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Pathogen profile

Xanthomonas axonopodis pv. *citri*: factors affecting successful eradication of citrus canker

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SUMMARY

Taxonomic status: Bacteria, Proteobacteria, gamma subdivision, Xanthomodales, *Xanthomonas* group, *axonopodis* DNA homology group, *X. axonopodis* pv. *citri* (Hasse) Vauterin *et al.*

Microbiological properties: Gram negative, slender, rod-shaped, aerobic, motile by a single polar flagellum, produces slow growing, non-mucoid colonies in culture, ecologically obligate plant parasite.

Host range: Causal agent of Asiatic citrus canker on most *Citrus* spp. and close relatives of *Citrus* in the family Rutaceae.

Disease symptoms: Distinctively raised, necrotic lesions on fruits, stems and leaves.

Epidemiology: Bacteria exude from lesions during wet weather and are disseminated by splash dispersal at short range, windblown rain at medium to long range and human assisted movement at all ranges.

Crop loss: Severe infections cause defoliation, blemished fruit, premature fruit drop, die-back of twigs and general debilitation of the tree.

Distribution: Citrus canker is not present in all subtropical to tropical regions of citriculture in the world, so considerable regulatory efforts are expended to prevent the introduction and spread of *X. axonopodis* pv. *citri* into areas in the Americas, Australia and elsewhere, with climates conducive to the disease.

Importance: Limited strategies exist for suppression of citrus canker on more susceptible cultivars. Blemished fruit are unmarketable and exposed fruit are restricted in market access. The economic impact of loss of markets is much greater than that from yield and quality reductions of the crop.

Useful websites: http://doacs.state.fl.us/canker, http://www.apsnet.org/education/lessonsplantpath/citruscanker/top.htm, http://www.apsnet.org/online/feature/citruscanker/, http://www.plantmanagementnetwork.org/pub/php/review/citruscanker/, http://www.abecitrus.com.br/fundecitrus.html, http://www.biotech.ufl.edu/PlantContainment/canker.htm, http://www.aphis.usda.gov/oa/ccanker/.

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INTRODUCTION

Rationale for eradication of citrus canker

Increasing international travel and trade have dramatically accelerated introductions of invasive species into agricultural crops (Anonymous, 1999). Systems for protecting agricultural industries have been overwhelmed by an unprecedented number of pests, especially plant pathogens. One of the most notable is *Xanthomonas axonopodis* pv. *citri* (syn. *X. citri* pv. *citri* Gabriel *et al.*, 1989), the bacterium that causes Asiatic citrus canker. Although *X. axonopodis* pv. *citri* directly reduces fruit quality and yield, the impact is worsened because the presence of citrus canker in an area triggers immediate quarantine restrictions, disrupting the movement of fresh fruit (Anonymous, 1997a,b). During the last 20 years in Florida, introductions of *X. axonopodis* pv. *citri* and eradication of citrus canker have received considerable attention, mainly because of the far-reaching national and international trade, political, legal and socio-economic implications of the eradication concept for exotics (Gottwald *et al.*, 2001; Schubert *et al.*, 2001).

X. axonopodis pv. *citri* is a candidate for plant pathogen eradication because the disease has fundamental features that make the concept feasible and highly desirable: (i) The bacterium is unable to survive outside of the host lesion for extended periods; (ii) The bacterium lacks an efficient vector (aside from humans); (iii) The characteristic raised lesions are easily identifiable, which permits relatively quick and accurate diagnosis; (iv) The host range of the bacterium is restricted to a perennial fruit tree crop of high value; (v) Many commercial varieties of citrus are moderately to highly susceptible, so disease control measures are only

modestly effective and relatively costly; (vi) The disease has been successfully eradicated previously during campaigns in Florida, Australia, South Africa. This review of citrus canker features current knowledge specifically relating to the ongoing attempt to eradicate and control the disease in Florida and South America, and is intended to compliment the recent reviews of the biology and molecular biology of the pathosystem (Brunings and Gabriel, 2003; Gottwald *et al.*, 2002a).

Brief history of canker eradication in Florida

Citrus canker has a long history in Florida. The disease was first found around 1912, spread throughout the south-eastern US on imported seedlings from Japan, and was declared eradicated from Florida and the adjacent states in 1933 (Schoulties *et al.*, 1987). Citrus canker was discovered again in Manatee County, Florida, south of Tampa Bay in 1986, and was declared eradicated by 1994 (Schubert *et al.*, 2001). Three years later the disease re-emerged, in the same area on the west coast of Florida where the 1980s outbreak had occurred. In the meantime, a new and separate infestation of citrus canker was discovered in urban Miami in 1995, with an estimated introduction date of 1992 or 1993 (Gottwald *et al.*, 1997; Schubert *et al.*, 2001).

When detected in Miami in 1995, the infected area was confined to 36.3 km^2 of residential properties south-west of the Miami International Airport. In response to the detections of citrus canker, a cooperative state/federal Citrus Canker Eradication Program (CCEP) was established between the Florida Department of Agriculture and Consumer Services, Division of Plant Industry and the USDA, Animal and Plant Health Inspection Service (APHIS). Despite extensive eradication efforts, which have resulted in the removal or cutting back of over 1.56 million commercial trees and nearly 600 000 infected and exposed door-yard citrus trees, the infected area has increased to 1701 km^2 as of March 2002. The quarantine area is presently over 2590 km^2 in south-east Miami-Dade and Broward counties and the state-wide quarantine areas encompass 3890 km^2 (Schubert *et al.*, 2001).

When citrus canker was rediscovered in commercial citrus in Manatee County on the west coast of Florida in June 1997, the eradication effort was resumed there. Subsequent disease outbreaks in residential and commercial citrus in Collier, Hendry, Hillsborough, Palm Beach, Martin, De Soto, Brevard, Orange and Monroe counties of Southern and central Florida were related predominantly to the inoculum reservoir in residential Dade and Broward counties. In some areas outside of south-east Florida, progress towards eradication reached a point of actually lifting the quarantine (Schubert *et al.*, 2001).

Citrus canker symptoms and disease

All above-ground tissues of citrus are at maximum susceptibility to infection by *X. axonopodis* pv. *citri* during the last half of the expansion phase of growth (Gottwald and Graham, 1992; Stall *et al.*, 1982a). Like many other bacterial diseases, the pathogen enters host plant tissues through stomates (Gottwald and Graham, 1992; Graham *et al.*, 1992a,b) and wounds (Koizumi and Kuhara, 1982). The optimum temperature for infection falls between 20 and 30 °C (Koizumi, 1977). Bacteria multiply 3–4 log units per lesion under optimum conditions and cells may emerge from stomatal openings in as little as 5 days to provide inoculum for further disease development (Fig. 1A). The earliest symptoms on leaves appear as tiny, slightly raised blister-like lesions beginning around 9 days post-infection (Fig. 1B). As the lesions age, they first turn light tan, then tan to brown, and a water-soaked margin appears, often surrounded by a chlorotic halo. The water-soaked margin may disappear as the lesions age, and is not as prominent on resistant cultivars. The centre of the lesion becomes raised and spongy or corky. These raised lesions from stomatal infection are typically visible on both sides of a leaf. Eventually, the centres of the leaf lesions become crater-like. Defoliation becomes a problem as the disease intensifies (Goto and Yaguchi, 1979; Gottwald *et al.*, 1988).

On twigs and fruit, citrus canker symptoms are similar: raised corky lesions surrounded by an oily or water-soaked margin. No chlorosis surrounds twig lesions but may be present on fruit lesions. Twig lesions on angular young shoots perpetuate the inoculum and prolong survival of *X. axonopodis* pv. *citri* in areas where citrus canker is endemic. If twigs are not killed back by girdling infections, the lesions can persist for many years, causing raised corky patches in the otherwise smooth bark.

Twig die-back, fruit blemishes and early fruit drop are economic impacts of the disease (Graham, 2001). Since the young growth provides the bulk of the susceptible tissues, vigorously growing trees are most threatened by *X. axonopodis* pv. *citri*. A well-managed citrus tree in Florida will undergo three to five growth flushes every growing season, each accompanied by a period of enhanced susceptibility. Coincidence of growth flushes and meteorological conditions in summer favour disease build-up on leaf flushes in subtropical Florida.

