Development of a Bio-PCR Protocol for the Detection of *Xanthomonas arboricola* pv. *pruni*

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Abstract

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A real-time SYBR Green I assay was developed and evaluated as a biological and enzymatic polymerase chain reaction (Bio-PCR) protocol for the detection of *Xanthomonas arboricola* pv. *pruni*. Suppression subtractive hybridization was used to generate a *X. arboricola* pv. *pruni*-specific subtracted DNA library, using *X. arboricola* pv. *corylina* as the driver strain. Primer pair 29F/R, designed from cloned sequence, showed no homology to GenBank sequences and amplified a 344-bp product in all *X. arboricola* pv. *pruni* isolates. Compared with other published *X. arboricola* pv. *pruni* primers, this primer pair was shown to be the only one capable of differentiating *X. arboricola* pv. *pruni* from all other *X. arboricola* pathovars. A real-time assay was developed and shown to be capable of detecting less than 10 CFU and 0.1 pg of DNA. Epiphytic bacteria isolated from plum tissue was used to further evaluate the specificity of the assay. A Bio-PCR protocol, developed for field evaluation, confirmed *X. arboricola* pv. *pruni* isolation from asymptomatic and symptomatic plum tissue over a 9-week period between host flowering and the first appearance of leaf and fruit symptoms in an orchard. Dilution plating enabled *X. arboricola* pv. *pruni* numbers to be quantified, providing supportive evidence for the usefulness of the Bio-PCR protocol in plant pathology and quarantine surveillance.

Bacterial spot of stone fruit (*Prunus* spp.) affects nearly all of the major stone-fruit-growing regions of the world. It is becoming an increasing problem for the stone fruit industry, with recent reports indicating the spread of this disease (5,11,16,18,20). The causal agent of bacterial spot, *Xanthomonas arboricola* pv. *pruni*, induces fruit and leaf lesions resulting in yield losses; however, it is the twig canker symptom which has long-term effects on the productivity of stone fruit trees (21). Control of this pathogen is currently limited to copper spray applications in areas already affected by the disease, and further spread has been restricted by strict quarantine regulation governed by the European and Mediterranean Plant Protection Organization (16).

Traditional plant pathology techniques are useful for the isolation and identification of *X. arboricola* pv. *pruni* from symptomatic and asymptomatic tissues. In recent years, significant novel facets in the life cycle of *X. arboricola* pv. *pruni* have been identified using these techniques. In Italy, *X. arboricola* pv. *pruni* was isolated from asymptomatic bud and leaf scars by culturing onto glucose-yeast-calcium-carbonate agar (GYCA), and indirect immunofluorescence staining was used to confirm and quantify *X. arboricola* pv. *pruni* (30). Survival of *X. arboricola* pv. *pruni* at the scar sites was later confirmed in Italy with studies involving the use of rifampicin- and chloramphenicol-resistant mutants (31). In South Carolina, United States, epiphytic *X. arboricola* pv. *pruni* was shown to be associated with asymptomatic twigs, leaves, buds, flowers, and fruit by culturing washings on *Xanthomonas* differential medium XPSM (25). The association of *X. arboricola* pv. *pruni* with a range of tissues suggests that improved control of this pathogen may be obtained with a greater understanding of the life cycle of *X. arboricola* pv. *pruni* by targeting treatments to crucial steps in its development. To facilitate such research, a molecular diagnostic test for *X. arboricola* pv. *pruni* is essential for specific and sensitive identification of this pathogen.

Development of a molecular diagnostic test for *X. arboricola* pv. *pruni* has proven difficult in the past, due to the close genetic relationships between *X. arboricola* pathovars (17,32). DNA:DNA homology studies indicated that *X. arboricola* pv. *pruni* shares 79% homology with the other *X. arboricola* pathovars: *corylina*, *juglandis*, *populi*, *celebensis*, and *poinsettiicola* type C (28). Two published primer pairs developed from repetitive extrogenic palindromic sequence and random amplified polymorphic DNA fragments are capable of detecting *X. arboricola* pv. *pruni* but also amplify *X. arboricola* pv. *corylina* and *X. arboricola* pv. *juglandis* (17,32) (M. Pagani, *personal communication*).

Suppression subtractive hybridization (SSH) has been shown to be useful for studies requiring differentiation between closely related bacteria (3,6,27). The method involves hybridizing the DNA from two organisms, a tester (organism of interest) and a driver (closely related organism), followed by polymerase chain reaction (PCR) amplification of the unhybridized tester-specific subtracted DNA (4). The method has been especially useful for bacterial differentiation at the strain level and it has been successfully applied to other *Xanthomonas* spp. (1,8,12). In this study, this method was used to identify *X. arboricola* pv. *pruni*-specific DNA from which an X. *arboricola* pv. *pruni*-specific primer was developed. The tester DNA was obtained from pathovar type strain *X. arboricola* pv. *pruni* ICMP 51 and the driver DNA from pathovar type strain *X. arboricola* pv. *corylina* ICMP 5726. Although *X. arboricola* pvs. *corylina* and *juglandis* share a high degree of DNA:DNA homology (89%) with *X. arboricola* pv.

