

Improvements in Sample Preparation and Polymerase Chain Reaction Techniques for Detection of *Xylella fastidiosa* in Grapevine Tissue

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The sensitivity of detecting of *Xylella fastidiosa* in ground grape tissue via standard polymerase chain reaction (PCR) procedures is often limited by inhibitory plant compounds. Aliquots of standardized bacterial suspensions in healthy grapevine extracts were used to create a 10-fold dilution series for testing the effect of immunocapture (IC) and nested primers (N) on PCR sensitivity. DNA from *X. fastidiosa* suspended in grape leaf extract was not amplified by non-IC-PCR using standard and nested methods. The use of dynabeads for the immunomagnetic separation of target bacteria cells in plant extract was shown to reduce the inhibitory effect dramatically and increase sensitivity 10,000-fold over non-IC-PCR methods. The IC procedure with standard PCR primers achieved a sensitivity of 10,000 cells/mL of grape leaf extract. N-PCR primers were also used in combination with the IC procedure and increased sensitivity an additional 1,000-fold over standard PCR primers. The combined IC-N-PCR procedure achieved a maximum sensitivity of 2 cfu/mL of bacteria concentration in grape leaf extract. This method also gave reliable results when samples were taken from *X. fastidiosa*-infected grapevine plants. A new method was also applied for specific amplification of genomic fragments of the bacteria with nucleic acid deposition on small pieces of charged nylon membrane by spotting crude infected grapevine sap (spot-PCR) in combination with the nested primers. Spot-nested (Sp-N)-PCR was able to achieve the same level of sensitivity as IC-N-PCR with known dilutions of *X. fastidiosa* in buffer. Sp-N-PCR was able to detect positive and negative controls, but its reliability in sensitivity tests suggests that it needs further study.

Key words: *Xylella fastidiosa*, disease detection, Pierce's disease, immunocapture

Pierce's disease (PD) of grape is caused by *Xylella fastidiosa*, a xylem-limited, gram-negative fastidious bacterium (Goheen et al. 1973, Hopkins and Mollenhauer 1973, Hopkins 1989, Wells et al. 1987). The bacterium also causes other plant diseases, including alfalfa dwarf (Goheen et al. 1973), almond leaf scorch (Mircetich et al. 1976), phony peach, plum leaf scald (French and Kitajima 1978, Kitajima et al. 1975), periwinkle wilt (McCoy et al. 1978), citrus variegated chlorosis (Chang et al. 1993), oleander leaf scorch (Purcell et al. 1999), pecan bacterial leaf scorch (Sanderlin and Heyderich-Alger 2000), and coffee leaf scorch (Li et al. 2001). However, strains of *X. fastidiosa* are pathogenically specialized. For example, periwinkle wilt strains do not cause severe disease in grape, although this strain multiplies in grape (Chang and Donaldson 2000, Hopkins 1989).

PD infection is severe and usually leads to death of susceptible *Vitis vinifera* and *Vitis X. labruscana* grapevines in two or three years. The foliar symptoms mimic extreme water stress and have been shown to be the result of xylem plug-

ging due to the bacteria, its secreted slime, and induction of plant gums, polysaccharides, and tyloses (Goodwin et al. 1988).

Xylella fastidiosa infection can be verified using standard bacteriological isolation techniques from symptomatic tissues, followed by serological testing of isolated colonies. However, such methods can be hindered by the specialized nutritional requirements of the bacterium and its relatively slow growth in culture. Additionally, cross-reaction among *X. fastidiosa* strains limits the use of available polyclonal antiserum for strain detection (Hill and Purcell 1995). These limitations prompted the use of DNA-based techniques to determine differences among *X. fastidiosa* strains (Banks et al. 1999, Chen et al. 1995, Da Costa et al. 2000, Henderson et al. 2001, Pooler et al. 1995) and to detect the bacterium from plant tissue (Minsavage et al. 1994). Nested-PCR with two sets of primers designed by cloning and sequencing specific random amplified polymorphic DNA (RAPD) products (Pooler and Hartung 1995) has been used to detect *X. fastidiosa* in insect vectors (Pooler et al. 1995). These techniques are limited because of the time required for purifying nucleic acid from grape tissue and are not practical when large numbers of samples need processing (Rowhani et al. 1995).