The bacterium multiplies in lesions in leaves, stems and fruit. When there is free moisture on the lesion surface, bacteria are released from an extracellular polysaccharide matrix (Fig. 1C) and dispersed to new growth by rain splash (Gottwald *et al.*, 1989; Pruvost *et al.*, 2002) and the force of windblown rain droplets (Goto, 1992; Timmer *et al.*, 1991). Rainwater collected from foliage with lesions contains between 10^5 and 10^8 cfu/mL (Goto, 1992; Stall *et al.*, 1980). Wind-driven rain is the main dispersal agent, and wind speeds ≥ 8 m/s (18 mph) aid in the penetration of bacteria through the stomatal pores or wounds made by thorns, insects such as the Asian citrus leafminer (*Phyllocnistis citrella*) (Fig. 2), and blowing sand. Water congestion of leaf tissues can be seen during rainstorms. Citrus foliage, fully water

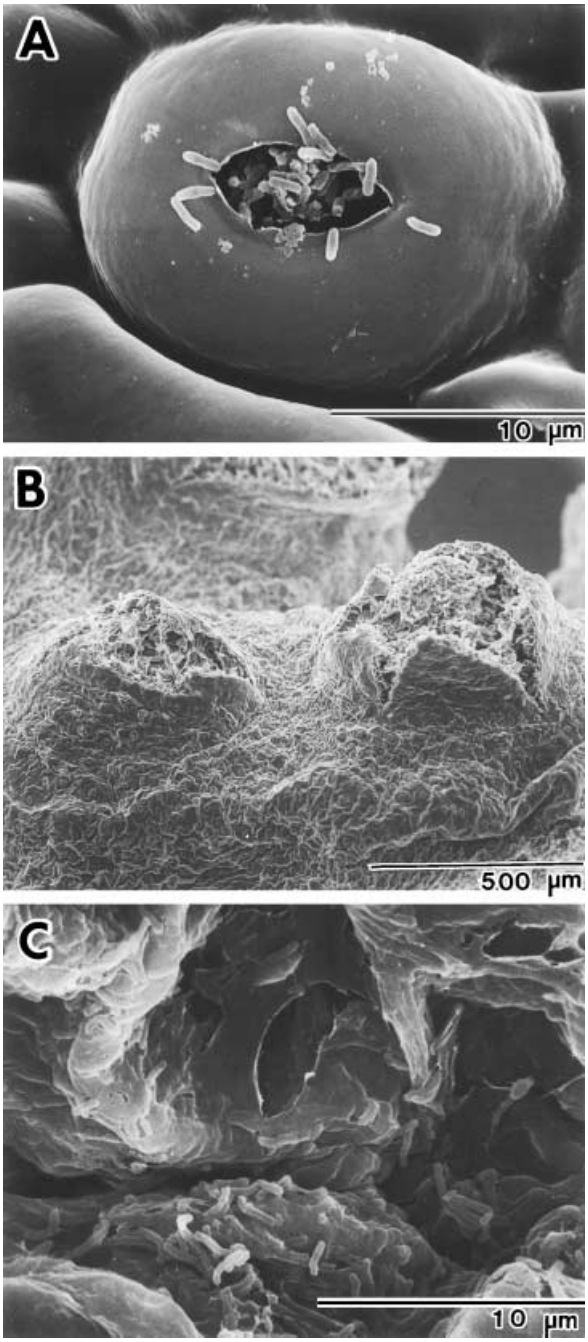

Fig. 1 Scanning electron microscopy of infections by *Xanthomonas axonopodis* pv. *citri* on the abaxial leaf surface of grapefruit (*Citrus paradisi*). (A) Bacterial egress from a stomatal opening 5 days after infiltration of the leaf mesophyll with 10^5 cfu/mL. (B) Eruption of infected mesophyll tissues through the epidermis to form a pustule 30 days after inoculation. (C) Bacteria embedded in a matrix of extracellular polysaccharide (EPS) from the surface of the lesion in (B).

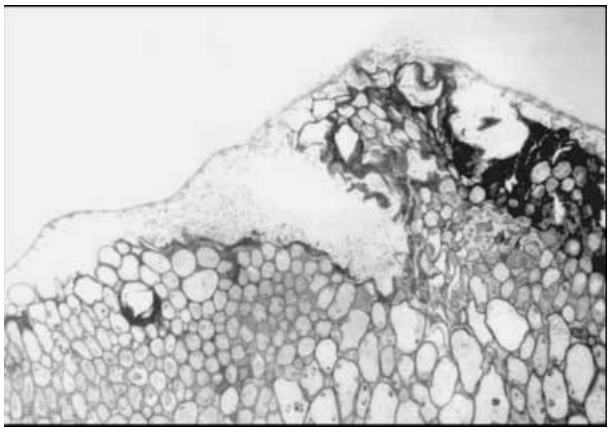

Fig. 2 Light microscope section of a leaf of grapefruit (*Citrus paradisi*) showing the lifting of the cuticle due to feeding by larvae of citrus leafminer, *Phyllocnistis citrella*. Bacteria were exuded into the subcuticular space from the canker lesion in the palisade parenchyma cell layer at the right.

soaked, contains approximately 7 µL/cm^2 of leaf area (Goto, 1992; Gottwald and Graham, 1992). Water congestion during inoculation with as few as 1–2 bacterial cells, forced through stomatal openings, can lead to infection and lesion formation (Gottwald and Graham, 1992; Graham *et al.*, 1992a).

Bacteria dispersed by wind blown rain were detected up to 32 m from infected trees in Argentina (Stall *et al.*, 1980). However, in Florida, evidence for a much longer dispersal associated with common thunderstorms has recently been documented (Gottwald *et al.*, 1997; Gottwald *et al.*, 2002). Strong circumstantial evidence points to occasional medium- to long-distance transport (10–15 km) by unusual storm events such as tornadoes and tropical storms (Gottwald *et al.*, 1997). Regional spread of the bacterium normally occurs by human movement of diseased or exposed citrus plant material or by the use of equipment contaminated by exposure to diseased citrus.

X. axonopodis pv. *citri* multiplies if the lesions are still expanding, and populations of bacteria produced per lesion are closely related to lesion size (Graham *et al.*, 1992a). The bacteria remain alive in the margins of the lesions in leaves and fruit until they fall. Bacteria also survive for up to several years in lesions on woody branches (Goto, 1992). Bacteria that ooze on to plant surfaces (Fig. 1) die within hours from desiccation and exposure to direct sunlight (Graham *et al.*, 2000). Exposed bacteria survive only a few days in soil, and a few months in plant refuse that has been incorporated into soil (Graham *et al.*, 1989). On the other hand, they can survive for many years in infected tissues that have been kept dry and free of soil (Goto, 1992).

X. axonopodis pv. *citri* persists from one growing season to the next in old lesions, especially those lesions formed late in the growing season (Koizumi, 1977). Bacteria remain viable as long as host cells in the vicinity of the lesion are alive, although the

bacterial population declines rapidly in low temperatures. Under tropical conditions this population drop does not occur, because bacterial growth continues at the margin of old lesions upon the resumption of favourable conditions (Pruvost *et al.*, 2002). Extracellular polysaccharide encapsulates the bacterial cells exuded on to the lesion surface and this aids in dispersal and survival (Fig. 1C; Goto and Hyodo, 1985). The persistence of *X. axonopodis* pv. *citri* for several weeks on non-host plant material and in the root zone of certain grasses under eradicated diseased trees has been reported in Japan (Goto *et al.*, 1975) and Brazil (Pereira *et al.*, 1978). However, there is no direct evidence that cells of *X. axonopodis* pv. *citri*, surviving in low numbers on weed hosts or in the soil, serve as a source of inoculum (Goto *et al.*, 1975). Attempts to detect surviving bacteria on various inanimate surfaces such as metal, plastics, cloth and processed wood in both shade and sun indicate that exposed inoculum dies within 24–72 h (Graham *et al.*, 2000). Once diseased leaves or fruit drop to the ground, the bacterial population declines to a non-detectable level in 1–2 months because of antagonism and competition with saprophytic micro-organisms (Graham *et al.*, 1989).