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pruni, *X. arboricola* pv. *corylina* was chosen as the driver strain based on work previously conducted in our laboratory (28,32). Zuli (32) examined repetitive extragenic palindromic sequences in *X. arboricola* pathovars and showed that the BOXAIR profile exhibited a high degree of similarity between pathovars *pruni* and *corylina* isolates compared with other pathovars, including *juglandis.*

Biological and enzymatic (Bio)-PCR involves culturing a pathogen prior to PCR amplification, making it a particularly useful method for increasing pathogen numbers to detectable levels in asymptomatic tissue and ensuring that only viable organisms are examined (7,14,22,23). An additional benefit of this method is that copper, a known PCR inhibitor which is routinely used to control bacterial spot, is excluded from the PCR reaction (9). The aim of this study is to demonstrate the application of an X. *arboricola* pv. *pruni*-specific Bio-PCR protocol in a field-based trial using both asymptomatic and symptomatic plant tissue.

Materials and Methods

Bacterial strains. The bacterial strains used during this study are listed in Table 1. All *X. arboricola* pv. *pruni* strains and *X. arboricola* pathovars, with the exception of *X. arboricola* pv. *fragariae*, were stored on Protect cyropreservation beads (Technical Consultant Services, Ltd., Heywood, Lancaster, UK) at −80°C. Two beads were placed onto GYCA plates, streaked, and incubated for 2 days at 28°C. *X. arboricola* pv. *fragariae* was supplied as a freeze-dried specimen, which was rehydrated in 200 µl of sterile water, streaked onto GYCA agar, and incubated for 2 days at 28°C.

DNA extraction. DNA was extracted from 2-day-old cultures for all bacterial strains described in Table 1. For the SSH procedure, the tester DNA (pathovar type strain *X. arboricola* pv. *pruni* ICMP 51) and driver DNA (pathovar type strain *X. arboricola* pv. *corylina* ICMP 5726) were extracted using the ChargeSwitch gDNA Mini Bacteria Kit (Invitrogen, Carlsbad, CA) and quantified using the Biospec mini spectrophotometer (Shimadzu Biotech, Kyoto, Japan). For primer specificity and sensitivity testing, the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI) "Isolation of genomic DNA from gram-positive and gram-negative bacteria" procedure was performed and DNA quantified by visual comparison with Hyperladder I (Bioline, London) following electrophoresis on a 2% (wt/vol) agarose gel in Tris-acetate-EDTA buffer and staining in ethidium bromide at 0.2 µg ml^{-1}.

SSH and identification of *X. arboricola* pv. *pruni*-specific DNA. SSH was performed using the BD PCR-Select Bacterial Genome Subtraction Kit (Clontech, Mountain View, CA) according to the manufacturer's recommendations. The secondary PCR products from the tester-specific subtracted DNA were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA), ligated into pGEM-T Easy (Promega Corp.), and the recombinant plasmids were transformed into XL10-Gold Ultracompetent cells (Stratagene, La Jolla, CA). Randomly selected clones were PCR amplified using both the M13 forward and reverse vector primers.

Table 1. Polymerase chain reaction (PCR) amplification of bacterial strains used in this study to evaluate the specificity of primer pair 29F/R, designed from DNA sequence obtained by suppression subtractive hybridization for the specific detection of *Xanthomonas arboricola* pv. *pruni*, compared with other published primers

Isolates[b]	Origin	Host plant[c]	PCR amplification with primer pairs[a]		
			29F/R	Zuli	Pagani
X. arboricola pv. *pruni*					
ICMP 51^T	Mt. Albert, New Zealand	Japanese Plum	+	+	+
ICMP 59	United Kingdom	n/a	+	+	+
ICMP 60	Argentina	Plum	+	+	+
ICMP 62	United States	Peach	+	+	–
ICMP 4288	Ontario, Canada	Peach	+	+	–
ICMP 6677	South Africa	Apricot	+	+	+
ICMP 7492	Sao Paulo, Brazil	Peach	+	+	–
QDPI–JNG 1	Queensland, Australia	Plum	+	+	+
QDPI–JNG 2	Queensland, Australia	Plum	+	+	+
QDPI–JNG 3	Queensland, Australia	Plum	+	+	–
QDPI–JNG 4	Queensland, Australia	Plum	+	+	–
QDPI–JNG 5	Queensland, Australia	Plum	+	+	+
QDPI–JNG 6	Queensland, Australia	Plum	+	+	+
QDPI–JNG 7	Victoria, Australia	Plum	+	+	+
QDPI–JNG 8	New South Wales, Australia	Nectarine	+	+	+
QDPI–JNG 9	Queensland, Australia	Plum	+	+	+
QDPI–JNG 10	Queensland, Australia	Plum	+	+	+
QDPI–JNG 11	Western Australia, Australia	Plum	+	+	–
QDPI–JNG 12	Queensland, Australia	Plum	+	+	+
QDPI–JNG 13	Queensland, Australia	Plum	+	+	+
QDPI–JNG 14	Queensland, Australia	Plum	+	+	+
QDPI–JNG 15	Victoria, Australia	Plum	+	+	+
QDPI–JNG 16	Victoria, Australia	Plum	+	+	+
QDPI–JNG 17	Queensland, Australia	Plum	+	+	+
QDPI–JNG 18	Victoria, Australia	Nectarine	+	+	+
QDPI–JNG 19	Queensland, Australia	Plum	+	+	+
QDPI–JNG 20	Queensland, Australia	Plum	+	+	+
B0003	Thulimbah, Queensland	Plum	+	+	+
B0028	Thulimbah, Queensland	Plum	+	+	+
B0030	The Summit, Queensland	Plum	+	+	+
B0144	Thulimbah, Queensland	Plum	+	+	+

(*continued on next page*)

[a] Amplification of an amplicon of expected size: + indicates amplification and – indicates no amplification. PCR conditions and product size described in text and by Zuli (32) and Pagani (17).

