National Diagnostic Protocol for Asiatic Citrus Canker, *Xanthomonas citri* subsp. *citri*

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This version of the National Diagnostic Protocol (NDP) for Asiatic citrus canker is current as at the date contained in the version control box on the front of this document.

NDPs are updated every 5 years or before this time if required (i.e. when new techniques become available).

The most current version of this document is available from the SPHDS website:
http://plantbiosecuritydiagnostics.net.au/resource-hub/priority-pest-diagnostic-resources/

Where an IPPC diagnostic protocol exists it should be used in preference to the NDP. NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website: https://www.ippc.int/core-activities/standards-setting/ispms

1 Introduction

Asiatic citrus canker is a bacterial disease affecting most citrus varieties, with grapefruit the most susceptible. The disease causes scab or crater-like lesions on the rind of the fruit, which reduce saleability. The causal agent is the bacterium *Xanthomonas citri* subsp*. citri*, (reclassified from "A" pathotype *Xanthomonas axonopodis* pv. *citri,* Schaad et al 2006*).* Canker has the capacity to devastate citrus industries because eradication is the only feasible method of disease control, but this is not always successful. Small outbreaks of canker have occurred in Australia several times in the past and rapid quarantine response and destruction of host material has to date ensured successful eradication. For example, in 1991 citrus canker was detected in an orchard in the Darwin area and despite the trees being destroyed, the disease was then found in a neighbouring orchard in 1993. Eradication was again carried out and after two years of intensive monitoring in the area, citrus canker was declared to be eradicated from the Northern Territory (OCPPO, 2003). More recently, citrus canker was detected in commercial orchards in Emerald, Queensland, in 2004. An intensive program of tree removal and monitoring was launched and the disease was declared eradicated in January, 2009 (http://www.daff.gov.au/animal-plant-health/pests-diseases-weeds/plant/eradication).

Correct identification of the causal agent is critical; however it can be confused with similar organisms. Incorrect identification in the USA prompted the removal of thousands of productive citrus that were infected only with citrus bacterial spot, a mild disease caused by the related but nonaggressive *X. alfalfae* subsp. *citrumelonis (*formerly *X. axonopodis* pv. *citrumelo*, Schaad et al 2006*).*

1.1 Host range

1.1.1 Primary hosts

The following host information is from CAB International (2002):

Aegle marmelos (bael fruit), *Citrus*, *Citrus aurantiifolia* (lime), *Citrus aurantium* (sour orange), *Citrus maxima* (pummelo), *Citrus hystrix*, *Citrus junos* (yuzu), *Citrus limon* (lemon), *Citrus limetta* (sweet lemon tree), *Citrus madurensis* (calamondin), *Citrus medica* (citron), *Citrus natsudaidai* (natsudaidai), *Citrus x paradisi* (grapefruit), *Citrus sunki* (sour mandarin), *Citrus reticulata* (mandarin), *Citrus reshni* (cleopatra mandarin), *Citrus sinensis* (navel orange), *Citrus tankan*, *Citrus unshiu* (satsuma), *Citrus reticulata* x *Poncirus trifoliata* (citrumelo), *Casimiroa edulis* (casimiroa), *Eremocitrus glauca* (Australian desert lime), *Limonia acidissima* (elephant apple), and *Poncirus trifoliata* (Trifoliate orange).

The following hybrids are also susceptible to varying degrees:

C. aurantiifolia x Microcitrus australasica (Faustrime), *C. limon x M. australasica* (Faustrimon), *C. madurensis x M. australasica* (Faustrimedin), *C. sinensis x Poncirus trifoliata* (Citrange), *C. paradisi x P. trifoliata* (Citrumelo) (Schoulties et al., 1987*), C. aurantifolium x P. trifoliata* (Citradia), *C. nobilis x P. trifoliata* (Citrandin), *C. unshiu x P. trifoliata* (Citrunshu), *Citrange x P. trifoliata* (Cicitrangle), *C. adurensis x Citrange* (Citrangedin), *C. deliciosa x Citrange* (Citrangarin), *C. unshiu x Citrange* (Citranguma), *Fortunella margarita x Citrange* (Citrangequat), *F. japonica x C. aurantiifolia* (Limequat), *C. maxima x C. aurantiifolia* (Limelo), *C. madurensis x C. aurantiifolia* (Bigaraldin), *C. maxima x C. sinensis* (Orangelo), *F. margarita x C. sinensis* (Orangequat), *C. nobilis* (Clementine) *x C. maxima* (Clemelo), *C. nobilis* (King of Siam) *x C. maxima* (Siamelo), *C. unshiu x C. maxima* (Satsumelo), *C. deliciosa x C. maxima* (Tangelo*), C. nobilis* (King of Siam) *x C. sinensis* (Siamor), *C. deliciosa x C. madurensis* (Calarin), *C. unshiu x C. madurensis* (Calashu).

Of the primary hosts listed, yuzu is highly resistant (Goto, 1992) and calamondins, *Cleopatra mandarin* and Sunki mandarin are immune (Reddy, 1997). Both *Fortunella japonica* and *F. margarita* are highly resistant (Goto, 1992).

1.1.2 Secondary hosts

Fortunella japonica (round kumquat), *Fortunella margarita* (oval kumquat).

A number of non-citrus hosts have also been recorded. Unless otherwise stated these are as listed by Peltier and Frederich (1920, 1924) who defined susceptibility on the basis of artificial inoculation in the greenhouse (G) and/or in the field (F):

Aeglopsis chevalieri (G), *Atalantia ceylonica* (G), *Atalantia citrioides* (G), *Atalantia disticha* (G) (Lee, 1918), *Chalcas exotica* (G), *Casimiroa edulis* (G, F), *Chaetospermum glutinosum* (G, F), *Clausena lansium* (G), *Citropsis schweinfurthii* (G), *Eremocitrus glauca* (G, F), *Evodia latifolia* (G), *Evodia ridleyei* (G), *Feronia limonia* [*Limonia acidissima*] (G), *Feroniella lucida* (G, F), *Feroniella crassifolia* (G), *Fortunella hindsii* (G, F), *Fortunella japonica* (G, F), *Fortunella margarita* (G, F), *Hesperethusa crenulata* (G, F), *Lansium domesticum* (G), *Melicope triphylla* (G), *Microcitrus australasica* (G, F), *Microcitrus australasica* var. *sanguinea* (G, F), *Microcitrus australis* (G, F), *Microcitrus garrowayi* (G, F), *Paramignya monophylla* (G), *Paramignya longipedunculata* (G) (Lee, 1918), *Poncirus trifoliata* (G, F), *Xanthoxylum clava-herculis* [*Zanthoxylum clava-herculis*] (G, F), *Xanthoxylum fagara* [*Zanthoxylum fagara*] (G, F) (Jehle, 1917). *Atalantia ceylanica*, *A. monophylla*, *Microcitrus australis*, *Feronia limonia* and *Severinia buxifolia* are immune (Reddy, 1997).

The following plants have been reported as susceptible to *X. citri* subsp. *citri*, however, the original descriptions were either not confirmed (U) or contradict those of other authors (C): *Aegle malmelos (*C), *Balsamocitrus paniculata* (U), *Feroniella obligata* (U), *Matthiola incana* var. *annua* (U) and *Toddalia asiatica* (C).

1.2 Transmission

The most destructive phase of the disease begins with spring rains, which lead dormant bacteria in a canker to multiply and spread. Canker bacteria enter actively growing shoots and leaves through natural openings and wounds. Fruit are also susceptible at an early stage of development and the first ninety days after petal fall are the most critical for fruit infection. Susceptibility of plant parts to infection can decline with maturity. Later infection results in the formation of small and inconspicuous pustules only. (OCPPO, 2003; CAB International, 2002).