Leafminer interaction in citrus canker epidemics

Prior to 1994, the citrus leafminer was restricted in distribution to south-east and south-west Asia (Cook, 1988). After the mid-1990s, the leafminer spread to most of the major citrus producing areas of the world and arrived in Florida in 1993 (Heppner, 1993), and was first reported in Brazil in 1996. In South Florida, the interaction between the leafminer feeding and citrus canker was immediately apparent as an exacerbation of disease. The leafminer infests young citrus flush including leaves and young stems. Young fruit, especially grapefruit, are also occasionally attacked. Leafminer larvae form feeding galleries in the epidermal cell layer of young leaves and other tissues, lifting and eventually tearing the cuticle (Fig. 2; Achor *et al.*, 1996). The feeding activities of the leafminer facilitate bacterial infections in three ways: first, the tearing of the cuticle opens the mesophyll of the leaf to direct bacterial infection when splash-dispersed or windblown-rain-dispersed bacteria come in contact with the leaf surface; second, leafminer wounds heal more slowly than mechanical wounds, allowing a longer period of exposure for bacterial infection; third, the leafminer larvae may become contaminated with bacteria and transport them through the feeding galleries. These processes result in a proliferation of mesophyll infections within the galleries (Bergamin-Filho *et al.*, 2000; Graham *et al.*, 1996). As leafminer-induced lesions expand, they rupture through the epidermis, and coalesce to form massive infections covering large areas of the leaf lamina. These lesions generate many times more inoculum compared to stomatal infections. Larger populations of bacteria exuded from leafminer-induced lesions promote spread and accelerate the dispersal process. No evidence has been presented that the adult insect, a moth, is a vector capable of moving the bacterium from plant to plant.

Wounding by leafminer accelerates the spread of citrus canker in Florida and Brazil (Bergamin-Filho *et al.*, 2000; Gottwald *et al.*, 1997, 2002a). The citrus canker epidemic greatly expanded in the State of São Paulo, Brazil after the leafminer was established in 1996. The number of disease foci increased from 25 in 1995 to 4180 in 1999, when the disease was detected in 299 856 trees. Consequently, 1737 545 trees underwent eradication. This rapid increase in the number of diseased trees was accompanied by a change in the spatial pattern of the epidemic. In Brazil, citrus canker exhibited strongly aggregated patterns from 1957 to 1995, whereas, post-1996 the pattern was less aggregated and satellite foci distant from the main focus of disease were common (Bergamin-Filho *et al.*, 2000). The change in the spatial pattern of the presence of the leafminer resulted in a lengthening of the disease gradient, because inoculum exuding from lesions could be picked up by wind-blown rain, disperse over long distances and cause infections. In the absence of leafminer damage to the cuticle, winds must reach 8 m/s to produce the impact force of inoculum-laden droplets sufficient for water congestion of foliage. When the tree canopy is wounded by leafminer, the inoculum can cause infections during rainstorms with relatively low wind speeds.

Dating the initiation of infection

In an eradication programme, it is important to determine how long the disease has been active at a particular site. Because the infection process on perennial citrus plants is well understood and occurs on tissues of a certain developmental stage, the approximate age of the lesions can be estimated (Gottwald *et al.*, 1992, 1997, 2002a,b). If symptoms are only detected on leaves of the most recent flush (the tissues most likely to be infected), the disease will have initiated only a few weeks or months before. On a well-fertilized, susceptible citrus variety in Florida, under environmental conditions conducive for disease, the expansion rate of leaf lesions was estimated to be 1 mm per month for the first 6–8 months. Leaf lesion expansion slows and stops at around this age. The maximum susceptible period of fruit enlargement is typically 90–120 days after fruit set (Graham *et al.*, 1992b), so lesions on enlarging fruit can be dated based on timing of bloom. Twig lesions are generally initiated after leaves and fruit have gone through one or more infection cycles. The appearance of fruit and twig lesions also assumes that an inoculum threshold has been reached on leaves to further advance the disease. Each growth flush leaves a distinctive node on the twig, thus enabling the determination of the age of the twig lesion. Older lesions on larger stems with brown bark can be dated by dendro-chronological methods, keeping in mind that each growth ring records a flush and not an annual ring. One difficulty

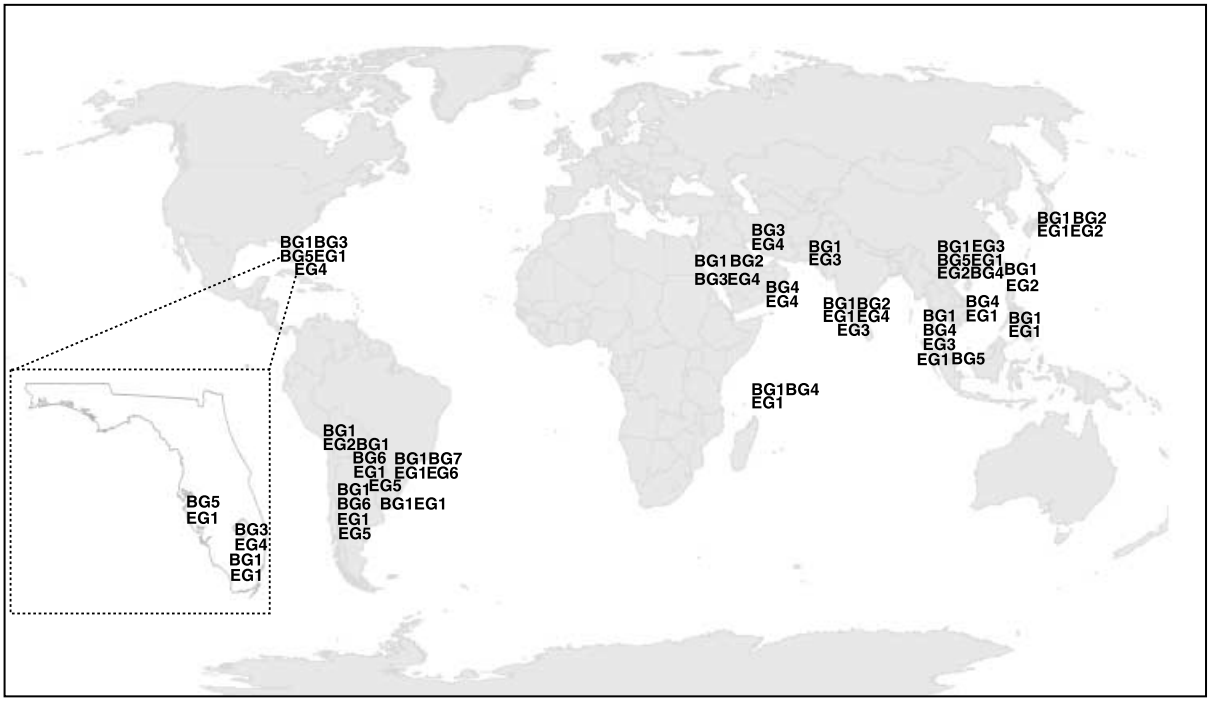

Fig. 3 Genotypes of *Xanthomonas axonopodis* pv. *citri* and *X. axonopodis* pv. *aurantifolia* from world-wide were identified by PCR amplification of repetitive sequences with BOX and ERIC elements. BG and EG genotype subgroups based on cluster analysis from fingerprints obtained after BOX-PCR and ERIC-PCR, respectively (Cubero and Graham, 2002).

is determining the number of flushes that have occurred on that particular diseased plant within the preceding growing season(s). On trees managed for commercial production, lesion age determination is more assured than on a residential tree, with varying levels of horticultural care.

Xanthomonads causing citrus canker diseases

There are distinct types of citrus canker disease caused by various pathovars and variants of the bacterium *X. axonopodis*. Because symptoms are generally similar, the separation of these forms from each other is based on host range and other phenotypic and genotypic characteristics of the strains.