[b] T, pathovar type strain for species; ACM, Australian Collection of Microorganisms, School of Chemistry and Molecular Biosciences, University of Queensland, St. Lucia, Australia; ICMP, International Collection of Micro-organisms from Plants, Lincoln, New Zealand; LMG, Culture Collection of the Laboratorium voor Microbiologie, Gent, Belgium; QDPI, Queensland Primary Industries and Fisheries, Applethorpe, Australia; CFBP, Collection Française de Bactèries Phytopathogènes, Angers, France; B, Queensland Primary Industries and Fisheries, Applethorpe Research Station, Australia.

[c] n/a = Not available.

Differential screening was carried out by Southern blot hybridization. *Rsa*I-digested tester and driver genomic DNA was digoxigenin (DIG)-11-dUTP labeled using the DIG High Prime DNA Labelling and Detection Starter Kit I (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. PCR products (150 ng) ranging in size from 800 to 1,300 bp were transferred to positively charged nylon membranes (Roche Diagnostics GmbH) under vacuum (Hoefer PR 648 slot blot manifold) and fixed to the membrane by UV cross-linking. Duplicate membranes were hybridized overnight to tester or driver DNA probes at 65°C, as described in the DIG application manual (Roche Diagnostics GmbH).

Clones showing differential or no hybridization to the driver DNA probe were sequenced by the Australian Genome Research Facility using M13 forward and reverse primers. The sequences were checked for homology to GenBank accessions using BlastN and BlastX nonredundant databases (http://www.ncbi.nlm.nih.gov/BLAST). Primer pair 29F/R was designed with the aid of Oligo Primer Analysis Software (version 6.71; Molecular Biology Insights).

Real-time PCR comparison with published *X. arboricola* pv. *pruni* primers. The real-time SYBR Green I primer pair 29F/R assay was set up in a 96-well plate with a 10-µl reaction mix per well containing a final concentration of 1× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.2 µM each primer (29F [5′-GTACCGCATTTCAGGCCGTCA-3′] and 29R [5′-AAGTAGCCAACGCGGAATTT-3′]; GeneWorks, Adelaide, SA, Australia), bovine serum albumin at 1 mg ml^{-1}, and 25 ng of template DNA. The ABI 7900HT sequence detection system (Applied Biosystems) was used with the following cycling conditions: 95°C for 10 min and 45 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s. The dissociation curve for each product was determined using the following protocol: 95°C for 2 min, 60°C for 15 s, and 95°C for 15 s.

DNA from each of the bacterial strains described in Table 1 was used as template to compare the real-time assay with the *X. arboricola* pv. *pruni* diagnostic PCRs described by Zuli (32) and the Pagani (17). Both the Zuli (32) and Pagani (17) protocols were followed as described except for the use of AmpliTaq Gold at 0.02 U $µl^{-1}$ and 1× GeneAmp PCR Buffer II in 25-µl PCR reactions.

Sensitivity of the real-time PCR. A serial dilution series ranging from 4.5×10^{8} to 4.5×10^{-1} CFU ml^{-1}, as determined by plate counting, was prepared in sterile water from a 2-day-old *X. arboricola* pv. *pruni* strain ICMP 51 culture. A serial dilution series of DNA was also made in sterile water ranging from 100 ng $µl^{-1}$ to 0.01 pg $µl^{-1}$. A 1-µl volume of each dilution was used as template for PCR using the real-time SYBR Green I primer 29F/R assay as described above. Both experiments were repeated twice.

Field evaluation of real-time PCR specificity. *Real-time PCR on mixed template.* Asymptomatic twigs were collected from plum trees, which had previously shown symptoms of bacterial spot, on five occasions between 29 September and 25 November 2004 at the Applethorpe Research Station (28° 37′ 21.61″ S, 151° 56′ 48.49″ E). Tissue was weighed and washed in up to 30 ml of sterile water for a period of 5 min. The wash was then serially diluted 10-fold to 1:10000 and a 100-µl volume of each dilution

Table 1. (*continued from preceding page*)