The purpose of this research was to compare the efficiency of three PCR-based techniques (standard PCR, nested PCR, and immunocapture PCR) at detecting *X. fastidiosa* in grape

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while avoiding DNA purification and colony-isolation techniques. This research also attempted to adapt an existing PCR protocol that combines immuno-capture (IC)- and nested (N)-PCR procedures for *X. fastidiosa* detection in insects (Hartung et al. 1996) to use with grape. Finally, a new method using a positively charged nylon membrane onto which plant sap is spotted and then analyzed in a two-step, single-tube N-PCR was evaluated for the detection of *X. fastidiosa* in grapevine tissue.

Materials and Methods

Bacterial culture conditions. The Stag's Leap isolate of *X. fastidiosa* (Hendson et al. 2001) was isolated from semilignified canes of PD-infected potted *Vitis vinifera* cv. Chardonnay, displaying irregular lignification symptoms or scorched leaves, by culturing on periwinkle agar medium (Davis et al. 1981) at 28°C. When well-established subcultured colonies developed (about four to five days), the bacteria were harvested by washing the surface of the agar with deionized water. The concentration of bacterial suspensions was standardized to an optical density of 0.25 at A_{600} , which was established to be 2×10^8 colony forming units (cfu)/mL by Minsavage et al. (1994) via dilution plating on periwinkle medium. Aliquots of the standardized bacterial suspensions were used to create 10-fold dilution series in 100 μ L of: (1) PBS (8 g NaCl, 0.2 g KH_2PO_4 , 11.5 g Na_2HPO_4 , 0.2 g KCl per 1 L and pH 7.4); (2) grapevine plant extracts prepared from freshly ground healthy leaves (0.5 g) in 10 vol (w/v) of extraction buffer [PBS, 2% polyvinylpyrrolidone (PVP, w/v) and 0.05% Tween 20]; (3) extraction buffer alone; and (4) water. The PCR experiments used a 10^{-1} to 10^{-8} (equivalent to 2×10^7 to 2 cfu/mL) dilution series. Negative controls consisted of the liquid dilution series without bacteria.

Standard PCR. Five- μ L aliquots of bacteria samples diluted in water, PBS buffer, or healthy grapevine extract were added to 50 μ L of final PCR mastermix containing 1X thermophilic buffer (supplied by manufacturer with the enzyme), 2 mM $MgCl_2$, 200 μ M of each dNTP, 120 nM of each primer, and 1U of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD). This mixture was overlaid with mineral oil and tubes were placed in a thermocycler (M.J. Research, Watertown, MA). Oligonucleotide primers designed from genomic DNA and specific for *X. fastidiosa* (RST31/RST33) and PCR profile were as reported by Minsavage et al. (1994). All PCR experiments described here and in subsequent sections were repeated two or three times to ensure results were reproducible.

Nested (N)-PCR. The same bacterial preparations for standard PCR were also used for N-PCR. Oligonucleotide primers specific to *X. fastidiosa* were designed by Pooler et al. (1995). Reaction mastermix for the first round of amplification was prepared in 30 μ L with external primers (272-1-ext: 5'-AGCGGGCCAAAACGATGCGTG-3' and 272-2-ext: 5'-AGCGGGCCAAAACGATGCGTG-3'), with other components at the same final concentration as previously described for single-round PCR, including 1U *Taq* DNA polymerase. The PCR-cycling parameters were initial denaturation

at 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min for 35 cycles. The second round of N-PCR used 5 μ L from the first reaction as template with internal (nested) primers (272-1-int: 5'-CTGCACTTACCCAATGCATCG-3' and 272-2-int: 5'-GCCGCTTCGGAGAGCATTCCT-3') and 1.5U *Taq* DNA polymerase. Amplification conditions were the same as established for the first round.

