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ScienceDirect
Journal of Microbiological Methods 73 (2008) 57–61
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

High throughput PCR detection of *Xylella fastidiosa* directly from almond tissues

Jianchi Chen [a,*], Sam Livingston [b], Russell Groves [a], Edwin L. Civerolo [a]

[a] *Agricultural Research Service, United States Department of Agriculture, Parlier, CA, United States*
[b] *Department of Plant Pathology, University of California, Davis, CA, United States*

Received 29 October 2007; received in revised form 22 January 2008; accepted 25 January 2008
Available online 5 February 2008

Abstract

Xylella fastidiosa, the causal agent of almond leaf scorch disease (ALSD), is currently re-emerging as a serious concern in California. Efficient pathogen detection is critical for ALSD epidemiological studies, particularly when a large sample size is involved. We here report a PCR procedure to detect *X. fastidiosa* directly from infected almond tissue without the laborious DNA extraction. Plant samples were prepared by freeze-drying and pulverized. Appropriate dilutions of the pulverized freeze-dried tissue (PFT) were determined to minimize the effect of enzyme inhibitors from plant tissue and retain PCR detection of *X. fastdiosa* cells at a single digit number level. This PFT-PCR procedure was evaluated by comparing to the *in vitro* cultivation method using 102 symptomatic samples and resulted in a predictive value of 90.8%. PFT-PCR was further applied to monitor the seasonal occurrence of *X. fastidiosa* from four selected almond trees in two orchards in 2005. The results matched with those of the cultivation method at 92.3%. Considering the simplicity and reliability, we conclude that PFT-PCR is a valuable option for high throughput rapid detection of *X. fastidiosa*.
Published by Elsevier B.V.

Keywords: Almond leaf scorch; Freeze-drying; High throughput detection; Pathogen detection; PFT-PCR; *Xylella fastidiosa*

1. Introduction

Almond leaf scorch disease (ALSD) is currently re-emerging in the San Joaquin Valley (SJV) of California, potentially threatening the production of this highly valued crop. The epidemiology of ALSD is poorly understood and the potential economic effects of the disease are unknown. ALSD is caused by *Xylella fastidiosa*, a nutritionally fastidious bacterium (Wells et al., 1987). Symptoms of ALSD are typically leaf marginal necrosis or leaf scorching. These symptoms are not a specific indication of ALSD and can be easily confused with salt toxicity and mineral deficiency commonly occurring in SJV (Mircetich et al., 1976). The primary diagnosis of ALSD relies on pathogen detection.

Isolation by pathogen cultivation *in vitro* is the most definitive and direct for pathogen detection and identification because whole bacterial cells and their biochemical and physiological properties are observed. However, cultivation of *X. fastidiosa* in the laboratory is time consuming, ranging from 3 to 20 days, and labor intensive, particularly when a large number of samples are involved. Serological methods target the unique properties of bacterial cell surface. Among them, enzyme-linked immunosorbent assay (ELISA) is commonly used and has a high throughput capacity (Sherald & Lei, 1991) because of the simplicity in sample preparation and the use of the 96-well plate format. However, production of antiserum is a complicated process and cross reactions between different *X. fastidiosa* strains remains to be a problem with the currently available antiserum.

PCR detection targets the variation of bacterial genomic DNA. The available whole genome sequences of *X. fastidiosa* strains (Bhattacharyya et al., 2002; Simpson et al., 2000; Van Sluys et al., 2003) make it feasible to design PCR primers at various levels of

* Corresponding author. USDA-ARS, Parlier, CA, 93648, United States. Tel.: +1 559 596 2924; fax: +1 559 596 2921.
E-mail address: jichen@fresno.ars.usda.gov (J. Chen).

0167-7012/$ - see front matter. Published by Elsevier B.V.
doi:10.1016/j.mimet.2008.01.011

specificity. Several specific PCR primer sets are currently available for *X. fastidiosa* detection including the most thoroughly tested RST31/33 primer set (Minsavage et al., 1994), derived from the RNA polymerase genomic locus, and those derived from 16S rRNA gene (Chen et al., 2005), an important taxonomical character for the description of *X. fastidiosa* (Wells et al., 1987). In contrary, sample preparation methods have been subjected to much less vigorous evaluation.

The most common PCR detection procedures for *X. fastidiosa* involve sample extraction to generate template DNA. However, the current DNA extraction procedure is very time consuming, severely reducing the high throughput capacity of a PCR technique. Efforts were made to simplify or omit the DNA extraction procedure by using plant sap for PCR DNA template, but the results were inconsistent (Banks et al., 1999; Minsavage et al., 1994). In this study, we developed and evaluated a PCR procedure to detect *X. fastidiosa* from pulverized freeze-dried tissues (PFT-PCR, namely) without the need for DNA extraction. Our hypothesis was that bacterial DNA would be released into detection buffer if the plant tissue was sufficiently pulverized and thereby provided adequate quantity and quality of template DNA for PCR analysis.