Isolates[b]	Origin	Host plant[c]	PCR amplification with primer pairs[a]		
			29F/R	Zuli	Pagani
B1539	Ballandean, Queensland	Plum	+	+	+
B1540	Ballandean, Queensland	Plum	+	+	+
B1541	Ballandean, Queensland	Plum	+	+	+
B1542	Ballandean, Queensland	Plum	+	+	+
B1825	Applethorpe, Queensland	Plum	+	+	+
QDPI–16	Australia	Plum	+	+	–
QDPI–18	Australia	Plum	+	+	–
QDPI–20	Australia	Plum	+	+	+
CFBP 5577	France	Peach	+	+	+
Canker P11 C1	Applethorpe, Queensland	Plum	+	+	+
Canker P4 C2	Applethorpe, Queensland	Plum	+	+	+
Fruit E6 F2	Applethorpe, Queensland	Plum	+	+	+
Fruit L14 F2	Applethorpe, Queensland	Plum	+	+	+
Fruit F4 F1	Applethorpe, Queensland	Plum	+	+	+
Fruit L3 F1	Applethorpe, Queensland	Plum	+	+	+
Leaf E8 L3	Applethorpe, Queensland	Plum	+	+	+
Leaf F4 L2	Applethorpe, Queensland	Plum	+	+	+
X. arboricola pv. *corylina*					
ICMP 5726^{T}	United States	Filbert	–	+	–
ICMP 449	Oregon, United States	Hazelnut	–	+	–
ACM 2135	Victoria	Hazelnut	–	+	–
ICMP 7081	United Kingdom	Hazelnut	–	+	–
ICMP 11956	France	Hazelnut	–	+	+
X. arboricola pv. *juglandis*					
ICMP 35^{T}	Mt. Albert, New Zealand	Walnut	–	–	–
ICMP 34	England, United Kingdom	Walnut	–	–	–
ICMP 10865	Valencia, Spain	Walnut	–	–	–
ICMP 11829	France	Walnut	–	+	+
ICMP 11955	Italy	Walnut	–	–	–
X. arboricola pv. *celebensis*					
ICMP 1488	Auckland, New Zealand	Banana	–	–	–
X. arboricola pv. *poinsettiicola*					
ICMP 6274	Mt. Albert, New Zealand	Poinsettia	–	–	+
ICMP 7180	Mt. Albert, New Zealand	Poinsettia	–	–	+
X. arboricola pv. *populi*					
ICMP 8923^{T}	Roggebotslusis, Netherlands	Euramerican poplars	–	–	–
ICMP 9367	New Zealand	Interamerican poplars	–	–	–
ICMP 11965	France	Necklace poplar	–	–	–
ICMP 11974	Italy	Euramerican poplars	–	–	–
X. arboricola pv. *fragariae*					
LMG 19145	Cesena, Italy	Strawberry	–	–	–

was spread onto agar plates containing GYCA medium in duplicate. Plates were incubated at 28 ± 2°C for 4 days. A loopful of growth representing all of the different bacterial colony forms present on each twig was added directly to extraction buffer (200 mM Tris-HCl [pH 7.5], 250 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate, and 2% polyvinyl-pyrrolidone) and the DNA extracted as per the Llop et al. (13) protocol. DNA was quantified by visual comparison with Hyperladder I following gel electrophoresis and adjusted to 25 ng μl^{-1}. A 1-µl volume of DNA was added to the PCR mix for amplification with the real-time SYBR Green I primer pair 29F/R assay described above.

Real-time PCR on individual colony forms. During 2005, five 6-year-old plum trees of the highly susceptible 'Friar' were chosen for study at the Applethorpe Research Station (26). Trees were growing in an open vase shape, had not been copper treated, and had previously shown bacterial spot symptoms. Sampling occurred on 12 occasions between 4 September and 27 November, between the state of plum "full bloom" (29) and the first observed appearance of bacterial spot fruit and leaf symptoms. During each sampling, an "epiphytic" sample consisting of a combination of fruit, leaves, and twigs was collected from each tree. Tissue was washed in a small volume of sterile water for 3 min. The wash was then serially diluted and plated onto GYCA medium as described above. A selection of individual colonies representing each colony form present on the plate were placed directly into extraction buffer as described above. A 1-µl volume of DNA was added to the PCR mix for amplification with the real-time SYBR Green I primer pair 29F/R assay described above.

Field evaluation of Bio-PCR. *Experimental trial site.* During the 2006–07 growing season, two trees from both the highly susceptible 'Laroda' and 'Friar' were chosen for study at the Applethorpe Research Station (26). The trees were 14 years old, had been trained in an open vase shape, had not been sprayed with copper, and had previously shown bacterial spot symptoms. Sampling occurred on nine occasions (5 to 9 days apart) between 4 September and 6 November 2006, between the period of plum full bloom (29) and the first observation of leaf and fruit symptoms on both cultivars for the growing season. Samples were bulked per cultivar and separated into three tissue types: "epiphytic," "asymptomatic leaf scars," and "cankers". The epiphytic sample was as described above. Both asymptomatic leaf scar and canker tissue came from twigs which had developed during the previous growing season. Asymptomatic leaf scars were defined as the bulbous area surrounding and including the leaf scar. The canker tissue included both the external and internal symptomatic tissues from the bacterial spot canker. For data analysis, sampling dates were defined as the number of weeks prior to the first observation of leaf and fruit symptoms, with the last sampling date being defined as the time when symptoms were observed for the first instance on both cultivars, indicated as week 0. A weather monitoring station was positioned within the experimental site and collected temperature and rainfall data at 15-min intervals.