Immunocapture (IC)-PCR. The immunocapture protocol followed Pooler et al. (1997) and Smart et al. (1998) with slight modifications. Serial dilutions of bacterial suspensions were prepared in 0.5 mL of water, healthy grapevine extracts, or extraction buffer (PBS/PVP/Tween 20) for immunomagnetic separation, and anti-*X. fastidiosa* rabbit immunoglobulin (supplied by B.C. Kirkpatrick, Plant Pathology, University of California, Davis) raised in rabbit against *X. fastidiosa* diluted 1:1000 (crude antiserum) in PBS/0.2% BSA (w/v) buffer. Dynabeads M-280 (6 to 7 $\times 10^6$ beads/mL) bound with sheep antirabbit IgG (DynaL A.S., Oslo, Norway) were used as paramagnetic beads to capture antibody-bound bacteria cells. The bead/bacteria complex was later suspended in 15 μ L of deionized water, and a 5- μ L aliquot was subjected to both standard and N-PCR.

IC-PCR detection of *X. fastidiosa* in inoculated grapevine samples. Two plants of Fairchild and BD12-49, PD-resistant interspecific hybrids from the southeastern United States were established in the greenhouse and inoculated with *X. fastidiosa*. These hybrids were selected because they were under test as potential parents in the PD-resistance breeding program. All plants were inoculated with 20 μ L of bacterial suspension at concentration of 2×10^8 cfu/mL in water. Leaf samples for PCR were collected 10 cm above and below the point of inoculation three months after the inoculation. About 0.5 g of petiole tissue was ground in the presence of PBS/PVP/Tween 20 in 1:10 ratio (w/v). One-mL aliquots were transferred into 1.5-mL microcentrifuge tubes and centrifuged at 14,000 rpm for 5 min to eliminate the unwanted components of the plant extract. The pellet was resuspended in 500 μ L of PBS/BSA for immunocapture separation. Further procedures for immunomagnetic separation were the same as those used for the bacteria serial dilutions. Five- μ L aliquots of the supernatant were used in standard PCR, IC-standard-PCR, and IC-N-PCR methods.

Spot (Sp)-N-PCR detection of *X. fastidiosa* in grapevine samples. Petioles and semilignified shoots from the PD-infected plants described above were collected and stored at 4°C. Samples were processed and deposited onto Hybond N+ membranes (Amersham Pharmacia Biotech, Piscataway, NJ) as described by La Notte et al. (1997) with slight modifications. Small pieces of the sample spotted-membranes (1.5 cm x 1.5 cm) were incubated with 100 to 150 μ L of releasing buffer (500 mM Tris-HCl, pH 8.3, 2% PVP-40, 1% PEG-6000, 140 mM NaCl, and 0.05% Tween 20) at 65°C for 30 min, and then immediately chilled on ice for 5 min. Five- μ L aliquots were used as a DNA template for amplification in standard PCR and N-PCR. The same samples were also processed for IC-PCR as described in the above sections.

Analysis of the amplification products. Ten- μ L aliquots from each reaction were electrophoresed in 1.2% agarose gels in 1X TBE buffer (890 mM tris-base, 890 mM boric acid, 25 mM EDTA, and pH 8.3). The gels were run at 5V/cm for 90 min and stained with ethidium bromide for 10 min. PCR products were visualized on an UV transilluminator and photographed using Polaroid 667 film.

Results

Bacteria detection with standard PCR. Standard PCR using the RST31/RST33 primers specifically amplified a 733-bp product of *X. fastidiosa* DNA from pure culture. The lowest detectable bacterial dilution was a five times diluted aliquot (10^{-5}) in water from the original bacterial suspension (2×10^8 cfu/mL). The bands from the first four dilutions in water were clearly visible on the gel; however, the band from the fifth dilution was faint (not shown). The DNA bands from bacterial suspensions in PBS had almost the same intensity as those obtained from bacterial suspensions in water. The sensitivity limit of the method was 2×10^3 cfu/mL (Figure 1). Bacterial suspensions were used to define the detection limits of PCR. However, when PCR was conducted with bacterial dilutions in grapevine leaf extract, DNA amplification was only evident for the highest bacteria dilution (10^{-1} : 2×10^7 cfu/mL) or 10^5 cells per reaction (Table 1). There were no positive amplification signals from the rest of the dilution series.