The Asiatic type of canker (Canker A), caused by the Asian strain *X. axonopodis* pv. *citri* (syn. *X. citri*, *X. campestris* pv. *citri*), is by far the most widespread (Fig. 3) and severe form of the disease. This is the strain that causes the disease most often referred to as 'citrus canker'.

Cancrosis B, caused by the B strain, *X. axonopodis* pv. *aurantifolii*, is a minor canker disease of diminishing importance on lemons in Argentina, Paraguay and Uruguay. Mexican lime, sour orange and pummelo are also susceptible. The cancrosis B strain can be easily differentiated from the Canker A strain, but not from the cancrosis C strain.

Cancrosis C, also caused by *X. axonopodis* pv. *aurantifolii*, was isolated in the 1970s from Mexican lime in Sao Paulo State, Brazil, but has rarely been encountered since then. The only other known natural host for this bacterium is sour orange.

Other forms of canker bacteria have been reported at times. Isolates such as A* were discovered in Oman, Saudi Arabia, Iran and India that produce Canker A-like lesions only on Mexican lime and that appear to be distinct from the common A strains (Vernière *et al.*, 1998; Mohammadi *et al.*, 2001). Minor genetic variations in citrus canker strains have been detected in the A strains in Florida and other citrus growing regions of the world, which may be exploited to identify their origin when introduced into new locations. For example, a newly described A strain variant in Florida, A^w, appears to come from south-west Asia (see below).

In 1984, a leaf spot of a rootstock cultivar, Swingle citrumelo (*Poncirus trifoliata* × *Citrus paradisi*), discovered in citrus nurseries in Florida was associated with a xanthomonad. Although the lesions on leaves, stems and fruits were not raised and canker-like in aspect, the new disease was called 'nursery canker' and the causal bacteria classified as *X. axonopodis* pv. *citri* strain type E (Schoulties *et al.*, 1987). Later the disease was renamed 'citrus bacterial spot' and the bacterium reclassified as *X. axonopodis* pv. *citrumelo* (Gabriel *et al.*, 1989; Graham and Gottwald, 1991; Graham *et al.*, 1990a,b).

Host range of canker A strain

The host range of *X. axonopodis* pv. *citri* A strain is broad, encompassing many citrus species and hybrids between citrus species and the citrus relative trifoliate orange *Poncirus trifoliata* (Gottwald *et al.*, 1993; Graham *et al.*, 1990a; Leite and Mohan, 1984). Among citrus cultivars and rootstocks, Asiatic citrus canker is most severe on grapefruit, some sweet oranges such as Hamlin, Pineapple and Navel, Mexican (Key) lime, and the hybrids of trifoliate orange used for rootstocks. These cultivars have proven very challenging or impossible to grow profitably in the presence of citrus canker in moist subtropical and tropical climates (Graham, 2001; Leite and Mohan, 1984). All other commercial cultivars of citrus, although varying in susceptibility, are susceptible enough that they must be removed in an eradication effort when they are diseased or exposed, especially where the citrus leafminer occurs. Civerolo (1984) lists a number of plants in the Rutaceae other than *Citrus* and *Poncirus* that can serve as hosts of *X. axonopodis* pv. *citri* under experimental conditions. These plants would not be expected to play any significant role in epidemiology where the disease is endemic and leafminer is absent, but serve as troublesome inoculum reservoirs in an eradication programme.

Structural and cellular aspects of host resistance

Two fundamental host determinants for citrus canker are the stage of leaf expansion and the resistance of mesophyll tissue (Gottwald and Graham, 1992; Graham *et al.*, 1992a). The susceptibility of tissues to bacterial ingress is greatest when leaves are one-half to two-thirds expanded, a stage of leaf development at which stomates open, but the leaf cuticle is not fully developed (Fig. 4). At this stage, leaves are most prone to water soaking. As the leaves continue to expand, the cuticle rapidly thickens (Fig. 4) and the forces required for water to infiltrate tissue increases dramatically (Graham *et al.*, 1992a). Concurrently, the mesophyll cell layer underlying the cutinized epidermis is undergoing an increase in resistance to bacterial establishment and growth (Stall *et al.*, 1982a). Lower bacterial populations develop in mesophyll tissues of less susceptible cultivars for both *X. axonopodis* pv. *citri* A strain and *X. axonopodis* pv. *citrumelo* (Fig. 5). In tissues of the susceptible host grapefruit, the A strain reaches its greatest populations 168 h following inoculation, at which time the bacterium is still growing in the intercellular spaces and causing cell disruption (Fig. 6A,B,C). Although grapefruit are considerably more resistant to citrus bacterial spot, *X. axonopodis* pv. *citrumelo* initially grows more rapidly than the A strain up to 72 h (Fig. 5). As the populations peak, *X. axonopodis* pv. *citrumelo* is enveloped by fibrils in the intercellular spaces and the population begins to slowly decline (Fig. 6D,E). In contrast, *Pseudomonas syringae* pv. *syringae* causes a hypersensitive-like reaction at the site of stomatal infection. Black pit is the fruit symptom and citrus

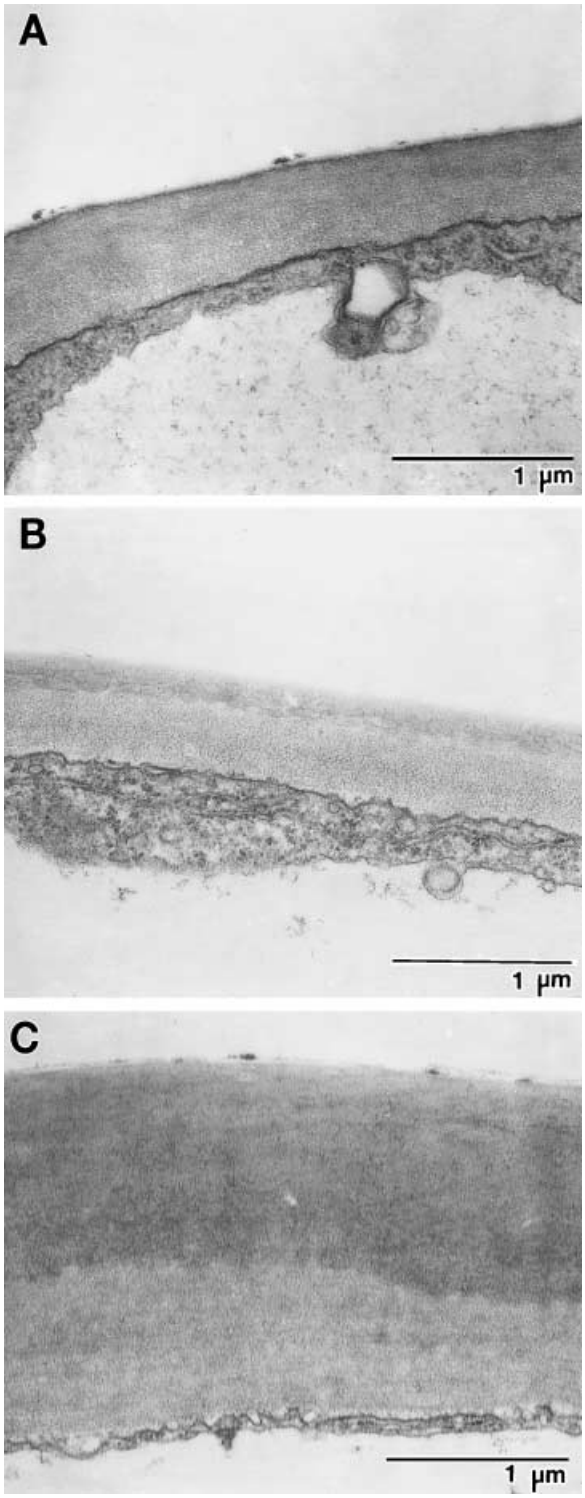

Fig. 4 Transmission electron microscopy of the abaxial leaf surface of grapefruit (*Citrus paradisi*) at various stages of expansion of immature leaves. (A) For leaves at 50% of full expansion, the cuticle is undeveloped on the surface of the epidermis cell wall. (B) For leaves at 75% expansion, the cuticle layer is approximately 15% of the thickness of fully expanded leaves. (C) The cuticle layer is completely developed on fully expanded leaves.