The citrus canker bacterium can survive for up to ten months in lesions on *Citrus,* and can also survive for long periods in diseased plant tissues including discoloured bark tissue of tree trunks and limbs, in plant debris, as an epiphyte on host and non-host plants, and as a weak saprophyte on straw mulch. Latent infections can occur on shoots infected in autumn just before dormancy. Overwintering lesions, especially those formed on angular shoots, are the most important source of inoculum for the following season (OCPPO, 2003; CAB International, 2002).

Dispersal of the citrus canker bacterium within a tree canopy is aided by free moisture. Wind-driven rain and water splash are the primary means of short-distance dispersal, within and between trees. Overhead or spray irrigation can escalate the rate of disease spread in an orchard (OCPPO, 2003; CAB International, 2002).

Birds and insects can also act as vectors, as can human workers, particularly when trees are wet. The Asian leafminer (*Phyllocnistis citrella* – present in Australia) can infest leaves and greatly increase the number of individual lesions. Infested lesions coalesce and form large irregular shaped lesions that follow the outlines of the feeding galleries.

Long-distance dispersal is most common when infected plant material is moved between areas, including budwood, rootstock seedlings and budded trees, and less commonly fruit or leaves. Movement of soil or straw mulch could also lead to spread of the disease (OCPPO, 2003; CAB International, 2002).

2 Taxonomic Information

SCIENTIFIC NAME: *Xanthomonas citri* subsp*. citri (*Gabriel et al 1989)

SYNONYMS: *Xanthamonas axonopodis* pv. *citri* (Hasse) Vauterin et al

Xanthomonas citri subsp. *malvacearum* (Hasse 1915)

Xanthomonas campestris pv. *citri* (Hasse 1915) Dye 1978;

Bacillus citri (Hasse); Holland 1920; *Bacterium citri* (Hasse) Doidge 1916; *Phytomonas citri* (Hasse) Bergey et al., 1923;

Pseudomonas citri Hasse 1915; *Xanthomonas axonopodis* pv.*aurantifolii* Vauterin et al., 1995; *Xanthomonas campestris* pv. *aurantifolii* Gabriel et al., 1989.

COMMON NAME: Asiatic citrus canker, Citrus canker

3 Detection

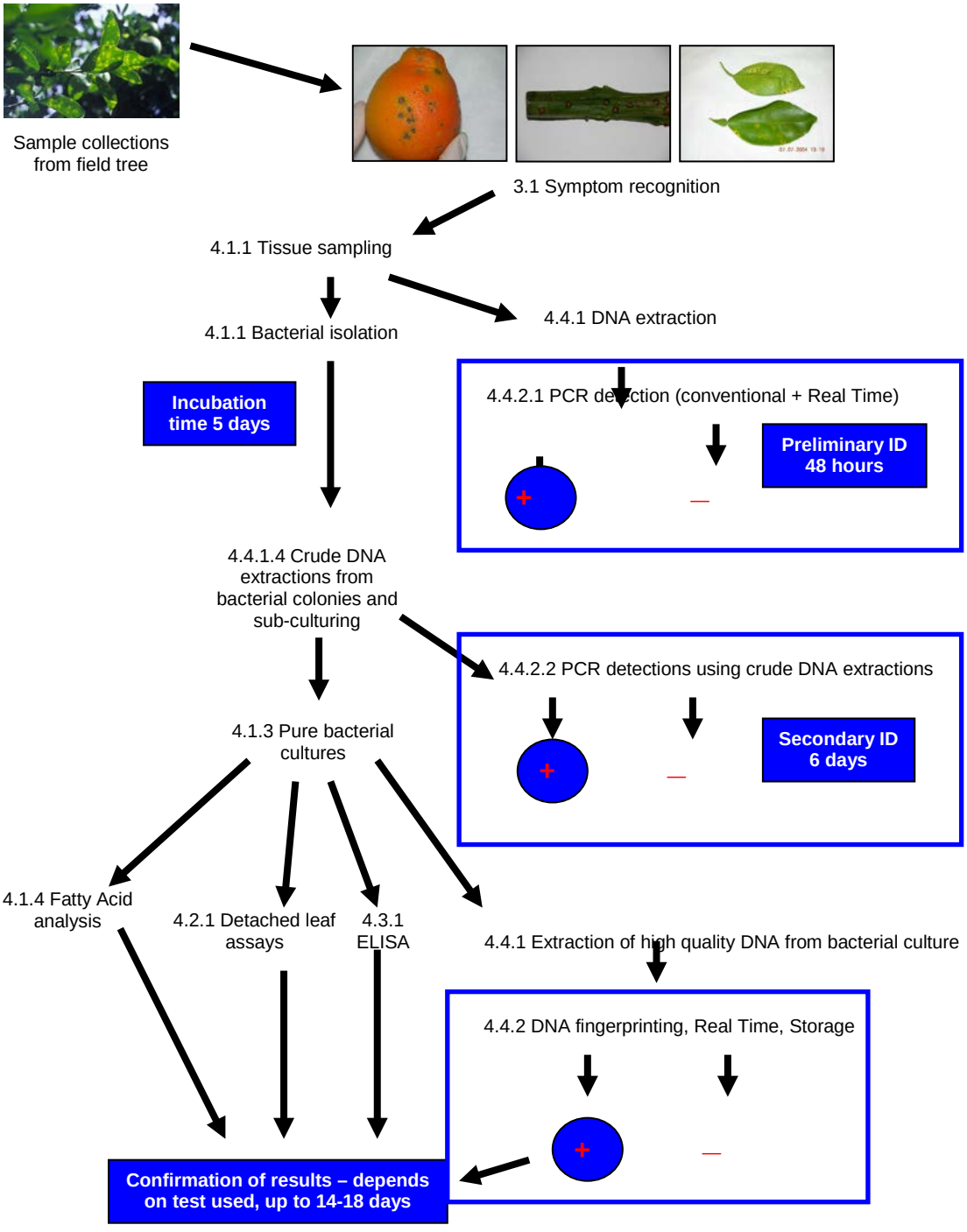
Best practice in diagnostics requires that results be obtained by more than one method. The minimum diagnostic process required is:

1. Observation of symptoms consistent with canker
2. Positive reaction of leaf lesions in multiplex PCR (possibly also other reactions)

Ideally, the same result should be obtained from two different laboratories, for each sample.

3. Other methods also that can also be used to confirm diagnosis are:
    i) isolation of bacteria with morphological and molecular characteristics consistent with *Xanthomonas citri* subsp*. citri*
    ii) FAME analysis of bacteria
    iii) pathogenic response on inoculation to susceptible citrus cultivars
    iv) Enzyme Linked Immunosorbent Assay (ELISA).

The following flow chart shows a typical diagnostic pathway for citrus canker.



3.1 Symptom description

The major symptom of citrus canker infection is the corky lesions that develop on the leaves, stems, shoots and fruit between 7-10 days after infection. In severe cases the disease also leads to shoot dieback, defoliation and fruit drop.

3.1.1 Appearance of lesions

The appearance of canker lesions can vary depending on the citrus variety, plant part affected and the age of the lesions (Figs 1-4). Lesions can be irregular in shape and appear atypical if found in association with a wound site (Fig 5) or citrus leafminer (*Phyllocnistis citrella*) feeding (Fig 6). Lesions can also appear atypical if trees are water stressed through drought or reduced irrigation. In this case the lesions can appear flatter than normal due to reduced turgor in the plant tissues.

The disease first appears as tiny, dark, slightly raised spots or lesions, usually on the lower leaf surface. As they age, they change colour from tan through brown to grey and protrude from both leaf surfaces. The lesions expand and become thick and spongy or corky, developing a water-soaked margin and often surrounded by a chlorotic halo (Figs 1, 2). The water soaked margin may disappear as lesions age and it is not as prominent on resistant host cultivars. Lesions are usually visible on both sides of the leaves and eventually the centres of the lesions become crater-like or may fall out, creating a shot hole effect. Infection can lead to early leaf fall (OCPPO, 2003; CAB International, 2002).