Bacteria detection with N-PCR and sensitivity of IC with the combination of standard PCR or N-PCR. N-PCR worked well when detecting serial dilutions of pure bacterial culture in water and PBS buffer (Figure 2), and its detection threshold was 2×10^2 cfu/mL. However N-PCR did not work well when bacterial dilutions were made in grapevine leaf extract and its detection threshold was 10^{-1} dilution (2×10^7 cfu/mL). Thus, the N-PCR sensitivity was one order of magnitude greater than that of standard PCR (Table 1).

When immunomagnetic separation (immunocapture, IC) and N-PCR were combined, specific amplification was obtained for concentrations of bacterial samples in grapevine leaf extract as low as a 10^{-8} dilution. Detection of bacteria in dilutions with water and PBS using IC-N-PCR had the same sensitivity as those in grapevine leaf extract (Table 2). When IC with standard PCR primers was used to detect bacteria in grapevine leaf extract, water, or extraction buffer alone, the sensitivity was reduced compared to reactions with nested primers. The lowest bacterial concentration amplified with the standard primers from the dilution series was 2×10^2 cfu/mL. The last three dilutions had reduced band intensity compared to the others (data not shown).

Efforts to detect the bacteria in samples from artificially inoculated grapevine plants with standard PCR and N-PCR (without immunocapture) were unsuccessful (data not shown). Assay of these samples with standard and N-PCR (without IC) resulted in inconclusive and inconsistent results. When the same samples were tested with IC-N-PCR, clear products of the expected size were produced (Figure 3). Four of the

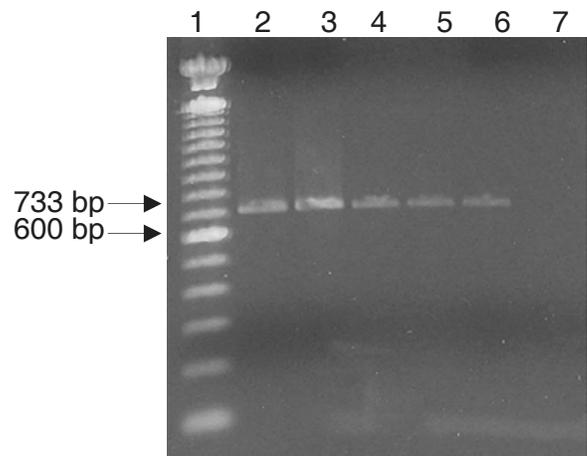


Figure 1 Agarose gel electrophoresis of the products (733 bp) from standard PCR using RST31 and RST33 primers with 10-fold serially diluted suspensions of *X. fastidiosa* in PBS. Lane 1, 100-bp DNA ladder; lanes 2 to 6, bacterial dilutions from 10^{-1} to 10^{-5} ; lane 7, water without bacteria.

Table 1 Comparison of detection sensitivity of standard PCR and N-PCR methods for *X. fastidiosa* in sample preparations.

Sample	Minimum concn detected by PCR (cfu/mL)	
	Standard	Nested
Water	2×10^3	2×10^2
PBS	2×10^3	2×10^2
Healthy plant extract in PBS/PVP/Tween	2×10^7	2×10^7
Negative control	none	none