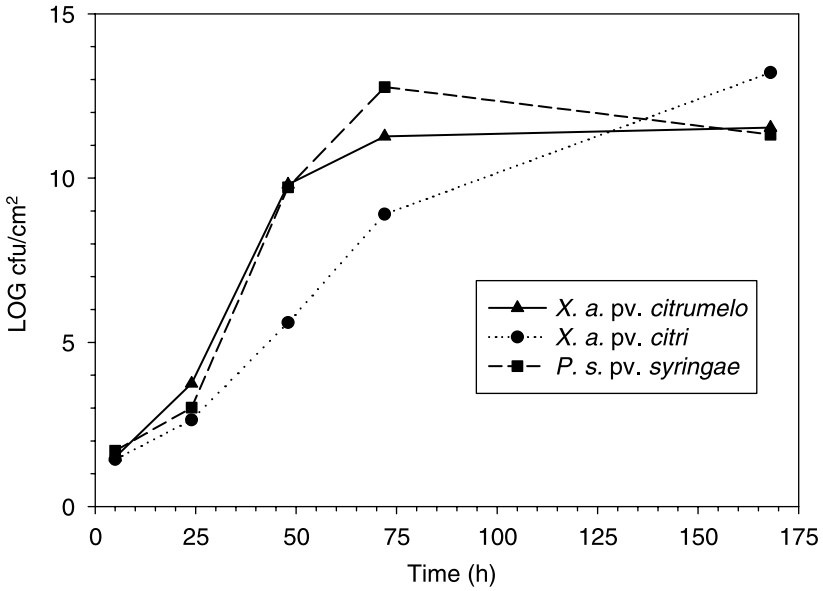

Fig. 5 Growth of *Xanthomonas axonopodis* pv. *citri*, *X. axonopodis* pv. *citrumelo* and *Pseudomonas syringae* pv. *syringae* in two-thirds expanded leaves of grapefruit (*Citrus paradisi*) after infiltration of tissues with 10^5 cfu/mL. Populations are expressed as log cfu/cm^2 of leaf surface.

blast is the leaf/twig symptom (Timmer *et al*., 2000). This bacterium grows more rapidly than the A strain (Fig. 5) and the lysis of cells adjacent to bacteria in the intercellular space occurs within 48 h (Fig. 6F).

Hosts with field resistance to strain A, such as mandarins, support populations 1–2 log units lower than in susceptible hosts (Graham *et al*., 1992a). Hence, an evaluation of mesophyll tissues in screening germplasm for resistance to citrus canker is considered of utmost importance (Gottwald *et al*., 1993).

Diagnosis of canker

X. axonopodis pv. *citri* is readily isolated from young lesions on fruit, leaves and stems, but bacterial populations decrease in older tissues, increasing the difficulty of recovery. Different media have been used for isolation, but KCB (kasugamycin-cephalexin-Bravo) semi-selective medium is most useful for isolating the *X. axonopodis* pv. *citri* from plant material (Graham and Gottwald, 1990). *X. axonopodis* pv. *aurantifolii* grows best on a modified nutrient agar medium for isolation and culture from citrus tissues (Canteros *et al*., 1985). Other yellow-pigmented bacteria may be isolated from citrus tissues, some of which belong to the genus *Xanthomonas* (Graham *et al*., 1990a,b). For disease diagnosis, bacterial isolates from plant material should be confirmed by inoculations of leaves in grapefruit and Mexican lime for A strains, lemon for B strains and Mexican lime for C strains, to reproduce the canker lesion phenotype.

Polymerase chain reaction (PCR) methods have been developed for the rapid and accurate identification of the bacterium isolated in culture and from extracts of lesions on leaves and fruits (Cubero *et al*., 2001; Hartung *et al*., 1993). The primers which were first used to detect and identify citrus canker strains were based on a plasmid sequence (Hartung *et al*., 1993; Hartung, 1992). However, these primers failed to detect the A strain variant, A^w, which was recently discovered in Palm Beach County, Florida. The A^w strain induces canker symptoms on a restricted range of citrus hosts, including Mexican lime and alemow (*C. macrophylla*) (Sun *et al*., 2000). New universal primers based on the *pth* gene, the primary virulence element in all citrus canker strains (Yang and Gabriel, 1995), are now available for the detection of all canker strains in Florida and elsewhere (Cubero and Graham, 2002).

Another approach for producing universal primers for canker producing strains utilizes specific sequences in the intergenic spacer (ITS) regions of 16S and 23S ribosomal DNAs. Variation in the ITS sequences allows the design of specific primers for A strains, to identify the A^w as an A strain, and to readily differentiate the A^w strain from the B and C strains of *X. axonopodis* pv. *aurantifolii*, even though these strains have a very similar host range (Cubero and Graham, 2002).

The routine application of PCR for the detection of plant pathogens in plant tissue is restricted by the presence of inhibitors that interfere with amplification of the target sequence (Wilson, 1997). This condition may result in a false negative diagnosis when the pathogen is actually present. The fidelity of the PCR amplification for identification of citrus canker is confirmed by the addition of an internal sequence that is complementary to a set of primers designed to amplify a specific region of the *X. axonopodis* pv. *citri* genome (Cubero *et al*., 2001). This internal control sequence, with primers designed by Hartung *et al*. (1996) and with those more recently described for *pthA* gene detection (Cubero and Graham, 2002) ensures that at least one product is obtained after PCR. The addition of this plasmid also permits estimation of the initial bacterial concentration in citrus tissues by competitive PCR (Cubero *et al*., 2001). Quantification by this