After infection of the leaves, symptoms typically spread onto twigs and eventually to branches. As the lesions age and thicken, they become corky and may develop a brown crater-like depression in the centre (Fig 3.). Cankers can occur on woody stems, bark may develop discoloured areas, and dieback of stems coupled with internal reddening can occur.

Infected fruit may develop scabs or pitting. Typical lesions initially resemble large oil glands on the rind. These gradually darken and become cork-like in texture (Fig 4). They are usually round, and can occur either singly or in groups. Fruit may drop prematurely. (OCPPO, 2003; CAB International, 2002).

Lesions on fruit that have been through the packing shed appear less corky and erumpent than lesions found on un-waxed fruit. During processing the top of the lesion is shaved off leaving a smooth, slightly raised dark spot, still with an irregular margin.

Figure 1: Typical symptoms of canker on leaves in the field (Copyright DPI NSW).

Figure 2 Symptoms of canker on leaves (Copyright DPI NSW)

Figure 3 Symptoms of canker on stem (Copyright DPI NSW).

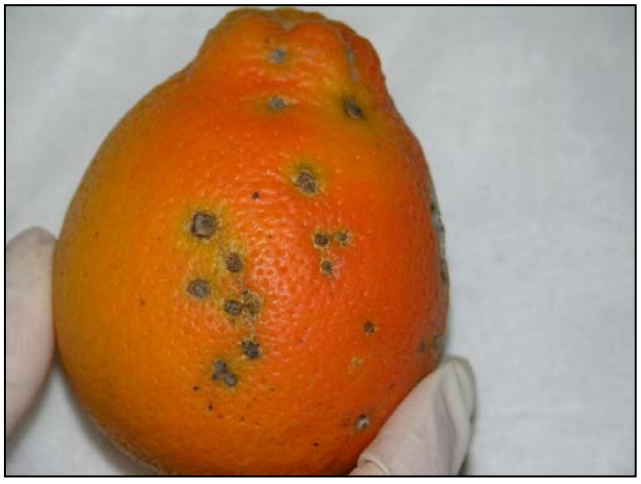


Figure 4 Symptoms of canker on tangelo fruit (Copyright DPI NSW).

Figure 5. Leaf lesions associated with wounding sites (Copyright QLD DEEDI).

Figure 6. Leaf lesions associated with citrus leafminer feeding (Copyright QLD DEEDI).

Size of lesions

Citrus leaves are highly susceptible to infection when 50-80% expanded. Lesions on the one leaf are often similar in size because the short time frame for susceptibility only allows for only one infection period. For infections through stomata or natural openings, lesions can vary in size between 5-10 mm depending on host susceptibility and number of infection cycles. Under dry conditions they remain corky or spongy in appearance. When exposed to wet conditions they rapidly enlarge and turn into flat lesions with a water-soaked appearance. (OCPPO, 2003; CAB International, 2002).

Lesions can vary in size on fruit because fruit rind is susceptible for a longer period than leaf tissue allowing more than one infection period to occur.

3.1.2 Comparison with similar diseases

Lemon scab

Lemon scab is a disease caused by the fungus *Elsinoë fawcettii* that occurs on lemons in the coastal citrus growing districts of Australia. The disease causes irregular scabby lesions to form on fruit, stems and leaves that are initially grey or pink and darken with age. Scab lesions are sometimes confused with those of citrus canker, but in general scab lesions are drier and more irregular than those of citrus canker and lack the characteristic yellow halo (Figure 7). Scab infection usually leads to a distortion of leaf growth that is unlikely to occur with citrus canker. Scab is readily differentiated from citrus canker by checking for bacterial ooze: it is absent in scab but copious in canker. Furthermore, citrus canker bacteria are readily cultured on standard media. Due to the similarity in appearance of lemon scab symptoms with citrus canker, samples should be tested by multiple methods to ensure false negative results are not recorded for the sample.

Figure 7 Typical symptoms of scab (Copyright DPI NSW).

Bacterial spot

Citrus bacterial spot caused by the bacterium *X. alfalfae* subsp. *citrumelonis* is a disease with a limited host range that is currently confined to citrus nurseries in Florida. The leaf lesions differ from canker in that they are flat, with water soaked margins and necrotic centres that often fall out (Figure 8). The disease rarely causes lesions to form on fruit.

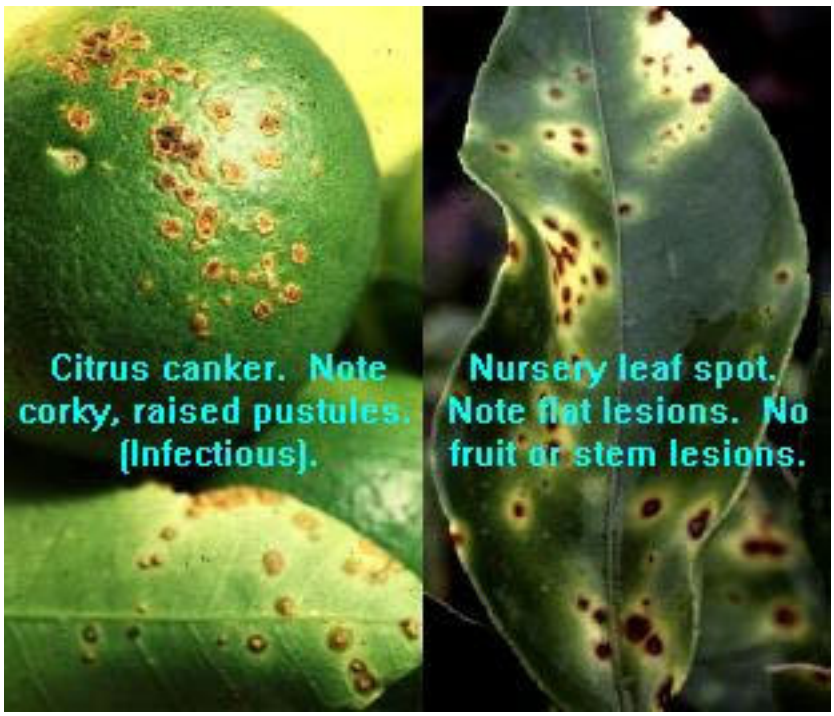


Figure 8 Citrus bacterial spot or Nursery leaf spot compared with citrus canker.
http://plantpath.ifas.ufl.edu/Newsletter/Newsletters/October99.PDF

4 Identification

Citrus canker has characteristic symptoms and diagnosis can often be confirmed using standard isolation and inoculation methods. The strains and/or subspecies of *X. citri* subsp. *citri* are less easily distinguished and molecular methods can be required for reliable differentiation.

The identification methods described here are a combination of molecular tests (the amplification of specific "signature" sequences using the polymerase chain reaction, or PCR) symptom evaluation, bacterial isolation and characterisation (including analysis of fatty acid methyl ester (FAME) profiles) and detached leaf assays.

