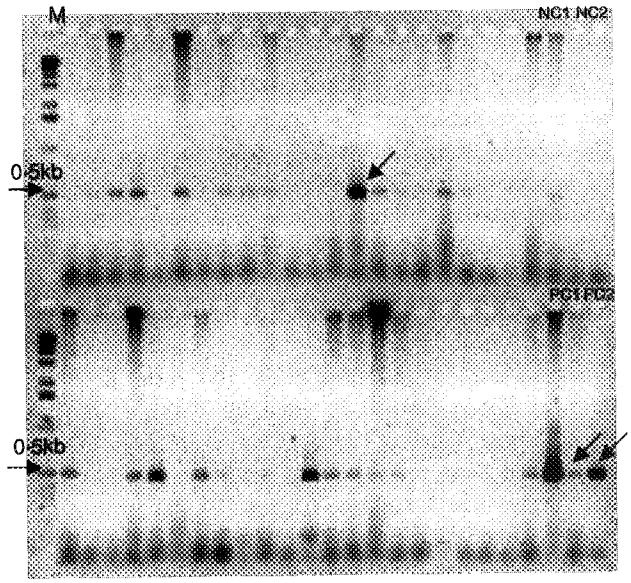

Fig. 2 Detection of *Xylella fastidiosa* in fed sharpshooter by nested-PCR using the CVC-1/272-2 int primer. Lanes: M, Ladder 1 kb DNA molecular size (Invitrogen); NC1, Negative Control (macerated head of healthy sharpshooter); NC2, Negative Control (PCR mix without DNA); PC1, Positive Control (DNA extracted from macerated head of healthy sharpshooter + 100 μl of cultured bacteria); PC2, DNA extracted from 100 μl of cultured bacteria. All the other lanes were loaded with amplified material from *X. fastidiosa* present in the head of sharpshooter fed on infected plants in the second round of PCR. Positive samples showed an amplified fragment of 500 bp indicated by arrow

Table 1 Results of the nested-PCR

Species	No. of insects	Positive PCR	Infected sharpshooters (%)
Dilobopterus costalimai	152	54	35.5
Oncometopia facialis	78	31	39.7
Bucephalogonia xanthopis	15	0	0
Acrogonia sp.	5	2	40.0
Total of individuals	250	87	34.8

the species *Oncometopia facialis* that had an acquisition access period (AAP) of 48 h were positive in nested-PCR reactions. When insects of *O. facialis* had an AAP of 1·5 h they tested negative for *X. fastidiosa*.

DISCUSSION

The extraction of high quality DNA, from plant, insect and environmental sample, that does not inhibit the PCR has been one of the main problems in using PCR as a diagnosis tool (Holben *et al.* 1988; Vega *et al.* 1993; Minsavage *et al.* 1994). The removal of phenolic compounds has been performed by the use of PVPP in DNA extraction from plant material to detect *Xanthomonas campestris* (Leite *et al.* 1995) and *X. fastidiosa* (Minsavage *et al.* 1994). Thus, the use of PVPP prior to DNA extraction from sharpshooter heads was an important step to obtain high quality DNA.

Procedures using Chelex 100 chelating resin have been used efficiently for DNA extraction of small samples of biological materials (blood, semen and hair) with forensic objectives (Walsh *et al.* 1991). DNA extracted by this procedure seems to contain less PCR inhibitors when compared with samples prepared using phenol–chloroform extraction (Walsh *et al.* 1991).

The mode of action of Chelex 100 resin in PCR is unknown. Singer-Sam *et al.* cited by Walsh *et al.* (1991) suggested that Chelex 100 acts by protecting DNA by chelating bivalent metal cations that can damage DNA. According to several authors, Chelex 100 may protect DNA liberated when boiling, or if high temperatures are used, to extract DNA from bacterial cells (Walsh *et al.* 1991). In addition, Chelex 100 can chelate many bivalent ions released in samples and, as the beads of Chelex 100 can be easily removed by centrifugation, the resin does not interfere with further amplification by PCR.

In asymptomatic plant material it was possible to detect up to 70 CFU ml^{-1} of *Erwinia amylovora* using the standard PCR method, while the sensitivity using nested-PCR assays was 0·7 CFU ml^{-1} (Llop *et al.* 2000). Using immunocapture and nested-PCR assays, Hartung *et al.* (1996) detected 10 cells per millilitre of *Xanthomonas axonopodis* pv. *citri* in plant extracts. In insect vectors it was possible to detect as few as five *X. fastidiosa* bacteria per sample (a single insect in a volume of 300 μl) by immunomagnetic separation and nested-PCR techniques (Pooler *et al.* 1997). It has been possible to increase the efficiency of detection of low concentrations of organisms in samples by using immunocapture and immunomagnetic separation techniques prior to nested-PCR. However, both techniques require antibodies that recognize the target bacteria and these methodologies are expensive and laborious.

In this study, the combination of an efficient, fast and less laborious DNA extraction procedure using Chelex 100 and nested-PCR assays resulted in a high degree of sensitivity that allowed a low concentration of bacteria to be easily detected in insect samples. At the same time, the methodology overcame the presence of agents that might have inhibited the PCR reactions.

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