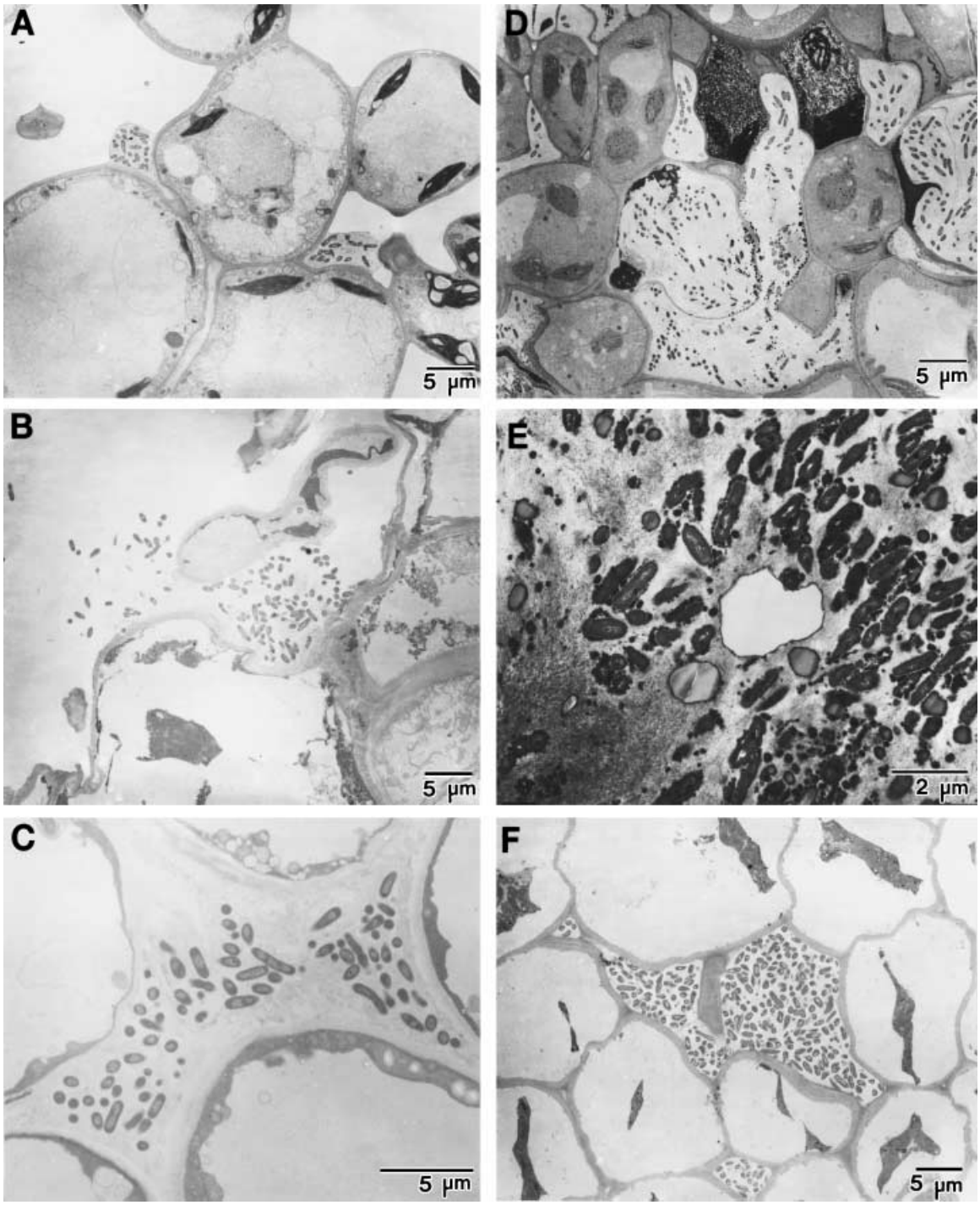

Fig. 6 Transmission electron microscopy of the mesophyll cells of grapefruit (*Citrus paradisi*) or Swingle citrumelo (*Poncirus trifoliata* × *C. paradisi*) at various times after infiltration of two-thirds expanded leaves with 10^5 cfu/mL of *Xanthomonas axonopodis* pv. *citri*, *X. axonopodis* pv. *citrumelo* or *Pseudomonas syringae* pv. *syringae*. (A) At 7 days after inoculation of susceptible grapefruit, *X. axonopodis* pv. *citri* is growing in the intercellular space in a matrix of extracellular polysaccharide (EPS). Adjacent host mesophyll cells are vacuolated but not disrupted. (B) At 14 days after inoculation of susceptible grapefruit, *Xanthomonas axonopodis* pv. *citri* cells are released from the EPS matrix. Adjacent host mesophyll cells are hypertrophied and collapsing. (C) At 7 days after inoculation of susceptible Swingle citrumelo, *X. axonopodis* pv. *citrumelo* cells have actively divided in the EPS matrix. (D) At 7 days after inoculation of resistant grapefruit, *X. axonopodis* pv. *citrumelo* has proliferated in mesophyll cells, and some adjacent cells are filled with an electron-dense material. (E) At 14 days after inoculation of resistant grapefruit, *X. axonopodis* pv. *citrumelo* cells are occluded in an electron dense, fibrillar matrix. (F) At 2 days after inoculation of hypersensitive grapefruit, *P. syringae* pv. *syringae* has proliferated in the intercellular space and the contents of mesophyll cells have lysed.

competitive PCR method is based on the accumulation of two PCR products from the target sequence and the internal control after a fixed number of cycles. The ratio of the amplified product generated from the target sequence to the product of the internal control is related to the bacterial concentration in the sample (Cubero *et al*., 2001).

Real time PCR has also been applied for quantitative PCR and for the rapid, on-site identification of bacteria in plant material (Mavrodieva *et al*., 2002; Schaad and Frederick, 2002). Real time PCR is not based in the accumulation of amplicons at the end of the reaction, but fluorescence detection of the point in time when amplification of the target is initiated. Approaches using of SYBR® green dye and Taqman® probes have been evaluated in conjunction with primers based on sequences from the *pth* and ribosomal genes (Cubero and Graham, 2002), as well as on a gene for the leucine responsive regulatory protein (*lrp*) (Cubero and Graham, 2003). In addition, protocols for Real Time PCR that include an internal control for *pth* primers have been developed. After a reaction using SYBR® green dye is performed, a dissociation curve is used to obtain the relative amount of PCR products from the target sequence compared to the internal control (Cubero and Graham, unpubl. results).

Identification of canker strain genotypes

Primers for rep-PCR with BOX and ERIC elements are used to separate strain types and to differentiate strains within the same pathotype (Cubero and Graham, 2002; Louws *et al*., 1999). This methodology is applied to evaluate the diversity of *Xanthomonas* strains causing canker in Florida and to relate these strains to isolates in a world-wide collection, to possibly establish their geographical origin (Cubero and Graham, 2002). rep-PCR confirms the inclusion of A^w strains from Palm Beach County among the A strains, and reveals diagnostic genotype differences among A strains in different geographical areas of Florida. This genotyping also confirms that isolates of the original outbreak in Manatee County in 1986, an infestation that was supposedly eradicated in 1994, are of the same genotype that re-emerged in 1996 (Cubero and Graham, 2002; Schubert *et al*., 2001). The specific fingerprint for the MA genotype of the A strain from Manatee County, Florida also matches that of unique strains from China and Malaysia (Fig. 3). The A strain genotype from the Miami (MI) outbreak matches strains from several geographical areas of the world including south-east Asia and South America. The MI genotype has been detected in several locations in south and central Florida and links the movement of plant material and other human activities to the Miami metropolitan area. The close relationship between the A^w the A* strains suggests that a common origin of these strains is south-west Asia. Overall, genotyping with rep-PCR validates the existence of at least three separate A strain introductions in Florida in the last 20 years (Cubero and Graham, 2002)

Importance of accurate canker strain identification

Although *X. axonopodis* pv. *citri* causes characteristic lesions in fruit, leaf and stem, reliable methods for pathogen detection, accurate identification of the symptoms and causal bacterium are crucial in a quarantine situation. Quarantine restrictions resulting from misdiagnosis of leaf spotting diseases as citrus canker may have far-reaching effects on the export of fruit from the affected areas to local and overseas markets. In 1981, a leaf and twig-spotting disease on Mexican lime in Colima, Mexico was named 'citrus bacteriosis' and the causal agent identified as *X. axonopodis* pv. *aurantifolii*, based on biochemical, serological and pathogenicity tests (Rodriguez *et al*., 1985). The disease lacked fruit symptoms and there were no leaf symptoms on grapefruit in adjacent orchards. The causal agent has now been identified as *Alternaria limicola* and the disease is known as 'mancha foliar de los citricos', or 'Alternaria leaf spot' (Palm and Civerolo, 1994; Timmer *et al*., 2000).

In 1984, a leaf spot of Swingle citrumelo discovered in citrus nurseries in Florida was associated with a xanthomonad and thought to be a new form of citrus canker (Schoulties *et al*., 1987). This action was taken partially in deference to the nomenclatural system for pathovars that assigns the name based on the host from which the bacterium was first isolated. The lesions on tissues from this leaf spot are never raised like lesions of other canker diseases and the bacterium was later determined to be distantly related to canker-producing strains by DNA homology and various RFLP analyses (Graham and Gottwald, 1991). The disease was named 'citrus bacterial spot' and the bacterium reclassified as *X. axonopodis* pv. *citrumelo* to clearly separate this minor, and most likely indigenous pathogen restricted to citrus nurseries (Gabriel *et al*., 1989; Graham and Gottwald, 1991; Graham *et al*., 1990a,b).

In both cases, before the causal agents were thoroughly characterized, the diseases caused serious economic losses through the destruction or quarantine of nursery trees and embargos on fruit movement.

Classification of Xanthomonads causing canker diseases

The taxonomy and classification in the genus *Xanthomonas* is constantly undergoing revision because of phytopathogenic diversity and continues to be controversial (Schaad *et al*., 2000; Vauterin *et al*., 2000). Genetic studies of xanthomonads causing citrus canker and citrus bacterial spot support the distinct symptomatology and histopathology of these diseases. Most techniques reveal that citrus bacterial spot is caused by a heterogeneous group of strains, while groups of canker strains are quite uniform genetically (Graham and Gottwald, 1991; Stall and Civerolo, 1991).