4.1 Isolation/culture techniques

4.1.1 Isolation of bacteria from field samples

Equipment & Reagents

Microscope
Scalpel blades
Tissues or Kimwipes
Microscope slides
Cover slips
Sterile disposable inoculation loops
Petri dishes
Nutrient agar (or other medium)
70 % ethanol (technical grade)
0.85% saline or sterile water

Method

1. Surface sterilise selected material (leaf, stem etc)
    a. Place clean absorbent cloth (tissue, Kimwipe or similar) on bench
    b. Hold plant material above cloth, spray with copious quantities of 70% ethanol, both sides of material, collecting ethanol on cloth
    c. Use cloth to physically wipe the selected lesion dry
    d. Place plant material on sterile slide or in sterile Petri dish
2. Excise selected lesion using sterile scalpel blade (minimise quantity of non-lesion excised, particularly important if sampling fruit).
3. Carefully use sterile scalpel blade to halve and then quarter the excised lesion.
4. Add a drop of sterile 0.85% saline or sterile water using a sterile transfer pipette, place cover slip over top.
5. Examine for bacterial ooze within two minutes of preparing the slide, however, if the lesion is very dry a longer exudation period, up to five minutes, may be necessary.
6. Label agar plate with sample number and date.
7. Allow two to five minutes for maximum efflux of bacteria into saline.
8. Use sterile disposable loop to collect small volume of exudate, inoculate agar plate.
9. Use each of four clean sterile disposable loops to serially streak the inoculum on the plate.
10. Incubate plate at 25-28 ^{o}C.
11. Check plates for bacterial growth after 5 days and as required thereafter.

4.1.2 Bacterial Storage

The commercially available medium known as Nutrient Agar (NA) (Oxoid) has been used in developing this standard. The use of a commercial medium should ensure quality control at point of manufacture and requires only the addition of water and autoclaving prior to use. Other media (eg. sucrose peptone agar) could also be used.

Cultures on NA will need to be subcultured once per week or viability will suffer. Cultures can be 'stored' for several weeks on GYCE medium. Longer term storage is achieved through the use of Protect beads (Oxoid) (or similar) stored at -80°C, freeze drying, or ultra low temperature storage of glycerol stocks.

4.1.3 Selecting, screening and subculturing candidate bacteria

Colonies of *X. citri* subsp*. citri* appear lemony yellow and very mucoid on NA and have a "sticky" texture when touched with loops etc. Colonies are slow growing (rarely visible before 4 days, more usually 5 or more) and so can be overgrown by other organisms.

The contaminating bacterium most frequently found on citrus material being screened for *X. citri* subsp*. citri* was *Pantoae agglomerans* (previously *Enterobacter agglomerans*.) This is a common environmental organism that is not linked to citrus canker. Colonies of *P. agglomerans* are drier, flatter and of a richer yellow than *X. citri* subsp*. citri*, and have a faster growth rate. It is therefore important that when seeking to isolate the canker bacterium, isolation plates should not be screened too soon.

Suspect colonies should be subcultured to a fresh plate and screened by PCR (using the procedure colloquially known as "pick and swizzle", see molecular methods) to confirm their identity. Colonies positive in PCR are subcultured once more to confirm purity, then grown for extraction of high quality DNA.

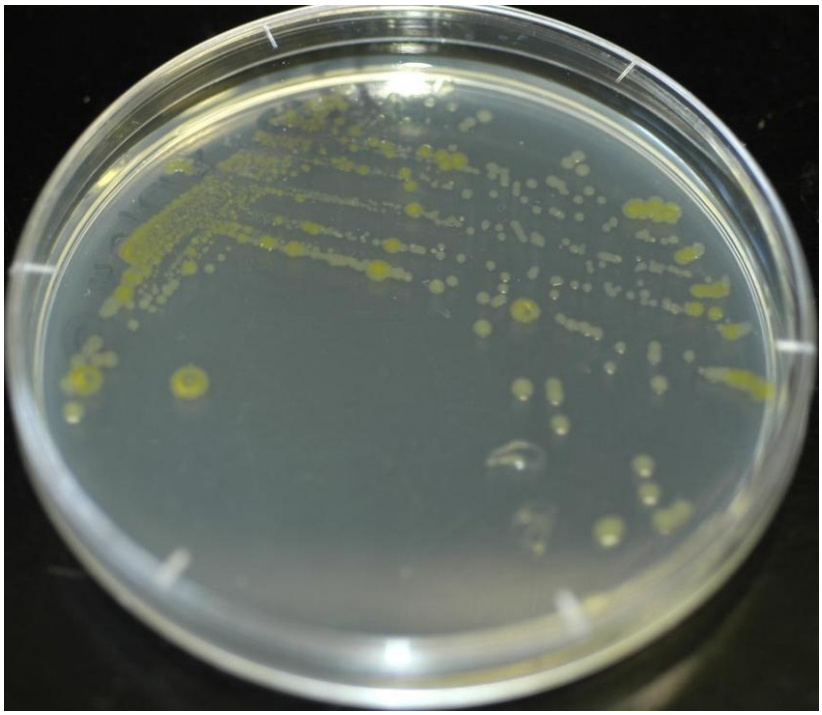


Figure 9 Colonies of *X. citri* subsp*. citri* (Pale yellow colonies) and *P. agglomerans* (Dark yellow colonies) on a nutrient agar plate.

4.1.4 FAME

Analysis of fatty acid methyl esters (FAME) clearly identifies bacteria typical of *X. citri* subsp*. citri.* Pure cultures should be subcultured to GYCE medium and transported (with permits) to an appropriate laboratory with the capability. Either Petri dishes or slopes can be sent but must be well sealed (with two layers of Parafilm or similar) and packaged securely to prevent any chance of breakage.

4.2 Biological methods

4.2.1 Detached leaf assays

Pathovars and pathotypes of *Xanthomonas citri* subsp. *citri* can be defined based on their pathogenicity to a range of citrus cultivars. Assessing the host specificity of pure isolates therefore provides a pertinent means to characterise them, particularly valuable in the case of an incursion.

A range of citrus hosts is inoculated and the response compared is to that produced on mock inoculated controls. We found the quickest and most distinct reaction was displayed by Carrizo citrange, 'Star Ruby' grapefruit and Imperial mandarin, suggesting these as the cultivars of choice in initial screening to confirm pathogenicity of new field isolates. Eureka lemon, West Indian lime and Symons sweet orange were slightly less responsive. Murcott tangor was the only host to show any differential response between isolates (slight variation in timing of initial response). The accession *Citrus hystrix* 'Eyles' (Kaffir lime) showed minimal response with any isolate tested and Nagami cumquat produced no response to any inoculation.

Equipment & reagents

Leaves of required cultivars
Sterile inoculating loops and spreaders
Pipettors and tips
Sterile 26 gauge needles
Nutrient agar plates
Water agar plates
1% sodium hypochlorite
Sterile distilled H_2O
Subaliquots of sterile water (10 ml)

Methods

Preparation of bacterial cultures

1. Inoculate the nutrient agar with pure bacterial isolates to be tested (refer to section 4.1.3).
2. Incubate cultures at 25°C for 5 to 8 days prior to inoculation.

Preparation of detached leaves

1. Collect fresh leaves of cultivars to be tested on the morning of inoculation (day 0). Select leaves that are soft and not yet fully expanded (ie. not mature).
2. Wash leaves for ten minutes in running water.
3. Soak leaves for 2 minutes in a fresh solution of 1% sodium hypochlorite
4. Rinse leaves three times in sterile distilled water
5. Air dry
6. Place each leaf, underside uppermost, on a water agar plate (one leaf per plate).