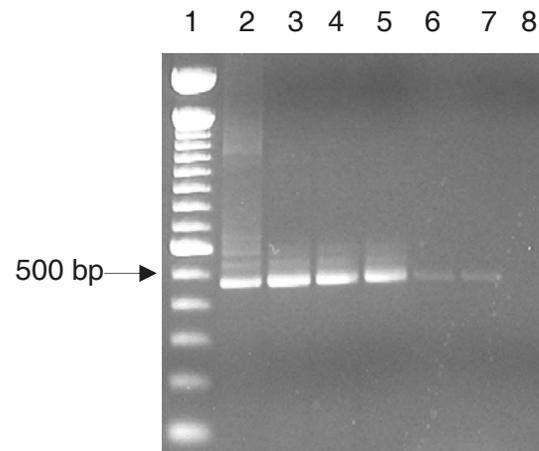
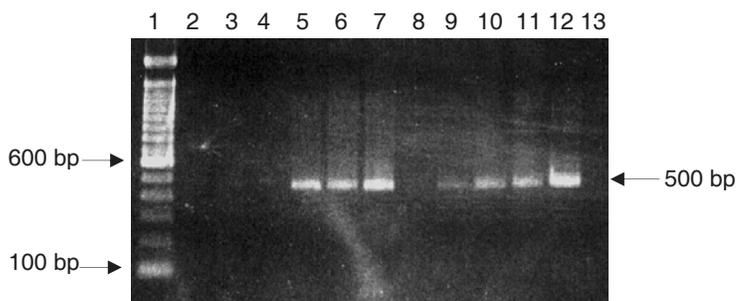
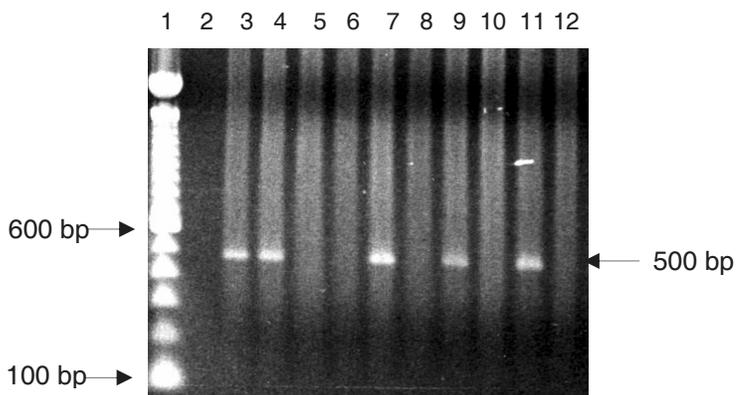


Figure 2 Agarose gel electrophoresis of the products from N-PCR with specific external and internal primer sets. A band of 500 bp was obtained after second round of amplification of DNAs from serially diluted suspensions of *X. fastidiosa* in PBS. Lane 1, 100-bp DNA ladder; lanes 2 to 7, bacterial dilutions from 10^{-1} to 10^{-6} ; lane 8, PBS without bacteria.

Table 2 Comparison of detection sensitivity of IC-standard-PCR and IC-N-PCR methods for *X. fastidiosa* in sample preparations.

Sample	Minimum concn detected by PCR (cfu/mL)	
	IC-standard	IC-N
Water	2×10^2	2
PBS/PVP/Tween buffer	2×10^2	2
Healthy plant extract in PBS/PVP/Tween	2×10^2	2
Negative control	none	none

**Figure 3** Agarose gel electrophoresis analysis of *X. fastidiosa*-specific N-PCR products with the combination of immunomagnetic separation of samples from inoculated grapevines. Samples were taken either 10 cm above the point of inoculation (A) or 10 cm below the point of inoculation (B). Lane 1, 100-bp DNA ladder; lane 2, Fairchild-1A; lane 3, Fairchild-1B; lane 4, Fairchild-2A; lane 5, Fairchild-2B; lane 6, BD12-49-1A; lane 7, BD12-49-1B; lane 8, BD12-49-2B; lane 9, BD12-49-2A; lane 10, infected Chardonnay; lane 11, infected Merlot; lane 12, concentrated bacterial suspension in grapevine leaf extract; lane 13, healthy control. Arrow indicates the specifically amplified 500-bp DNA fragment.**Figure 4** Agarose gel electrophoresis of *X. fastidiosa*-specific PCR products following spotting of sap samples on nucleic acid membrane and N-PCR from inoculated grapevine samples. Samples were taken either 10 cm above the point of inoculation (A) or 10 cm below the point of inoculation (B). Lane 1, 100-bp DNA ladder; lane 2, healthy grapevine sample; lane 3, BD12-49-1A; lane 4, BD12-49-1B; lane 5, Fairchild-1A; lane 6, Fairchild-1B; lane 7, Fairchild-2B; lane 8, Fairchild-2A; lane 9, BD12-49-2A; lane 10, BD12-49-2B; lane 11, infected Chardonnay (positive control); lane 12, water control.

eight samples were positive, and two of these, Fairchild-2B and BD12-49-2A, were from nonsymptomatic but inoculated vines. Only BD12-49-1A and 1B had typical PD symptoms. Detecting *X. fastidiosa* above and below the point of inoculation was inconsistent because leaf petioles were used as sample tissue. Repeated testing and comparisons of IC-N-PCR test results from petioles and stem pieces have found that sampling from stem tissues produces consistent results above and below the point of inoculation. However, the use of petioles as sample tissue can produce inconsistent results based on the uneven movement of *X. fastidiosa* through vascular bundles to leaves (data not presented).