Confirmation of *X. arboricola* pv. *pruni* isolation from plant tissue using Bio-PCR. For both asymptomatic leaf scars and canker tissue, whole twigs were surface sterilized in 75% ethanol for 2 min, washed for 5 s in sterile distilled water, and placed into 1% sodium hypochlorite for 3 min, followed by three 5-s washes in sterile distilled water. Asymptomatic leaf scars and cankers were then excised from the twig with a flame-sterilized "v"-shaped linoleum cutting tool. The tissue was crushed with a hammer in a sterile petri dish between two sterile sheets of filter paper (Whatman No. 1), weighed, and resuspended in 10 ml of sterile water. Each suspension was incubated for 1 h at 28°C with shaking on a rotary shaker at 200 rpm (Bioline). For the epiphytic sample, twigs, leaves, and fruit were not surface sterilized and were only shaken for 3 min. The suspensions were serially diluted 10-fold up to a maximum dilution of 10^{-7}. A 100-µl volume of each suspension was spread onto agar plates containing XPSM medium in triplicate for the undiluted suspension and three sequential dilutions between 10^{-1} and 10^{-7}, chosen on the basis of results obtained during the previous week of the study. Plates were incubated at 28 ± 2°C for 6 days. Putative *X. arboricola* pv. *pruni*-like colonies from plates containing between 30 and 300 colonies were counted. Four individual putative *X. arboricola* pv. *pruni*-like colonies (shiny, convex, opaque, and grayish white in color) were isolated from the XPSM plates to confirm *X. arboricola* pv. *pruni* isolation. Two of the putative *X. arboricola* pv. *pruni*-like colonies were streaked onto GYCA agar and incubated at 28 ± 2°C for 4 days to confirm characteristic *X. arboricola* pv. *pruni* colony phenotype (shiny, convex, and buttercup yellow with a butyrous consistency) on this medium. The other two putative *X. arboricola* pv. *pruni*-like colonies were placed into 100 µl of sterile water and individually tested by PCR with the *X. arboricola* pv. *pruni*-specific real-time SYBR Green I primer pair 29F/R assay described above. Once *X. arboricola* pv. *pruni* isolation was confirmed, the number of CFU g^{-1} of tissue was determined using the averaging colony count calculated from the triplicate plates for the dilution counted. The number of CFU g^{-1} of tissue was transformed using log (1 + CFU) g^{-1} of tissue fresh weight for graphical representation of the data.

Results

Development and specificity of primer pair 29F/R designed from DNA sequence obtained by SSH and its comparison with other published *X. arboricola* pv. *pruni* primers. A putative *X.*

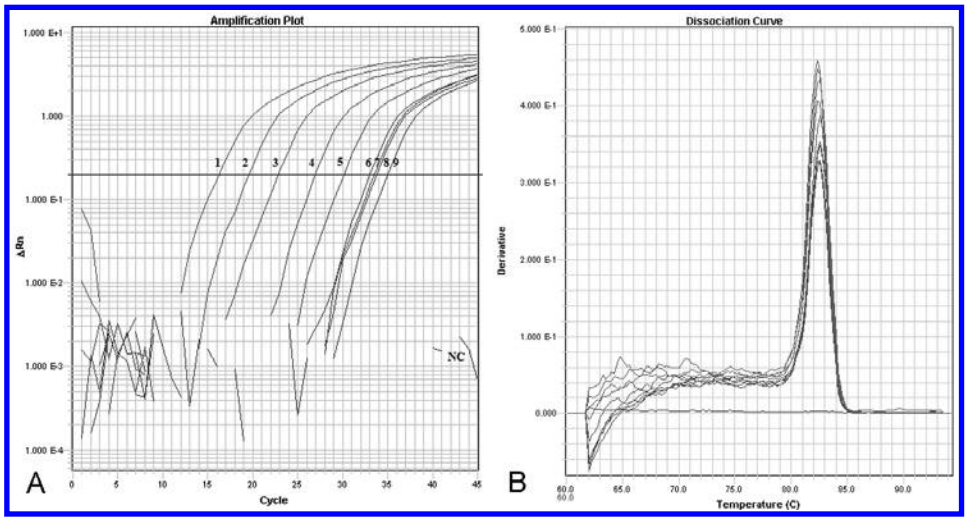

Fig. 1. Sensitivity of real-time SYBR Green I polymerase chain reaction (PCR) assay with primer pair 29F/R assessed by 10-fold serial dilutions of a cell suspension of *Xanthomonas arboricola* pv. *pruni* strain ICMP 51. **A,** Real-time amplification curve of different concentrations of DNA and a threshold set at 0.2. Line 1–9: 10-fold dilution of *X. arboricola* pv. *pruni* strain ICMP 51 cell suspension (4.5×10^8 to 4.5 CFU ml^{-1}); NC: PCR-negative control containing no DNA. **B,** Dissociation curve analysis.