Inoculation of leaves

1. For inoculation of the negative (water-only) control place 10 µl of sterile distilled water aseptically onto one leaf of each cultivar, at six separate sites on each leaf.
2. Use a sterile 26 gauge needle to gently prick the surface of each leaf at the location of the droplet of sterile water, five times each.
3. Thoroughly resuspend a small amount of each bacterial culture in 10 ml sterile distilled water. (the amount is not critical as long as it is similar for each isolate being tested).
4. Place 10 µl of each bacterial suspension onto one leaf of each cultivar, at six separate sites on each leaf.
5. Use a sterile 26 gauge needle to gently prick the surface of each leaf at the location of the droplet of bacterial suspension, five times each. Use a separate needle for each isolate.
6. Wrap plates in cling wrap and incubate at 25 °C in a growth cabinet or an incubator equipped with white lights.
7. To demonstrate that the aliquot of sterile water used as the negative control is uncontaminated, serially dilute 10 µl to 1 ml in sterile distilled water, and then 10 µl to 1 ml in sterile distilled water. Spread 50 µl of the final dilution over each of two NA plates.
8. For bacterial enumeration and to confirm viability, similarly dilute 10 µl of each bacterial suspension to 1 ml in sterile distilled water, and then 10 µl of this to 1 ml in sterile distilled water. Spread 50 µl of the final dilution over each of two NA plates.
9. Incubate plates at 25 °C and count bacterial colonies on day 5 or 6.

Interpretation of Results

1. Examine leaves for response from day 4 using a dissecting microscope.
2. Ensure leaves inoculated with sterile distilled water show no response beyond wound repair.
3. Record responses (may help to assess these descriptively initially then convert to a numerical scale).

The scale used at EMAI to score the pathogenicity response of all cultivars except the accessions of *C. glauca*, following their inoculation with isolates of citrus canker:

0 = as per water (negative control) inoculations, usually wound repair (representative example shown in Figure 10)

1 = swelling of cells evident at site of inoculation (representative example shown Figure 11)

2 = beginning of callus formation, and/or crystalline callus at one or two sites of inoculation

3 = white crystalline callus at all sites of inoculation, some chlorosis (representative example shown Figure 12)

4 = advanced lesions, crystalline callus tending to brown/tan, water soaking, marked chlorosis (Figure 13).

4. To confirm viability, count bacterial colonies for the different isolates after 5 days, calculate colony forming units (cfu) per NA plate and convert to cfu/ml inoculum.
5. Record symptoms on the leaves periodically until no further changes are observed.

Figure 10 Reaction at day 16 on Symons sweet orange (susceptible control) inoculated with sterile distilled water. The reaction is limited to wound repair; pathogenicity score 0.

Figure 2 Reaction at day 16 on *Citrus hystrix* inoculated with *X. citri* subsp. *citri* isolate. Reaction swelling at inoculation site; pathogenicity score 1).

Figure 3 Reaction at day 16 on Carrizo citrange inoculated with *X. citri* subsp. *citri* isolate. White crystalline callus developed on all inoculation points; pathogenicity score 3.

Figure 4 Reaction at day 26 on Carrizo citrange inoculated with *X. citri* subsp. *citri* isolate. Advanced, tan coloured callus with chlorosis and water soaking; pathogenicity score 4.

4.3 Biochemical methods

4.3.1 ELISA

The commercially prepared Agdia 1000 Bacterial Reagent set for Indirect ELISA (alkaline phosphatase label) (supplied by Tas Ag ELISA & Pathogen Testing Service) specific for *Xanthomonas campestris* pv. *citri* was used with a few alterations. Its specificity was tested against a range of *Xanthomonas* species (see Fig 16) Specificity was achieved if the antigen was bound to the plate at 4°C but not at 37°C as recommended.

Method used:

1. Grow pure cultures on Nutrient agar plates for 3-5 days.
2. Initial wash step:
   a) Collect a loopful of culture from the plate, resuspend in 1 ml of PBS
   b) centrifuge at 10,000 rpm for 2 mins
   c) discard supernatant
   d) repeat.
3. Resuspend cells in 100ml of coating buffer and apply to the ELISA plate.
4. Incubate at 4 °C over **two nights**.
5. Follow manufacturer's instructions.

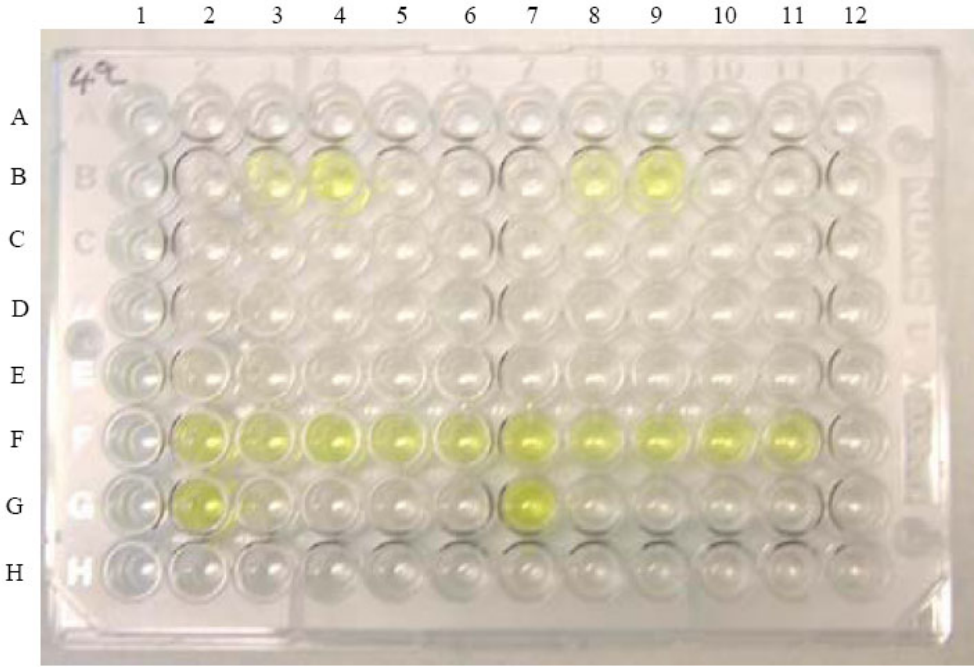


Figure 5 Photograph of the ELISA plate.

Outside wells are empty and samples analysed are duplicated. Wells B2 and B7 are the blank controls. Wells B3 and B8 are the ½ positive controls, B4 and B9 are the positive controls (supplied with the antibody). Wells F1-F6, F7-F11, G2 and G7 are the *Xanthomonas citri* subsp. *citri* isolates from Queensland 2004. The other wells on the plate are negative controls, and include *Xanthomonas axonopodis* pv*. citrumelo* in well and *X. campestris* pv*. aberrans*, *armoraciae, barbareae, campestris, carotae, cucurbitae, fici, incanae, malvacearum phaseoli* and *raphani*, *Xanthomonas axonopodis* pv*. vesicatoria* and *Xanthomonas oryzae* pv*. oryzae*.

4.4 Molecular methods

Molecular protocols designed overseas to detect the pathogen (Hartung et al., 1993) are already established in the literature. However, some assays do not detect *X. fuscans* subsp*. aurantifolii (*formerly *X.* pv*. aurantifolii* pathotypes B and C) or certain strains of *X. citri* subsp. *citri* (including the Aw strains from Florida). Recently, Cubero and Graham (2002) developed improved methods that target a gene involved in virulence (of all citrus canker strains) and a region that is specific for identification of pathotype A strains. These methods are recommended for use in this diagnostic standard in parallel with an "amplification" control that identifies extracts recalcitrant to reaction because of the presence of inhibitors. This eliminates the possibility of "false negative" results. The method reported here has been validated against a wide selection of reference isolates, including the more "atypical" strains (A* and Aw).

4.4.1 Extraction of DNA

4.4.1.1 Reagent recipes

TNE Buffer

25 mM Tris-Cl, pH 7.6
100 mM NaCl
100 mM EDTA

Store at room temperature

TE Buffer

1 mM EDTA, pH 8.0
10 mM Tris-Cl, pH 8.0
Store at room temperature.

PBS (10x):

1.37 M NaCl
27 mM KCl
43 mM $Na_2HPO_4.7H_2O$
14 mM KH_2PO_4

Autoclave.
Store at room temperature.
Dilute to 1 x with sterile water for use, store at room temperature in sterile bottle.