In the late 1980s, strains associated with canker A were proposed as a new species, *Xanthomonas citri*, whereas types B and C, as well as strains causing citrus bacterial spot remained within *X. campestris* as pathovars *aurantifolii* and *citrumelo*, respectively (Gabriel *et al*., 1989). Schaad *et al*. (2000) proposed a reclassification that places citrus canker and citrus bacterial spot strains within *Xanthomonas* as species *citri* (A strains), *aurantifolii* (B and C strains) and *citrumelo* (citrus bacterial spot strains). However, other authors rejected this new proposal, citing insufficient data to justify the removal of these strains from the species *axonopodis* (Vauterin *et al*., 2000; Young *et al*., 2001). Most recently, Brunings and Gabriel (2003) proposed the retention of *X. citri* as the species that includes only citrus canker strains (A and B–C). This was proposed to prevent further abuse of placing other citrus-associated xanthomonads in pathovar designations on the basis of 'host from which first isolated', as occurred with citrus bacterial spot strains in Florida nurseries.

Recent analysis in our laboratory of a transcription regulator gene, the leucine responsive regulatory protein (*lrp*) confirms a close relationship of all citrus xanthomonads with other host specific strains in the species *X. axonopodis* such as those affecting tomato, pepper, cotton, ficus and dieffenbachia (Cubero & Graham, 2003). Comparative analysis of *lrp* gene also discriminates among A, B and C types of citrus canker strains and differentiates narrow (A* and A^w) and wide host range A strains (Cubero & Graham, 2003).

Analysis of the *lrp* gene furthermore reveals that A strains are most closely associated with strains of *X. axonopodis* pv. malvacearum that affect cotton, confirming the results obtained by other techniques (Egel *et al*., 1991), while *Xanthomonas* producing citrus bacterial spot are more related with strains affecting dieffenbachia, ficus, tomato or pepper. These results suggest that citrus bacterial spot, as well as other xanthomonad diseases cited above, resulted from the adaptation of very closely related xanthomonad strains to a different host. In contrast, citrus canker would be considered a host specific disease caused by strains containing *pthA* pathogenicity genes that are differentially adapted to *Citrus* spp. (Brunings and Gabriel, 2003). This idea is supported by the fact that citrus bacterial spot is a disease caused by a diversity of strains with differential aggressiveness that represent a heterogeneous group, while the citrus canker group displays a higher homology among its members (Egel *et al*., 1991).

Analysis of the *lrp* gene also shows the closer relationships that exist among pathovars in *X. axonopodis* as compared with *X. campestris*, supporting the most widely accepted reclassification of the genus (Vauterin *et al*., 2000).

Recently, the genomes of *X. campestris* and *X. axonopodis* pv. *citri* have been sequenced (da Silva *et al*., 2002). In a whole-chromosome alignment, 70% of the total genes were orthologous and suggested a common ancestor. Only 15.4% of the genes in *X. campestris* were not present in *X. axonopodis* pv. *citri* and 18.5% of the genes in *X. axonopodis* pv. *citri* were not found in *X. campestris* (da Silva *et al*., 2002). Because the use of different techniques revealed a higher homology of *X. axonopodis* pv. *citri* with other *Xanthomonas* as compared with *X. campestris*, it is expected that a higher percentage of complete genome homology would also be encountered with other *Xanthomonas* currently included in the species *axonopodis*. Genomic analysis of strains very closely related to *X. axonopodis* pv. *citri* and pv. *aurantifolii*, currently in process, will permit a deeper understanding of not only the phylogenetic relationships among different *Xanthomonads*, but the evolution of different diseases and molecular mechanisms of pathogenesis. Brunings and Gabriel (2003) provided a detailed discussion of the completed sequence's potential for testing additional hypotheses for pathogenicity and regulatory genes present in the genome.

Epidemiology of citrus canker

Opportunities to evaluate the epidemiology of the disease in Florida have been infrequent because citrus canker is exotic and under eradication. However, the rapid assessment of a disease epidemic in 1990 in an orchard in south-central Florida provided the first documented spread of citrus canker over longer distances associated with thunderstorms (Gottwald *et al*., 1992). An August 1989 thunderstorm, with high winds and heavy rainfall, resulted in the dissemination of inoculum and the establishment of four foci of infection in the orchard that ranged from 230 to 810 m from the infected source trees.

No regional studies of citrus canker spread in urban environments were conducted until the largest outbreak of citrus canker in US history extended through urban Dade and Broward counties (Metropolitan Miami and Fort Lauderdale) of Florida. When the outbreak was first discovered, the disease was limited to a 32 km^2 area south-west of the Miami International Airport. In January 1996, a severe rainstorm with tornadoes passed through this infected area on a south-west to north-east track. By midsummer of 1996, canker had spread 9.6–11.2 km to the north-east and encompassed a 223 km^2 area (Gottwald *et al*., 1997, 2001). Such storms occur when fronts stretch diagonally across Florida with prevailing winds to the north-east along the frontal boundary. Weather-driven spread and occasional human movement have both contributed to the continual expansion of the citrus canker epidemic northward up the east coast through Florida's most dense residential area and southward into the commercial lime growing areas below Miami.

Until recently, the scientific basis for the eradication effort of citrus canker was a study in Argentina that documented bacterial dispersal up to 38.1 m during rainstorms associated with wind (Stall *et al*., 1980). In Florida, this finding became the basis for regulatory policy for the destruction of presumptive 'exposed trees' within a 38.1 m (125 ft) radius of a diseased tree (Schubert

et al., 2001). In São Paulo State, Brazil a distance of 30 m, was used to define exposed trees in a canker eradication program similar to Florida's. Despite application of the '125 ft rule' by the Florida's Citrus Canker Eradication Program (CCEP), the disease outbreak continued to increase size in south-east Florida urban areas and the bacterium spread to numerous commercial citrus across south Florida (Schubert *et al*., 2001). There was rising concern that the '125 ft rule' was insufficient for eradication in an urban setting. To address these concerns, a cooperative research study CCEP, USDA-Agricultural Research Service, and University of Florida was established in August 1998.

The study was conducted in five areas in Miami to measure the distance of dispersal of A strain and to provide a biologically sound basis for defining the radius of exposure of trees to citrus canker under urban conditions (Gottwald *et al*., 2002a,b). Distances between each newly diseased tree and all prior focal trees were calculated and the maximum distances of spread ranged from 12 to 3474 m, indicating a broad continuum of distance for bacterial spread was possible (Gottwald *et al*., 2002a,b; Schubert *et al*., 2001).

The results of this study were examined by a group of scientists, regulators and citrus producers familiar with the disease. Based on measurements of disease spread, they selected a distance of 1900 ft (579 m) as a radius that would encompass the majority of newly infected trees resulting from a prior infection focus infection that can occur within a 30 day period. The study and the resulting determination of the 579 m distance served as the scientific basis of the removal of exposed trees around foci of infection practised in Florida at this time and was placed into a law passed by the Florida legislature in May 2002 (Gottwald *et al*., 2002a,b).

A few south Florida residents requested an administrative hearing to halt the eradication programme that they felt was protecting commercial citrus at the expense of residential trees. A Broward County Circuit Court Judge placed an injunction on eradication of non-infected citrus in residential areas in May 2002, essentially halting the eradication programme in urban south Florida for 8 months until this ruling was overturned by the 4th District Court of Appeals in January of 2003. Subsequent legal challenges to eradication have not resulted in further injunctions. The case will now be heard by the Florida Supreme Court in an attempt to finalize the dispute and relieve the lower courts of numerous legal challenges.