Detection of *X. fastidiosa* in grapevine by Sp-N-PCR. Satisfactory amplification was obtained when samples from artificially inoculated plants were spotted onto a charged nylon membrane and run with the N-PCR conditions detailed above. A lower temperature and a longer incubation time (65°C for 30 min) were more effective than the conditions reported by La Notte et al. (1997) for thermal release of the template (Figure 4). Amplification was unsuccessful when standard PCR primers and conditions were used, although the same spotted membranes were used for all reactions. To test the sensitivity of the Sp-N-PCR, bacterial dilutions in water and in release buffer were used to run the reaction on separate membranes. However, inconsistent results, unrelated to the dilution series, were obtained with positive and negative controls. This inconsistency may have been caused by the lack of genomic DNA deposition from suspension culture on the membrane or because nucleic acids could not be released into the buffer. In terms of sensitivity, most samples that tested positive with IC-N-PCR also produced positive signals with Sp-N-PCR.

Discussion

Specificity, sensitivity, and versatility are three reasons that PCR has generated considerable interest as a diagnostic tool for *X. fastidiosa* detection and identification. However, PCR has its limitations. In the present study, we compared different PCR techniques (standard PCR, nested (N)-PCR, and immunocapture (IC)-PCR) and used a more sensitive process by combining IC- and N-PCR, previously developed to detect *X. fastidiosa* in insects (Hartung et al. 1996). The experiments conducted here used pure bacterial cultures to define the sensitivity of the tested methods. It is difficult to extract DNA in good quantity and quality from grapevine tissues because plants are rich in phenolic compounds, polysaccharides, and other inhibitors. The creation of standard samples with known bacterial numbers allowed estimates of the average detection threshold and comparison of PCR methods.

Standard PCR primers were capable of detecting *X. fastidiosa* dilutions in water or PBS at 2×10^3 cfu/mL cor-

responding to about 10 cells/reaction; however, the sensitivity of standard PCR was reduced when the bacteria were diluted in grapevine leaf extract. When the same dilution preparations were used for N-PCR, *X. fastidiosa* was detected at a concentration of 2×10^2 cfu/mL in water or PBS, which was 10 times more sensitive than standard PCR. When N-PCR was used to detect bacterial dilutions in grapevine leaf extracts its sensitivity was further reduced. Crude grapevine plant extract can be either further processed or diluted in water or buffer as reported by Minsavage et al. (1994) to help reduce plant inhibitors during PCR reactions. They could detect *X. fastidiosa* genomic DNA when the plant extract was diluted 100-fold or more with buffer or water. *Xylella fastidiosa* was detected in dilutions of plant extract in buffer (at about 1×10^2 cfu/mL) with the addition of sodium ascorbate and acid-washed PVPP (polyvinylpyrrolidone). However, these large dilution factors reduce the ability to detect low titer amounts of *X. fastidiosa*, as reported in tests with herbaceous hosts (Purcell and Saunders 1999).

