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SHORT NOTES

Specific identification of *Xylella fastidiosa* using the polymerase chain reaction (*)

Xylella fastidiosa Wells, Boligala, Hung, Weisburg, Mandelco-Paul *et* Brenner is a fastidious, xylem-limited bacterium, causal agent of several plant disease syndromes of quarantine significance (Anonymous, 1989; Davis, 1992). Since the direct isolation of this slow-growing organism can be difficult, sensitive and specific molecular tools are expected to help its early detection in latently infected propagation material. Prior to setting up a rapid and reliable diagnostic assay, the potential use of polymerase chain reaction (PCR) (Mullis and Faloona, 1987) was investigated.

Two specific PCR primers were derived from variable regions V1 and V6 (Gray *et al.*, 1984) of the 16S rRNA sequence of *X. fastidiosa* strain ATCC 35880 (Wells *et al.*, 1987), available from the GenBank database with the accession number M26601. The 5' ends of the two primers correspond to positions 71 and 458 in this sequence, respectively. Primer sequences were: XF1 (direct) 5'-CAGCACATTGGTAGTAATAC-3' and XF6 (reverse) 5'-ACTAGGTATTAACCAA-TTGC-3'. Bacteria, including five strains of *X. fastidiosa* (PD 89-1, isolated from grapevine, kindly supplied by D.L. Hopkins, University of Florida, Leesburg, FL; ATCC 35870, isolated from almond; ATCC 35871, isolated from hybrid plum; ATCC 35873, isolated from American elm; CB-9 isolated from citrus [Hopkins, 1988]), *Xylophilus ampelinus* 071-12 and 420-10 (kindly supplied by M.M. López, Instituto Valenciano de Investigaciones Agrarias, Moncada, Spain), *Xanthomonas campestris* pv. *vesicatoria* NCPPB 701, *Pseudomonas solanacearum* NCPPB 2200, *P. syringae* pv. *syringae* NCPPB 1770, *Escherichia coli* ATCC 25645 and an unidentified, endophytic grapevine bacterium (IPV-BO 2586-2D), with morphological and growth-rate characteristics similar to those of *X. fastidiosa*, were plated on PD 3 agar medium of Davis *et al.* (1980).

Bacterial suspensions were prepared in sterile distilled water from 3 to 8 day-old cultures; using a spectrophotometer, the suspensions were adjusted to $A_{640\ nm}$= 0.1. Two µl of each untreated suspension were added to a PCR reaction containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µg/ml gelatine, 0.16 mM each dNTP, 600 ng each primer and water to 47.6 µl, overlaid with two drops of mineral oil. PCR reaction mixtures were heated to 95°C for 10 min, then the temperature was lowered to 80°C and 2.4 µl of 0.5 U/µl *Taq* polymerase (Boehringer, Mannheim, Germany) were added to each tube. Amplification was carried out in a Perkin-Elmer 480 Thermal Cycler with 30 cycles of: denaturation (94°C) for 45 s, annealing (60°C) for 30 s and extension (72°C) for 30 s. The final extension step was 10 min at 72°C. Two µl of each suspension were also added to a PCR reaction for the general amplification of eubacterial 16S rDNA, using the primer pair fD1-rP1 in the conditions described by Weisburg *et al.* (1991), with a modification consisting of heating the reaction at 96°C for 10 min prior to the addition of the enzyme. An aliquot (15 µl) of each PCR

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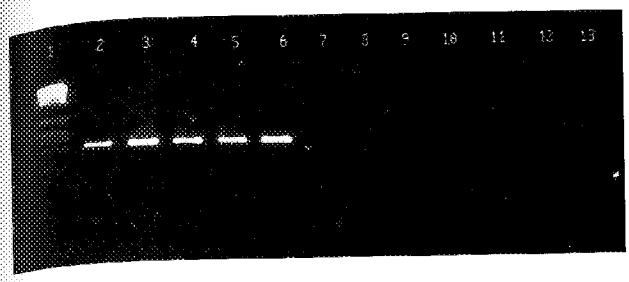

Fig. 1- Agarose gel electrophoresis of the products of the amplifications carried out in the conditions described in the text using primers XF1-XF6 and suspensions of the following bacteria as a source of DNA template: *X. fastidiosa*, PD 89-1 (lane 2), ATCC 35870 (lane 3), ATCC 35871 (lane 4); ATCC 35873 (lane 5), CB-9 (lane 6), *Xylophilus ampelinus* 071-12 (lane 7), 420-10 (lane 8); *Xanthomonas campestris* pv. *vesicatoria* NCPPB 701 (lane 9); *Pseudomonas solanacearum* NCPPB 2200 (lane 10); *P. syringae* pv. *syringae* NCPPB 1770 (lane 11); *Escherichia coli* ATCC 25645 (lane 12); unidentified bacterium IPV-BO 2586-2D (lane 13). Lane 1: Molecular marker VI (Boehringer).

product was run in an agarose gel (1% for general amplification, 4% for specific amplification) electrophoresis in TBE (45 mM Tris-borate, 1 mM EDTA) buffer.