Integrated approaches for prevention and control of citrus canker

Countries where citrus canker does not occur, or has been eradicated, rely on quarantine measures to prevent the introduction and establishment of *X. axonopodis* pv. *citri*. Historically, citrus canker has been introduced from Asian countries into the USA, South Africa, New Zealand and Australia. In newly established outbreaks, programmes that started immediately are successful in the eradication of citrus canker (Broadbent *et al*., 1992). Although many citrus producing countries prohibit the importation of plant material from canker-endemic areas, outbreaks continue to occur in new areas of Florida, South America and Australia (Broadbent *et al*., 1992; Schubert *et al*., 2001).

In urban areas, under eradication, diseased and potentially exposed trees within 579 m are removed and destroyed. Trees are cut down by chainsaw, and the debris moved to the street where commercial wood chipping machinery reduces the trees to pieces generally < 10 cm in size. The 'chipped' debris is exhausted into a covered trailer, transported to a landfill, dumped, and covered with soil. Some fine particles escape the covered trailers during the chipping process and when the trailers are emptied at the landfill. Air sampling experiments have been conducted to test the escaping debris for viable *X. axonopodis* pv. *citri* bacteria. In a few cases, when some infected trees were chipped, a few cells were detected in the escaping debris. Similar studies at landfills detected viable cells in the particulate matter escaping downwind from trucks dumping chipped material (Gottwald *et al.* unpublished). The duration of survival of the bacteria in such debris has not been tested. However, the lack of susceptible citrus within 579 m of any infected tree in an urban setting and within much greater distances of any landfill, makes the significance of such bacteria-laden debris for further spread of the disease questionable. Certainly if infected trees were allowed to remain in an area, the inoculum they would produce and disperse within a very short time would vastly outnumber those few viable cells that escape during tree destruction and disposal.

In regions where *X. axonopodis* pv. *citri* is endemic, integrated control measures rely heavily on the planting of resistant varieties of citrus. In south-east Asia, where climatic conditions are favourable for epidemics, the dominant cultivars grown are mandarins. Citrus canker will not be a serious problem until more susceptible sweet oranges are introduced into disease prone areas of Japan and China (Kuhara, 1978). In Brazil, eradication programmes have been ongoing since the 1950s to control the spread of *X. axonopodis* pv. *citri* across the largest sweet orange production area in the world: São Paulo State. In contrast, nearby regions of Paraná State, Brazil and Misiones and Corrientes, Argentina have practised an integrated programme for the prevention and control of citrus canker in sweet oranges (Leite and Mohan, 1990). Guidelines specify management practices for citrus canker, and the marketing of fresh fruit and nursery stock: (i) nurseries are only to be located in areas free of citrus canker; (ii) orchard production areas are designed to prevent or reduce the risk of citrus canker epidemics through the establishment of windbreaks, the use of preventive copper sprays, construction of fences to restrict the access to the orchard; (iv) disinfection sprays are applied for orchard machinery and harvesting equipment, and orchard workers comply with measures for thorough disinfection

of clothes, shoes and gloves; and (v) Fresh fruit for internal and export markets is subject to rigorous inspection protocols for freedom of citrus canker symptoms on fruit in orchards and sanitation treatments in the packing-house.

Field screening has been conducted world-wide to evaluate the reaction of varieties to citrus canker under local environmental conditions (Koizumi, 1985; Leite and Mohan, 1990). Due to their high susceptibility, grapefruit, Mexican lime, and several early midseason sweet oranges (e.g. Navel, Hamlin) are not recommended for planting, unless very intensive control programmes are to be undertaken (Leite and Mohan, 1990). Screening programmes have identified selected mid- and late-season sweet oranges, mandarin hybrids (tangerines, tangelos, tangors) and Tahiti lime with an acceptable level of resistance to citrus canker. These cultivars may be susceptible in the young stages and require sprays for control of citrus leafminer to prevent damage to emerging leaf flushes that predisposes them to infection. Adult trees flush less frequently, reducing leafminer activity to an acceptable level of resistance that allows for effective disease management through an integrated programme, including windbreaks and chemical control (Gottwald and Timmer, 1995; Leite and Mohan, 1990).

Copper-based bactericides are a standard control measure for citrus canker world-wide (Koizumi, 1985; Leite and Mohan, 1990). Copper reduces bacterial populations on leaf surfaces, and multiple applications are needed to achieve adequate control on susceptible hosts (Stall *et al.*, 1980). Copper-based spray programmes are effective when targeted to the spring leaf flush to protect leaves from the one-half to full expansion stage over a period of 2–4 weeks (Graham *et al.*, 1992a; Stall *et al.*, 1982b). Fruit are susceptible as they grow from 2.0 to 6.0 mm in diameter for a period of 90–120 days, depending on citrus species (Graham *et al.*, 1992b). When the incidence of canker infection on spring leaves is reduced, the subsequent infection of fruit is reduced, provided that the treatments are repeated during the summer months as the fruit continue to expand (Kuhara, 1978; Stall *et al.*, 1982b). Since copper diminishes infection by contact effect on bacteria on surfaces, the effectiveness of copper spray programmes is overcome by rains with wind that introduce bacteria directly into stomates (Gottwald and Graham, 1992; Gottwald and Timmer, 1995). In addition to their partial effectiveness under wind-blown rain conditions, copper bactericides have other possible disadvantages after long-term use, including resistance to copper in xanthomonad populations (Rinaldi and Leite, 2000) and the accumulation of copper metal in soils with potential phytotoxic and environmental effects (Alva *et al.*, 1995). Other contact bactericides tested, including antibiotics, are not as effective as copper-based products (Leite and Mohan, 1990; Timmer, 1988) and the development of antibiotic resistance within xanthomonad populations has occurred (Ritchie and Dittspongpitch, 1991).

Induced systemic resistance (ISR) is the process of active resistance dependent on the host plant's physical or chemical barriers, activated by biotic or abiotic inducing agents (Kessmann *et al.*, 1994). Such resistance is active against many organisms, including bacteria and fungi. A wide range of compounds, such as benzothiadiazoles, salicylic acid and harpin protein, are known to be effective inducers of plant resistance to diseases (Romero *et al.*, 2001; Wei *et al.*, 1992). Several mechanisms for ISR may operate simultaneously to control the disease, reducing the risk of development of pathogen resistance (Tally *et al.*, 1999). The compounds acibenzolar-S-methyl, a benzothiadiazole, registered as 'Actigard' in the USA and 'Bion' in Europe and South America (Syngenta Crop Protection), and harpin protein, a *hrp* gene product registered as 'Messenger' (Eden Bioscience) are marketed for the control of certain xanthomond diseases. ISR activity could potentially be deployed early in the season to slow bacterial growth in rapidly developing leaves to complement the protectant activity of Cu. However, early season sprays of ISRs in combination with copper have not proved effective for the control of citrus canker in Southern Brazilian orchards wherein copper is moderately to highly effective (Graham and Leite, unpubl. data).

CONCLUSION

In the last 90 years, scientific, industry and regulatory personnel world-wide have made recommendations to eradicate citrus canker, based on cost/benefit ratios for the planned actions (Broadbent *et al.*, 1992; Schubert *et al.*, 2001). Compared to 'living with canker', the calculations clearly indicate that eradication is a wise choice if the disease is caught early and the action is taken swiftly and decisively (Muraro *et al.*, 2000). However, cost estimates of an eradication programme become difficult to predict if survey, detection, and/or control are delayed for any reasons: including grower/public resistance, legal actions, or financial limitations (Gottwald *et al.*, 2001). Moreover, the cost associated with the lost revenues due to legislated quarantines of fresh fruit by other citrus producing states and countries, also defies accurate quantification. Decisions guiding programmes to eradicate are made by specific advisory committees consisting of industry representatives, citrus production experts, plant pathologists, regulators and sometimes, ordinary citizens (Schubert *et al.*, 2001). In scientific circles, the concept of plant disease eradication has lost credibility because of the tendency to underestimate the costs and overestimate the benefits. Although much is now known about the behaviour of citrus canker in Florida, overwhelming non-scientific issues remind us that an effective eradication programme depends heavily on voluntary compliance and cooperation of the regulated parties. Only a citrus industry and general public well informed of the scientific basis for eradication is prepared to accept the onerous aspects of an eradication effort.

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