arboricola pv. *pruni*-specific subtracted DNA library was obtained using SSH. Southern blot analysis identified 29 putative *X. arboricola* pv. *pruni*-specific subtracted DNA clones which exhibited no or differential hybridization to driver (*X. arboricola* pv. *corylina*) genomic DNA. DNA from all of these clones was sequenced and analyzed by comparison with the GenBank database (2). Primer pair 29F/R was designed from a DNA sequence which did not show similarity to sequences deposited in GenBank. The sequence has been deposited in GenBank as accession number HN268887. The real-time SYBR Green I primer pair 29F/R assay amplified DNA from all of the *X. arboricola* pv. *pruni* strains tested and did not amplify any of the other *X. arboricola* pathovars (Table 1). When the PCR products were analyzed on an agarose gel, the expected amplicon size of 344 bp was evident for all the *X. arboricola* pv. *pruni* strains (*data not shown*). The dissociation curve showed that the *X. arboricola* pv. *pruni* amplification product had a melting temperature of 82°C (*data not shown*).

The detection limit of this real-time PCR assay was determined using dilution series for both an X. *arboricola* pv. *pruni* bacterial cell suspension and purified DNA. Template was amplified in all dilutions of *X. arboricola* pv. *pruni* bacterial cells, with a lower limit of 4.5 CFU ml^{-1} detected (Fig. 1). However, between 4.5 × 10^3 and 4.5 CFU ml^{-1}, the cycle threshold remained between 33 and 35 cycles (Fig. 1A). The limit of detection for *X. arboricola* pv. *pruni* genomic DNA was 0.1 pg per reaction (*data not shown*). All *X. arboricola* pv. *pruni* amplicons yielded a single strong peak at 82°C on the dissociation curve (Fig. 1B).

The primer pair designed by Zuli (32) amplified DNA from all of the *X. arboricola* pv. *pruni* strains examined, yielding the expected 180-bp amplicon (*data not shown*); however, DNA from all of the *X. arboricola* pv. *corylina* strains also yielded the 180-bp amplicon, as did DNA from *X. arboricola* pv. *juglandis* strain ICMP 11829 (Table 1).

The Pagani (17) primers yielded the expected 950-bp amplicon for DNA from *X. arboricola* pv. *pruni* isolates (*data not shown*). However, 8 of the 44 *X. arboricola* pv. *pruni* strains could not be amplified by this primer pair (Table 1). DNA from *X. arboricola* pv. *corylina* strain ICMP 11956, *X. arboricola* pv. *juglandis* strain ICMP 11829, and both of the *X. arboricola* pv. *poinsettiicola* isolates yielded amplicons of a size similar to that of the *X. arboricola* pv. *pruni* isolates (*data not shown*).

Field evaluation of real-time PCR. Washings plated onto GYCA medium from the surface of asymptomatic twigs showed evidence of a range of bacteria present at the time of sampling. However, *X. arboricola* pv. *pruni*-like colony forms were always present on plates at each sampling. The real-time SYBR Green 1 primer pair 29F/R assay amplified *X. arboricola* pv. *pruni* template from within mixed template at each sampling with products demonstrating a melting temperature of 82°C (*data not shown*).

In total, 143 single bacterial colonies were isolated from washings containing the epiphytic bacterial populations present on the surface of plum twigs, leaves, and fruit. In all, 87 colonies showed the typical *X. arboricola* pv. *pruni* phenotype on GYCA agar and produced amplification products which had a melting temperature of 82°C using the real-time SYBR Green 1 primer pair 29F/R assay (*data not shown*). The remaining 56 colonies were not phenotypically *X. arboricola* pv. *pruni*-like on GYCA agar and, although no attempt was made to identify these bacteria, 14 visually distinct colony phenotypes were evident from this collection. Of these colony types, 11 were not amplifiable by the real-time assay; however, 3 colony phenotypes (golden yellow, white/yellow, and pale yellow colony forms) produced late amplification reactions (>35 cycles at a threshold of 0.2) and their products had melting temperatures of 79, 88, and 91°C, respectively (Fig. 2).

Bio-PCR evaluation. The Bio-PCR protocol confirmed the isolation of epiphytic *X. arboricola* pv. *pruni* and *X. arboricola* pv. *pruni* associated with asymptomatic leaf scar and canker tissue. Occasionally, colonies not phenotypically *X. arboricola* pv. *pruni*-like grew on XPSM agar; however, these colony forms were low in number and did not interfere with plate counts. *X. arboricola* pv. *pruni*-like colonies from XPSM plates were confirmed as being *X. arboricola* pv. *pruni* because they always grew with the anticipated phenotype on GYCA agar, and the PCR amplification products in the real-time SYBR Green I assay for each colony had a melting temperature of 82°C (*data not shown*).

The first leaf and fruit bacterial spot symptoms were observed on both plum cultivars 9 weeks into the trial, on 6 November 2006, and this was defined as week 0. Development of *X. arboricola* pv. *pruni* populations associated with asymptomatic leaf scars, cankers, and epiphytic populations over these 9 weeks are shown in Figures 3, 4, and 5, respectively. *X. arboricola* pv. *pruni* populations associated with asymptomatic leaf scars ranged from 2.58 × 10^4 to 1.06 × 10^9 CFU g^{-1} of tissue in Friar and 2.47 × 10^3 to 4.36 × 10^{11} CFU g^{-1} of tissue in Laroda over this time frame (Fig. 3). A sudden decrease in *X. arboricola* pv. *pruni* population size by more than five orders of magnitude, 7 weeks prior to leaf and fruit symptoms, was evident for both plum cultivars (Fig. 3). The average weekly temperature recorded at the trial site also dropped during this week, potentially suggesting a link with the *X. arboricola* pv. *pruni* population trend (Fig. 3).