2. Materials and methods

2.1. Sample collection and preparation

All almond leave samples were collected from commercial orchards in SJV of California. The leaf sample was first used for bacterial isolation, and, then, the same piece of sample was used for PFT-PCR. Two evaluation procedures were used. The first procedure was to test the predictive capacity PFT-PCR to the bacterial cultivation method, and the second procedure was to test if PFT-PCR could be used to monitor the population dynamic of *X. fastidiosa* in selected trees in two orchards during a growing season.

For the first evaluation, samples were collected from 102 almond trees in an orchard in Fresno County, California, in September, 2004. Briefly, small branches showing leaf scorching symptoms were excised, placed in labeled plastic bags, and transported in an ice cooler to the SJV Agricultural Sciences Center, Parlier, California. Upon arrival, samples were stored at 4 °C and processed within 24 h. One symptomatic leaf was selected to represent one tree. For the second evaluation, two almond orchards in Fresno County, California, were selected in 2005 based on the presence of ALSD in 2004. One previously known ALSD tree and one tree which did not show any leaf scorching symptoms were selected from each almond orchard. The branching patterns of each tree were mapped. Leaf samples were collected from labeled scaffolds in May and June when no leaf scorching symptoms were seen and in September when symptoms were obvious. Sample collection and processing were identical to that of the first evaluation.

2.2. Bacterial isolation and cultivation

The previously described procedure (Chen et al., 2005) was followed. Briefly, petioles of approximate 2 cm length were separated from leaves and surface sterilized in 10% sodium hypochlorite for two minutes followed by three successive rinses in sterile, distilled water. An incision was made in the center of the petiole. Xylem sap was expressed aseptically using a pair of flame-sterilized, needle-nose pliers onto the sterile surface of a Petri dish. A drop of PW broth (Davis et al., 1981) was immediately added and mixed with the sap. One loopful of the sap mixture was then streaked onto PW medium solidified by Gelrite (Sigma-Aldrich, Inc. St. Louis, Mo) and incubated at 28 °C. The appearance of opalescent colonies was monitored using a binocular microscope for up to 40 days. Candidate isolates were transferred onto new PW-G and confirmed as *X. fastidiosa* by PCR using the RST 31/33 primers (Minsavage et al., 1994) and 16S rDNA primers (Chen et al., 2005).

2.3. Freeze-drying and sample pulverization

Almond leaves or petiole leftovers from isolation experiment were placed in a labeled paper envelope and freeze-dried in the Freezone 2.5 Freeze Dry System (Labconco Corp., Kansas City, Mo) overnight. Almond petioles were sufficiently dried overnight (>12 h) in the freeze-dryer following the manufacturers' recommendation (Temperature <-40 °C; and Vacuum $<1.33\times10^{-3}$ mBAR). The dried samples were used immediately or stored at 4 °C in plastic bags. Individual freeze-dried leaf petioles with approximate length of 2 cm were placed into 2 ml microtubes with sterile ceramic beads and pulverized to a fine powder in a Fast-Prep machine (FP120, Qbiogene, Inc. Carlsbad, CA) for 20 s. The dried tissues were easily pulverized by FastPrep machine within 20 s. The pulverized freeze-dried tissue or PFT was suspended in 500 µl of TE buffer and used for PCR after dilution. PFT particle sizes were measured microscopically using a micrometer.

2.4. PCR procedure

PCR reaction (25 µl) was carried out using the TaKaRa TaqTM (Hot Start Version) kit (Takara Bio Inc., Seta 3-4-1, Otsu, Shiga, 520-2193, Japan). The reaction mixture contained: 2.5 µl of 10×DNA polymerase buffer, 2.5 µl of dNTPs (2.5 mM of each dNTP), 0.5 µl of each of the 10 µM forward and reverse primers, 1 µl of diluted petiole suspension, 0.2 µl of *Taq* DNA polymerase (5 U/µl) and 18.3 µl of H_2O. The multiplex PCR procedure (Chen et al., 2005) was used for *X. fastidiosa* detection. Briefly, primers Teme150fc (5′ tctaccttat cgtgggggac 3′) and Teme454rg (5′ aacaactagg tattaaccaa ttgcc 3′) specific to G-genotype and primers Dixon454fa (5′cttttgttg gggaagaaaa 3′) and Dixon1261rg (5′ tagctcaccc tcgcgagatc 3′) specific to A-genotype, were used for PCR amplification in an MJ Research Tetrad II DNA engine with an initial denaturing at 96 °C for 10 min, followed by 30 cycles consisting of: denaturing at 96 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The amplification products were stored at 4 °C. The amplified DNAs were resolved through 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. To further confirm that *X. fastidiosa* DNA was amplified, PCR amplicons were directly sequenced using