5 x TBE buffer:

450 mM Tris base
450 mM Boric acid
10 mM EDTA (pH 8.0)

Store at room temperature.
Dilute to 1 x TBE with milli-Q water prior to use.

Loading dye:

1 x TE buffer
0.25% Bromophenol Blue
0.25% Xylene cyanol FF

30% (v/v) Glycerol

Store at room temperature.

Water agar

15 g agar/litre, autoclaved.

Store at 4°C

20% SDS

20 g SDS per 100 ml sdH_2O.

Store at room temperature.

4.4.1.2 Extraction of DNA from leaves using phenol/chloroform

Equipment

Pipettors and tips
Razor blades
Sterile 1.5 ml microcentrifuge tubes
Sterile plastic pestles
Vortex
Microcentrifuge
Fridge/Freezer

Reagents

TNE buffer

TE buffer

Ethanol: 95-100 % ethanol, analytical grade. *Store at room temperature.*

20 % SDS: *Check for precipitate, resuspend with gentle heat if neces*sary.

Phenol: Ultra pure, saturated with TE. (MP Biomedicals). *Store at 4°C.*

Chloroform: BDH Laboratory Supplies

Iso-amyl alcohol: MP Biomedicals

Method

1. For each sample, aliquot 500 µl TNE into a 1.5 ml microcentrifuge tube,
2. Select a suspect lesion (section 3.1.1) and chop finely with a razor blade. Drop pieces into 1.5 ml microcentrifuge tube containing 500 µl TNE buffer and crush finely using a sterile plastic pestle.
3. Add 400 µl TE saturated phenol and 100 µl 20% SDS to the microcentrifuge tube, mix well.
4. Vortex tubes briefly then stand at room temperature for up to 30 mins.
5. Add 250 µl chloroform: isoamyl-amyl alcohol (24:1, prepare freshly), vortex.
6. Centrifuge the tube for 15 mins at 13,000 *g*.
7. Carefully recover supernatant, about 450 µl. Take extreme care to avoid the material at the interface. Transfer to a clean 1.5 ml microcentrifuge tube.

8. Add 900 µl of 95-100% ethanol.
9. Invert tube gently to mix, a DNA precipitate should form immediately.
10. To maximise yield, incubate samples overnight at 4^{o}C or put on ice for 30 mins (optional).
11. Collect DNA by micro centrifugation for 15 mins at 13,000 *g*.
12. Discard supernatant.
13. Wash pellet with 100 µl 70% ethanol.
14. Air dry pellet for 10 - 30 mins at room temperature (preferably in a BSC).
15. Resuspend pellet in 100 µl TE buffer.
16. Store at 4^{o}C for fingerprinting or - 20^{o}C if using in a PCR.

4.4.1.3 Extraction of DNA from leaf lesions using the "soak" method

Equipment

Pipettor and tips
Sterile scalpel blade
Sterile 1.5 ml microcentrifuge tubes
Microcentrifuge

Reagents

PBS (1 x)

Method

1. Use a sterile scalpel blade to excise a citrus canker lesion (Section 4.1.1).
2. Cut lesion into four, transfer to sterile microcentrifuge tube.
3. Add 100 µl sterile 1 x PBS
4. Shake at 250 rpm for 1 hour.
5. Store crude extract at -20^{o}C.
6. Microcentrifuge before using supernatant in PCR.

4.4.1.4 Extraction of DNA from bacterial colonies using the "Pick and Swizzle" method

Equipment

Pipettor and tips
Sterile disposable inoculation loops
Sterile 1.5 ml microcentrifuge tubes
Microcentrifuge
Dry heating block

Reagents

Sterile distilled H_2O
Nutrient agar plate

Method

1. Aseptically "pick" a suspect bacterial colony using a sterile disposable loop.

2. Touch the loop to an identifiable point on the surface of a sterile NA plate - inoculates the bacteria to a master plate for subsequent growth.
3. Transfer the loop (still contains sufficient bacteria) to a 1.5 ml microcentrifuge tube containing 100 µl of sdH_2O, "swizzle" the loop to resuspend the bacteria.
4. Incubate the suspension at 95°C for 5 minutes.
5. Centrifuge the suspension at 13,000 *g* for 5 mins to pellet debris.
6. Use 1 µl supernatant as DNA template for PCR.
7. Incubate NA "pick plate" overnight.
8. Colonies that are positive in PCR can then be identified on the "pick plate" and subcultured pending further analysis.

4.4.1.5 Extraction of high quality DNA using a commercial kit

A range of kits are available to extract high quality DNA from bacteria, such as is required for DNA fingerprinting, archiving etc. The EMAI laboratory uses the DNeasyÓ Tissue extraction kit (Qiagen), although others would also be suitable.

Colonies confirmed positive in PCR (by "pick and swizzle") are subcultured to produce pure isolates. Pure cultures are extracted from NA plates according to the manufacturer's recommendations and instructions. Good quality DNA of sufficient quantity was extracted without using the optional second elution from the columns.

4.4.2 PCR Methods

Reagents nominated in this standard work effectively in our hands but other brands may deliver equal success. Extracts of bacteria known to be *X. citri* subsp*. citri* were used as positive controls in all reactions.

Diagnosis of *X. citri* subsp. *citri* is made using primers to the pathogenicity gene *pthA*, designed by Cubero and Graham (2002), in a multiplex reaction with "universal" primers to the bacterial 16S rDNA gene (Weisburg *et al.*, 1991) (Table 1). The latter primers serve firstly as an amplification control, because all field samples carry environmental bacteria, and the primers cross react with chloroplast DNA, and so all samples should produce the 16S fragment if PCR has been successful. Given that the target for these primers must also be extracted from the sample, rather than being added to the purified extract prior to PCR, it also serves as a control to demonstrate the nucleic acids were successfully extracted from the original sample.

Extracts that fail to produce the 16S band may be recalcitrant to amplification and the reaction should be repeated. Samples that are positive for *X. citri* subsp*. citri* will amplify both the the *pthA* and 16S fragments.

Two other PCR-based assays have recently been described for detection of *X. citri* subsp. *citri* (Coletta-Filho *et al*., 2006; Park *et al*., 2006). As part of maintaining the standard, the laboratory at EMAI has recently compared these primers to those of Cubero and Graham (2002) and Hartung *et al*. (1993). The specificity of the assays was assessed against a collection of isolates (*citri* As, A*s, Aws, C, Es, *malvaceraurm*, other *Xanthomonas* species) and their respective limits of detection sensitivity determined by serial dilution of control extracts. Neither of these sets was found to offer improved sensitivity or specificity so there is no advantage to changing to these primers for detection.

Table 1: Primer sets used for the detection of *Xanthomonas citri* subsp*. citri*

Target gene	Primer Name	Sequence (5'-3')	Reference
pth-A	*DLH 1*	TTGGTGTCGTCGCTTGTAT	Hartung *et al.* (1993)
	DLH 2	CACGGGTGCAAAAAATCT	
16S rDNA	*FD1*	AGAGTTTGATCCTGGCTCAG	Weisburg *et al*. (1991)
	rP2	ACGGCTACCTTGTTACGACTT	
pth-A	*J-pth 1*	CTTCAACTCAAACGCCGGAC	Cubero and Graham, 2002
	J-pth 2	CATCGCGCTGTTCGGGAG	
Conserved BOX regions	*BOX A1R*	CTACGGCAAGGCGACGCTGACG	Koeuth *et al.* 1995
pth-A	*VM3*	GCATTTGATGACGCCATGAC	Mavrodieva *et al.* 2004
	VM4	TCCCTGATGCCTGGAGGATA	

4.4.2.1 PCR detection from plant extracts using GeneReleaser.

Plants intrinsically contain a range of substances that, if not effectively removed during extraction, will inhibit PCR. The effect of inhibitors remaining in DNA extracts can be reduced by dilution, although the dilution required must be determined empirically.