Overall, the *X. arboricola* pv. *pruni* populations increased within cankers in both plum cultivars prior to the first observation of leaf and fruit symptoms during the 2006–07 growing season (Fig. 4). Interestingly, *X. arboricola* pv. *pruni* populations suddenly

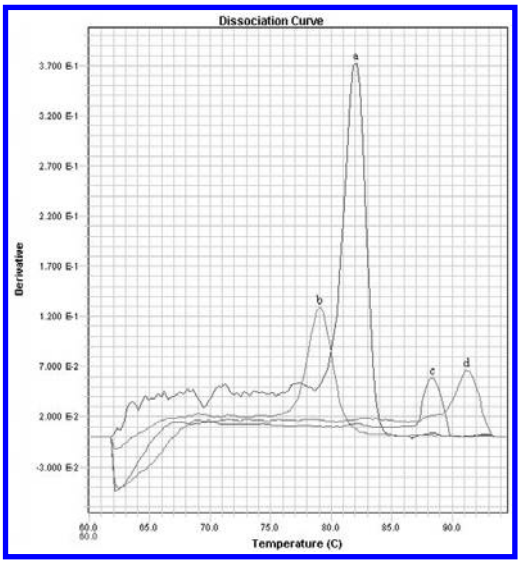

Fig. 2. Dissociation curve of polymerase chain reaction (PCR) amplification products from isolates of *Xanthomonas arboricola* pv. *pruni* (a), golden yellow colony form (b), white/yellow colony form (c), and pale yellow colony form (d) from glucose-yeast-calcium-carbonate agar using the real-time SYBR Green I PCR primer pair 29F/R assay.

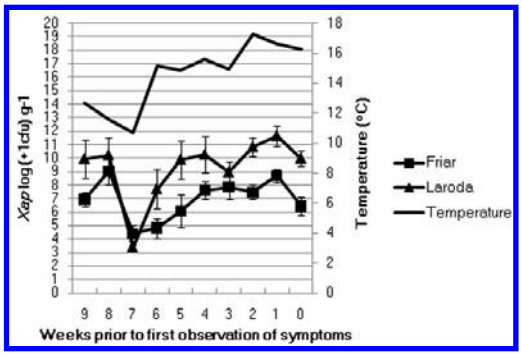

Fig. 3. Numbers of *Xanthomonas arboricola* pv. *pruni* log (1+CFU) g^{-1} asymptomatic leaf scar tissue fresh weight over the 9 weeks prior to the first observation (week 0 = 6 November 2006) of leaf and fruit symptoms on 'Friar' and 'Laroda' plum during the 2006 season compared with the average weekly temperature during this time. Values are means of counts of CFU on three plates for each plum cultivar and the standard error (black bar) is indicated for each sample.

dropped by seven orders of magnitude to below detectable levels 8 weeks prior to the observation of symptoms on Friar and five orders of magnitude to below detectable levels 7 weeks prior to the observation of symptoms on Laroda (Fig. 4). Between weeks nine and seven, there was a drop in average weekly temperature at the trial site, which may have influenced a drop in *X. arboricola* pv. *pruni* populations at this site in both plum cultivars. *X. arboricola* pv. *pruni* numbers increased to 5.67×10^8 CFU g^{-1} of tissue in Friar 5 weeks prior to the first symptoms and 4.14×10^8 CFU g^{-1} of tissue in Laroda 2 weeks prior to appearance of the first symptoms (Fig. 4).

Epiphytic *X. arboricola* pv. *pruni* populations generally increased steadily over the 9-week period (Fig. 5). However, the epiphytic *X. arboricola* pv. *pruni* population on Laroda was at least two orders of magnitude higher than on Friar for the majority of the sampling dates (Fig. 5). The highest epiphytic *X. arboricola* pv. *pruni* population measured was 6.27×10^7 CFU g^{-1} of tissue on Friar and 5.52×10^9 CFU g^{-1} of tissue on Laroda (Fig. 5). This peak in the *X. arboricola* pv. *pruni* population associated with Laroda may be related to an increase in temperature at this time (Fig. 5). No correlations between rainfall and *X. arboricola* pv. *pruni* populations in any of the tissues examined were evident (*data not shown*).

Discussion

X. arboricola pv. *pruni* has recently been reported on apricot and plum in Switzerland (20) and almond in Europe (18). To confirm *X. arboricola* pv. *pruni* identification, these authors conducted a range of tests on symptomatic tissue, including the molecular-based *X. arboricola* pv. *pruni*-specific PCR designed by Pagani (17). Although the Pagani (17) primer pair amplified *X. arboricola* pv. *pruni* DNA in all of the infected tissues examined in these two instances, testing of this primer pair during the experiments outlined within this article shows a lack of primer binding in some *X. arboricola* pv. *pruni* isolates and cross-specificity with other *X. arboricola* pathovars. Therefore, the requirement of an X. *arboricola* pv. *pruni*-specific molecular-based test is essential for a reliable diagnosis of new outbreaks of this pathogen in the future.