Promising results were obtained from the combination of IC- and N-PCR. This combination was the most sensitive method for *X. fastidiosa* detection in water, extraction buffer, and grapevine leaf extract. The IC-N-PCR method was able to detect bacterial concentrations as low as 2 cfu/mL and was 1,000 times more sensitive than standard PCR and 100 times more sensitive than N-PCR in buffer suspensions. This method was previously used to detect *X. fastidiosa* in insect vectors by Pooler et al. (1997), who reported detecting five bacteria per sample. The removal of bacteria from samples by IC reduced the negative effects of grape tissue DNA inhibitors. The immunomagnetic bead–bacteria complexes are held by magnetic force while nontarget cells and inhibitory compounds are washed away. N-PCR increased the possibility of amplifying the accumulated amplicon by using primers that annealed within the previously amplified products. This technique has also been successfully applied in the detection of other plant bacteria (Expert et al. 2000, Walcott and Gitaitis 2000). Hartung et al. (1996) combined IC- with N-PCR and were able to detect a single cell of *Xanthomonas axonopodis* pv. *citri* per μL , increasing the detection threshold 100 times. The combination of immunocapture and standard PCR primers applied to bacteria suspensions in water, extraction buffer, or healthy grapevine extract did not result in the same detection sensitivity as IC combined with nested primers. The IC-standard-PCR sensitivity level remained 10 times higher than standard PCR, but 10 times lower than IC-N-PCR. The results from IC-standard-PCR with water and PBS-diluted bacteria suspension were similar to those from N-PCR alone with the same diluted bacteria (Figure 3), except when bacteria were diluted in grapevine extract (Figure 4).

The IC-N-PCR method has additional advantages. This method uses the same buffer as that used with enzyme-linked immunosorbent assay (ELISA), allowing the same sample preparation to be assayed with both techniques. The inclusions of detergents in the buffer probably allows release of the template bacterial DNA, by degradation of bacterial cell

wall. The buffer composition also allowed samples to be stored for at least one month at -80°C and allowed stored samples to be used several times without degradation of the bacteria (data not presented). The main advantage in using the same extract for ELISA and IC-N-PCR is the integration of both techniques in certification or breeding programs that require *X. fastidiosa* detection. In this way, a preliminary assay can be performed by ELISA while sample extracts are kept in the freezer. After ELISA results are known, uncertain samples can be rechecked by IC-N-PCR. Previous work with IC-N-PCR was only directed at detecting *X. fastidiosa* in potential insect vectors (Hartung et al. 1996). Its application to and sensitivity at detecting *X. fastidiosa* in grape tissue was unknown. The results presented here found that the technique is practical as a routine method for detecting *X. fastidiosa* in breeding programs, in certification schemes, and for quarantine programs. Finally, IC-N-PCR eliminates phenol/ CHCl_3 extractions. However, immunomagnetic separation requires greater levels of attention and care to avoid cross-contamination among samples.

Because spotting of the bacterial cells diluted in water and/or release buffer was not successful, it was not possible to estimate Sp-N-PCR sensitivity. Repeated tests to determine this technique's sensitivity resulted in different responses, and further modification and optimization are necessary before the technique can be reliably used to detect *X. fastidiosa* in plants. However, promising results were obtained in comparisons between Sp-N-PCR and IC-N-PCR; both techniques gave the same response while testing a series of known negative and positive grape tissue samples (Figures 3 and 4). It appeared that plant extract helped in capturing of bacteria cells on the membrane, but further work is needed to validate Sp-N-PCR use. The Sp-N-PCR technique has several potential advantages. It accelerates the testing process by reducing the time required for sampling and detection. Membranes can be prepared in a few minutes and stored at 4°C up to one month before use. The technique also allows sampling of suspect plants at remote locations, followed by testing at a distant laboratory, thus providing potential advantages to certification and quarantine programs by preventing unwanted movement of *X. fastidiosa* in grapevine stock. The greatest potential of Sp-N-PCR is its efficient and sensitive detection of *X. fastidiosa* in breeding programs and field surveys.

Conclusion

Standard PCR methods were able to detect *X. fastidiosa* at relatively low concentrations when diluted in buffer but were only able to detect *X. fastidiosa* at the highest concentration when diluted in grapevine extract. Sensitive and reliable detection of *X. fastidiosa* in grape tissue extracts was only achieved with the addition of immunocapture. The combined IC-N-PCR procedure was the most sensitive and reliable detection method and was able to detect 2 cfu/mL of bacteria in grape leaf extract. This method was also used to detect the bacteria in leaf samples from inoculated grapevines

and was successful at detecting *X. fastidiosa* from inoculated but nonsymptomatic plants. The Sp-N-PCR technique was as sensitive as IC-N-PCR, but it was not as reliable and needs modification before it can be used to detect *X. fastidiosa* in breeding programs and field surveys.

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