PCRs with primers fD1-rP1 carried out using all bacterial suspensions as sources of DNA template gave the amplification of an about 1500 bp DNA fragment (not shown), demonstrating that 16S rDNA was available for enzymatic amplification in all samlpes. Only the *X. fastidiosa* suspensions produced a detectable amplification product when used as sources of DNA template in PCRs carried out with the specific primer pair XF1-XF6 (Fig. 1). The amplification product was a single DNA fragment of about 400 bp, a value which is consistent with the distance between regions V1 and V6 in the 16S rRNA sequence determined for *X. fastidiosa* (Wells *et al.*, 1987). Five tenfold dilutions were made of the suspension of *X. fastidiosa* strain PD 89-1 ($A_{640\ nm}$= 0.1) and 2 µl of each dilution were used for PCR, in order to assess the sensitivity threshold of the assay. The number of *X. fastidiosa* cells per ml of the suspension at A_{640nm}= 0.1 was determined with indirect immunofluorescence staining (IFAS), by counting the cells on each spot of multispot slides, and resulted 3.8 x 10^7 cells/ml^{-1}. A faint band was detected as a product of the amplification carried out on the third dilution (Fig. 2), and the detection limit of the assay was estimated to be 7.6 x 10 bacteria.

The amplification of a *X. fastidiosa* DNA sequence by PCR was obtained; the sensitivity of the assay, using pure bacterial cultures, was shown to be higher than that of serological techniques, combined with poly- and monoclonal antibodies (Hopkins, 1984; Nomé *et al.*, 1980; Wells *et al.*, 1987). Low specificity and/or loss of the antigenic determinant(s) in the target organism can be linked with conventional serology and these problems highlight the need for new molecular identification methods. However, the research on the molecular biology of *X. fastidiosa* is in its infancy (Goodwin, 1989); a DNA probe that hybridized with 3 out of 5 strains of *X. fastidiosa* was developed by Jimenez and Davis (1987), and different plasmid DNA probes were used by Leite *et al.* (1993) to characterize grapevine and non-grapevine isolates of the bacterium.

The PCR assay presented here allowed the rapid and specific identification of *X. fastidiosa* strains, including isolates from different sources, by the amplification of part of the 16S rRNA gene using the primer pair XF1-XF6. Because of its role in the protein synthesis, the 16S rRNA is a phylogenetically well conserved molecule (Woese, 1987); it is therefore unlikely that the primer pair described in this report may fail to identify other,

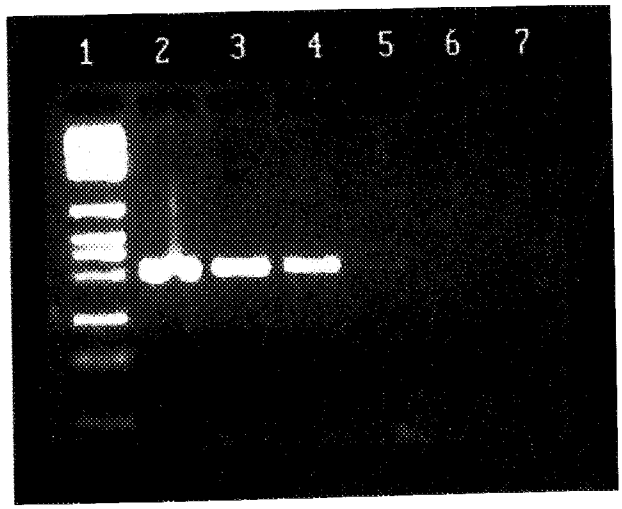

Fig. 2 - Agarose gel electrophoresis of the products of the amplifications carried out in the conditions described in the text using primers XF1-XF6 and two µl of suspensions of *X. fastidiosa* corresponding to: 7.6 x 10^4 cells (lane 2), 7.6 x 10^3 cells (lane 3), 7.6 x 10^2 cells (lane 4), 7.6 x 10^1 cells (lane 5), 7.6 cells (lane 6). Lane 1: Molecular marker VI (Boehringer). Lane 7: negative control (no DNA).

naturally occurring strains of the pathogen or cross-react with taxonomically distant related organisms.

Our results encourage application of this PCR based identification method in the setting up of a reliable diagnostic assay for the detection of *X. fastidiosa* in infected tissues.

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G. Firrao and C. Bazzi *
Dipartimento di Biologia Applicata alla Difesa delle Piante, Università degli Studi, Udine, Italy
* *Istituto di Patologia Vegetale, Università degli Studi, Bologna, Italy*

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