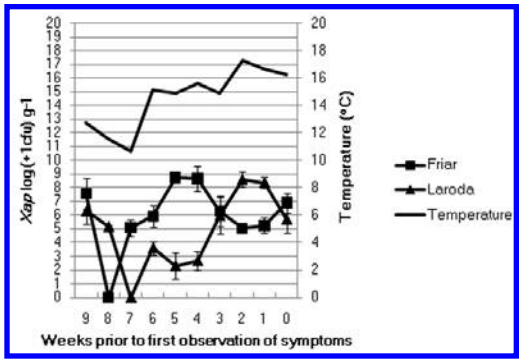

Fig. 4. Numbers of *Xanthomonas arboricola* pv. *pruni* log (1+CFU) g^{-1} canker tissue fresh weight over the 9 weeks prior to the first observation (week 0 = 6 November 2006) of leaf and fruit symptoms on 'Friar' and 'Laroda' plum during the 2006 season compared with the average weekly temperature during this time. Values are means of counts of CFU on three plates for each plum cultivar and the standard error (black bar) is indicated for each sample.

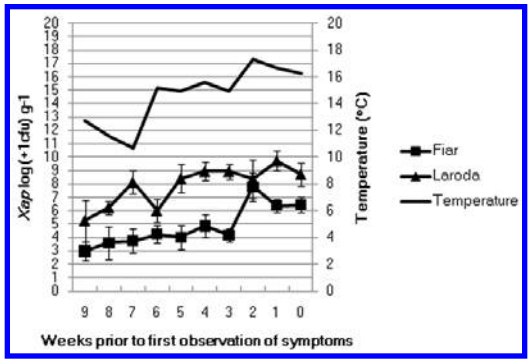

Fig. 5. Numbers of epiphytic *Xanthomonas arboricola* pv. *pruni* log (1+CFU) g^{-1} tissue fresh weight over the 9 weeks prior to the first observation (week 0 = 6 November 2006) of leaf and fruit symptoms on 'Friar' and 'Laroda' plum during the 2006 season compared with the average weekly temperature during this time. Values are means of counts of CFU on three plates for each plum cultivar and the standard error (black bar) is indicated for each sample.

Sources of *X. arboricola* pv. *pruni* inoculum within plum trees in Applethorpe, Queensland appear to include epiphytic *X. arboricola* pv. *pruni* as well as *X. arboricola* pv. *pruni* associated with asymptomatic leaf scars and cankers on plums. *X. arboricola* pv. *pruni* associated with each of these tissue types has been previously demonstrated but a study examining all of these tissue types at the same time has not been previously attempted (25,30,31). The *X. arboricola* pv. *pruni* populations associated with these sites and the influence weather has on these populations warrants further investigation using larger sample numbers over a greater time period. This type of study is now possible with the availability of the *X. arboricola* pv. *pruni*-specific Bio-PCR protocol. Alternatively, to speed up the assay, the PCR component of the method could potentially be utilized as a quantitative real-time PCR assay using fresh tissue, if this tissue had not been previously treated with copper. However, the limit of detection of the assay will be significantly higher due to the clustering of cycle thresholds for lower CFU levels, which is not an uncommon phenomenon for real-time PCR assays (10,15).

Spurious nonspecific backgrounds showing up as late-cycle amplifications is not unusual with SYBR Green real-time PCR (19). Melt-curve analysis is routinely used in this type of assay (24). Non-*X. arboricola* pv. *pruni* PCR products are clearly differentiated from true *X. arboricola* pv. *pruni* amplification products using the melt-curve analysis step built into the real-time SYBR Green I primer pair 29F/R assay and, therefore, the assay appears to be specific for the detection of *X. arboricola* pv. *pruni*. Further validation of this assay is described in the thesis by Ballard (2). In Table 1, there is a set of eight canker, fruit, and leaf bacterial spot symptom isolates which were PCR amplified by the real-time SYBR Green I primer pair 29F/R assay and Zuli (32) and Pagani (17) protocols. These eight isolates were also examined with the BIOLOG GN microplate system, 16S rRNA gene sequencing, and a detached leaf assay, providing further support of the specificity of primer pair 29F/R.

The aim of this study was to develop a Bio-PCR protocol for the detection of *X. arboricola* pv. *pruni*. The SSH method used to obtain tester-specific DNA from a pathovar type strain of *X. arboricola* pv. *pruni* using *X. arboricola* pv. *corylina* as the driver proved a successful approach. The detection of epiphytic *X. arboricola* pv. *pruni* and *X. arboricola* pv. *pruni* populations associated with asymptomatic leaf scars and cankers over a 9-week period prior to the first observation of leaf and fruit symptoms in the orchard provided proof-of-concept for using the Bio-PCR protocol in future epidemiological studies. A greater understanding of the life cycle of this pathogen and an effective method for the early detection of *X. arboricola* pv. *pruni* may help in improving control strategies for bacterial spot in the future.

Acknowledgments

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