GeneReleaser is a proprietary product designed to improve amplification by non-specifically sequestering cell lysis products that may inhibit polymerases. Its use circumvents the need to dilute plant DNA extracts prepared by the phenol/chloroform method, prior to use. If GeneReleaser is not available the reaction should be completed using diluted extracts, initially suggest using a 1:100 dilution in water.

Equipment

Pipettors and tips
0.2 ml PCR tubes
Ice
BSC
Microwave
Vortex
Gloves
Power pack
Thermal cycler
Gel tank
Gel Documentation system with UV light

Reagents

dNTPs (Promega)
Taq polymerase (Invitrogen)
10x buffer (Invitrogen)
GeneReleaser (BioVentures Inc., Murfreesboro, USA)
Reagent grade mineral oil for overlay.
1 x TBE buffer
1% (w/v) Agarose gel: 1 g DNA grade agarose per 100 ml 1x TBE
Loading dye

Molecular weight marker
Ethidium Bromide (Sigma) staining solution (0.8µg/ml).

Method

In Pre-PCR cabinet

1. Label sterile 0.2 ml PCR tubes.
2. Prepare "Master Mix" (without the Taq polymerase) as per Table 2.

Table 2: Master Mix for *J-pth1&2* primers in a multiplex reaction with 16 SrDNA primers (*FD1 and rP2)* to screen DNA extracted from leaf lesions

Reagents (initial concentration)	**Volume in each PCR tube (µl)**	**Final Concentration**
sdH_2O	6.55	
10x buffer	2.5	1x
dNTPs (2mM)	2.5	0.2 mM
$MgCl_2$ (50 mM)	1.5	3.0 mM
Forward primer *J-pth 1* (10 µM)	0.25	100 nM
Reverse primer *J-pth 2* (10 µM)	0.25	100 nM
Forward primer *FD1*(10 µM)	0.125	50 nM
Reverse primer *rP2* (10 µM)	0.125	50 nM
Taq polymerase (5U/µl)	0.2	1 U
DNA template	1.0	No dilution (with GeneReleaser)
GeneReleaser	10.0	
Total reaction volume	25.0	

3. Store "Master Mix" on ice in sterile 1.5 ml centrifuge tube.
4. Add 1 µl of sterile dH_2O to the first negative control tube.

In BSC

5. Vortex the GeneReleaser then add 10 µl to the labelled PCR tubes.
6. Add 1 µl DNA template to the appropriate tubes.
7. Add 1 µl of positive control DNA the appropriate tube(s).
8. Add 10 µl of mineral oil on top of each tube to prevent evaporation
9. Heat reactions for 7 mins on HIGH in microwave.
10. Place reactions tubes on ice slurry to cool.
11. Add the Taq polymerase to the Master Mix in the BSC.
12. Aliquot the 14 µl Master Mix to the PCR tubes.
13. Add 1 µl of sterile dH_2O to the second negative control tube.
14. Cycle the tubes with PCR programme in Table 3.

Table 3: Summary of the thermal cycling conditions for PCR's used in this standard

Target in PCR	Initial denatu-ration (1 cycle only)	Denatu-ration	Annealing	Extension	Number of cycles	Final extension (1 cycle only)	Fragment length
Multiplex	94/5min	94/30"	64-60/30" Touchdown	72/45"	5	72/5mins	16S rDNA 1500bp, pthA 197bp
		Then 94/30"	60/30"	72/45"	35		
J-pth	94/5min	94/30"	58/30"	72/45"	30	72/5mins	pthA 197bp
BOX	94/5min	94/30"	50/30"	72/1min	40	72/10mins	N/A
Real Time	94/2min	94/20"	57/20"	72/40"	30	N/A	150bp
Hartung *et al*	94/3min	94/40"	54/40"	72/40"	35	72/5mins	222bp

15. Mix 8 µl of each PCR sample with 2 µl loading dye.
16. Load samples and 1Kb+ ladder onto separate wells of 1 % (w/v) agarose gel.
17. Electrophorese in 1x TBE at 90 volts for approximately 40 mins.
18. Stain the gel in Ethidium Bromide staining solution, according to local Standard Operating Procedure.
19. Visualise and photograph gel using the Gel Documentation System.

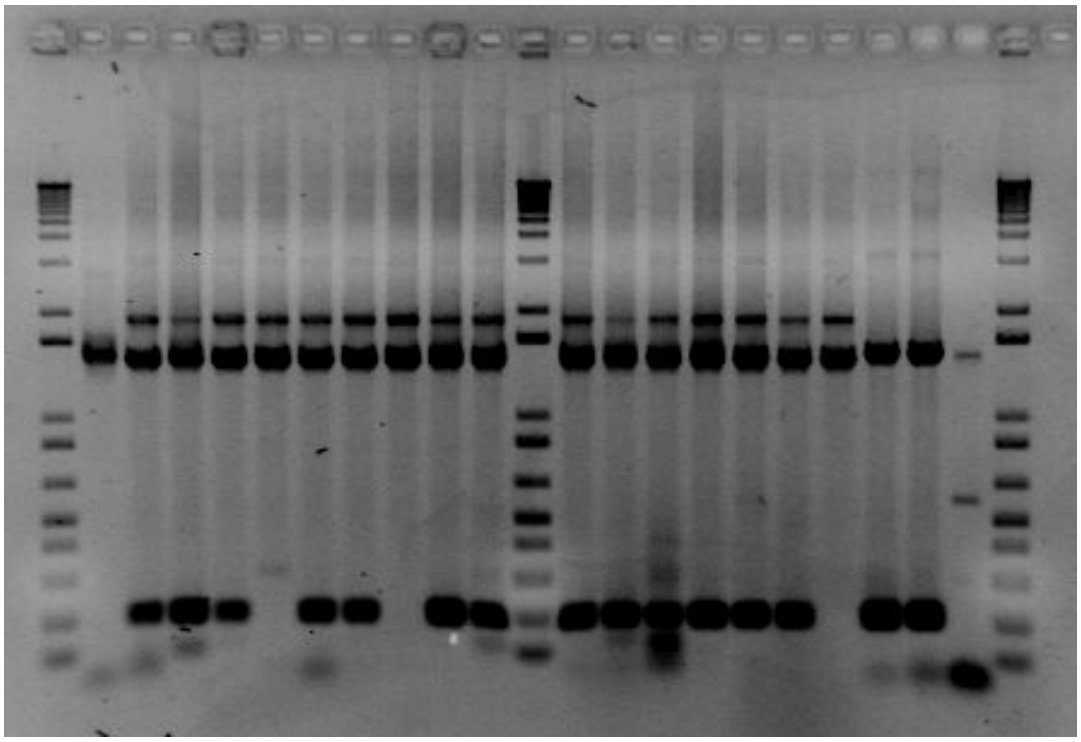


Figure 6 Agarose gel showing PCR products generated from samples using primers J-pth 1& 2 in a multiplex reaction with FD1 and rP2. DNA molecular weight marker, 1 Kb+ ladder (lanes 1, 12 and 23), positive controls (lanes 20 and 21), negative controls (lanes 2 and 22), diagnostic samples (lanes 3-11 and 13 to 19).

4.4.2.2 PCR detection from suspect bacterial colonies without GeneReleaser.

To screen bacterial colonies using the "pick and swizzle" method, or to confirm that high quality DNA extracted from bacterial cultures using Qiagen extraction are *Xanthomonas citri* subsp. *citri*, DNA is reacted with the J-pth primers of Cubero and Graham (2002), without the need for GeneReleaser. DNA extracted using a Qiagen extraction will give best results if diluted: dilute an aliquot 1:100 in sterile distilled water prior to reaction.

Method

In Pre-PCR cabinet

1. Label sterile 0.2ml PCR tubes.
2. Prepare "Master Mix" (without the Taq polymerase) as per Table 4.

Table 4: Master Mix for *J-pth1&2* primers to screen DNA extracted from bacteria

Reagents (initial concentration)	**Volume in each PCR tube (µl)**	**Final concentration**
sdH_2O	9.65	
10x buffer	1.5	1x
dNTPs (2mM)	1.5	0.2 mM
$MgCl_2$ (50 mM)	0.9	3.0 mM
Forward primer *J-pth 1* (10 µM)	0.15	100 nM
Reverse primer *J-pth 2* (10 µM)	0.15	100 nM
Taq polymerase (5U/µl)	0.15	0.75 U
DNA template	1.0	1:100 dilution
Total reaction volume	15.0	

3. Store "Master Mix" on ice in sterile 1.5ml centrifuge tube.
4. Add 1 µl of sterile dH_2O to the first negative control tube.

In BSC

5. Add the Taq polymerase to the Master Mix in the BSC.
6. Aliquot the 14 µl Master Mix to the PCR tubes.
7. Add 1 µl of sterile dH_2O to the second negative control tube.
8. Cycle the tubes with PCR programme in Table 3.
9. Mix 8 µl of each PCR sample with 2 µl loading dye.
10. Load samples and 1Kb+ ladder onto separate wells of 1 % (w/v) agarose gel.
11. Electrophorese in 1x TBE at 90 volts for approximately 40 min.
12. Stain the gel in Ethidium Bromide staining solution, according to local Standard Operating Procedure.
13. Visualise and photograph gel using a gel documentation System.

4.4.2.3 DNA fingerprinting

If required, DNA fingerprinting can be performed to differentiate genotypes of the canker organism, as described in Cubero and Graham (2003). This is sometimes required for surveillance or tracing programs, but is not suitable for use as for identification, particularly in isolation. Further information can be obtained by contacting the authors, but briefly, high quality DNA extracts are prepared using the DNeasyTM method and diluted for use in PCR with the BOXA1R primer (Table 1). Products can be resolved on a 25 cm agarose gel or polyacrylamide gels. High quality DNA (extracted using the DNeasyÓ method) is required for this procedure. An aliquot of the DNA is diluted 1:100 in sterile distilled water prior to use as template.

4.4.2.4 Real Time PCR

This method is adapted directly from Mavrodieva *et al* (2004). The amplification targets the *pth-A* gene and the amplicon is detected using SybrGreen dye. Increased fluorescence of SybrGreen is evident as the amplicon is produced, the identity of the amplicon is confirmed by it characteristic melting point around 88 °C.

This work used the ABsolute™ QPCR SYBR ® Green Mix (ABgene, UK) and a Rotor-gene 2000 from Corbett Research but other systems would probably work as effectively. DNA prepared from lesions by either the phenol chloroform or the "soak" method and from bacteria by "pick and swizzle", phenol chloroform or DNeasyÓ extraction methods were all suitable for use as templates in Real time PCR.

The specificity of these primers to *X. citri* subsp. *citri* was confirmed in supporting research. DNA templates prepared from other citrus pathogens (eg *Candidatus* Liberibacter (huanglongbing), *Guignardia citricarpa, Guignardia mangiferae, Alternaria species, Mycosphaerella species*) and other species of *Xanthomonas* did not result in any amplification in these assays.

Equipment

Pipettors and tips
0.2 ml PCR tubes
Ice
Gloves
Real Time Thermal cycler

Reagents

Primers *VM3* and *VM4* (as per Table 1) and ABsolute™ QPCR SYBR ® Green Mixes (or other system for Real time PCR using SybrGreen)

Method

In Pre-PCR cabinet

1. Label sterile 0.2 ml PCR tubes.
2. Add water, $MgCl_2$ and "Master Mix" into a sterile 1.5 ml centrifuge tube according to Table 5.
3. Store master mix on ice.
4. Add 1 µl of sterile dH_2O to the first negative control tube.

Table 5: Master Mix for Real Time PCR using primers *VM3* and *VM4*

Reagents (initial concentration)	Volume in each PCR tube (µl)	Final concentration
sdH_2O	8.0	
$MgCl_2$ (50 mM)	0.2	0.5 mM
Forward primer *VM 3* (10 µM)	0.4	0.2 µM
Reverse primer *VM4* (10 µM)	0.4	0.2 µM
2x Sybr MM	10.0	1x
DNA template	1.0	1:100 dilution
Total reaction volume	20.0	

In BSC

5. Add the SybrGreen cocktail to the Master Mix.
6. Aliquot 19 µl Master Mix to the PCR tubes.
7. Add 1 µl diluted DNA template to the appropriate labelled tubes.
8. Add 1 µl of positive control DNA to the appropriate tube(s).
9. Add 1 µl of sterile dH_2O to the second negative control tube.
10. Cycle the tubes with Real Time PCR programme in Table 3.

4.4.2.5 PCR detection using Hartung's primers

PCR can also be conducted using the primers of Hartung *et al* (1993). These primers do not detect all strains of *Xanthomonas citri* subsp*. citri* but can still be used as an extra confirmation of most A-type strains. The composition of the Master Mix and PCR program for this primer set are shown in Tables 6 and 3 respectively.

Table 6: Master Mix for Hartung's primers (*DLH 1* and *DLH 2)*

Reagents (initial concentration)	Volume in each PCR tube (µl)	Final concentration
sdH_2O	2.25	
10x buffer III	2.5	1x
dNTPs (2mM)	2.5	0.2 mM
$MgCl_2$ (50 mM)	1.5	3.0 mM
Forward primer *DLH 1*(10 µM)	2.5	1.0 µM
Reverse primer *DLH 2*(10 µM)	2.5	1.0 µM
Taq polymerase (5U/µl)	0.25	1.25U
DNA template	1.0	
Gene Releaser	10.0	
Total reaction volume	25.0	

The PCR-based assays recently described in Coletta-Filho *et al*., 2006 and Park *et al*., 2006 could also be used in this role but they do not offer any advantage with respect to specificity or sensitivity over those of Cubero and Graham (2002) and Hartung *et al*. (1993).

5 Contact points for further information

DPI NSW
Elizabeth Macarthur Agricultural Institute
Woodbridge Rd, Menangle NSW 2568
PMB 8, Camden NSW 2570
Phone: +61 2 4640 6333
Fax: +61 2 4640 6300

Other Australian laboratories with experience in aspects of diagnosis of Xac.

Primary sources of testing in addition to EMAI, DPI NSW.

- Dr John Thomas, DEEDI led the testing in Qld after the initial two months. He has led the Qld team in their analysis of all submitted samples - clinical exam, microscopy, molecular testing and bacterial isolation.
- Dr Cherie Gambley, DEEDI, has extensive experience in all aspects of citrus canker diagnostics, epidemiology and biology.

Others with experience in particular areas:

- Dr Vanessa Brake's (AQIS) lab has experience with canker - exam/micro/molecular/isolation.
- Pat Barkley has extensive experience with citrus canker in the field and has consulted to the Qld government and industry during the outbreaks of 2004.

6 Acknowledgements

The information presented here was extracted from the **Citrus Canker Diagnostic Manual** (produced by DPI New South Wales for Plant Health Australia and authored by Deborah Hailstones, Aida Ghalayani, Michelle Flack and Nerida Donovan)

The protocol was reviewed by DEEDI Qld.

7 Suppliers

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Tas Ag ELISA & Pathogen Testing Service 13 St Johns Ave New Town Tas 7008 Ph: (03) 6233 6845 Fax: (03) 6278 2716	

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