Copper sensitivity in Tasmanian populations of the walnut blight pathogen *Xanthomonas arboricola* pv *juglandis*

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Statement

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Abstract

Walnut blight, caused by *Xanthomonas arboricola* pv *juglandis* (*Xaj*), is a major concern for the walnut industry world wide, including Australia. The disease has been responsible for significant yield loss of more than 50%, especially in early leafing varieties of walnut in Tasmania. The disease causes necrotic lesions on all above ground parts of the tree that are green and succulent. Walnut blight is currently controlled in Tasmania by the application of copper containing bactericides as protective sprays at 7-14 days intervals from bud burst. Copper is sometimes applied up to ten times per growing season, and this spray program does not always provide a satisfactory level of disease control.

The major objective of this study was to estimate the range of copper sensitivity among a broad sample of *Xaj* isolated from blight lesions on walnut fruit from Tasmania. To achieve this objective, the identity of each bacterial isolate was determined by their phenotype on semi-selective media and by analysing their fatty acid profile by GC-FAME (Gas Chromatograph Analysis of Fatty Acids Methyl Esters). The identity and genetic variation among bacterial isolates was investigated further by application of a DNA fingerprinting technique called rep-PCR.

According to GC-FAME analyses, 32 of 45 isolates of bacteria from symptomatic walnut fruit appeared to be *Xanthomonas arboricola.* The frequent isolation of *Xanthomonas arboricola* from blight lesions on walnut fruit suggested that this organism was associated with the disease. The phenotypic characters of 67 strains of bacteria isolated from symptomatic walnut fruit were studied on two semiseletive media: Brilliant Cresyl Blue Starch (BS) medium and Succinate Quinate (SQ) medium. The semi-selective nature of these media was revealed because some non Xanthomonads gave a positive reaction on both media and two isolates of *Xanthomonas,* putatively *X. arboricola*, did not appear to metabolise quinate on SQ media. Genetic variation was observed among isolates of *Xaj* from Tasmania and there was limited evidence to suggest these isolates could be distinguished from non-Xanthomonads using rep-PCR.

Copper sensitivity was quantified for 37 isolates of *Xanthomonas arboricola* (putatively *Xaj*), isolated from blight lesions on walnut fruit from commercial orchards and home gardens in Tasmania. Each bacterial isolate was exposed to copper sulphate *in vitro*, at varying concentrations up to 1.2 mM or 76 μg Cu^{2+}/ml of liquid medium. All three isolates of *Xanthomonas arboricola* from home gardens and five isolates from commercial walnut orchards were highly sensitive to copper, with strain mortality occurring at ≤ 0.2 mM copper sulphate. Application of a quadratic model to the copper dose response curve enabled estimation of the lethal dose of copper sulphate for 50% mortality of bacterial cells (LD 50) and the minimum lethal dose (MLC). At least two isolates of *Xanthomonas arboricola* from commercial walnut orchards were considered copper tolerant with an MLC that was greater than 0.5 mM or 32 μg Cu^{2+}/ml copper sulphate. This is the first report of copper tolerant strains of the walnut blight bacterium from Australia and the southern hemisphere. The results highlight the urgent need to reduce inputs of copper in commercial walnut production by development of more sustainable solutions for managing bacterial blight.

Literature review

1.1 Introduction

Walnut blight, incited by the bacterium *Xanthomonas arboricola* pv *juglandis* (*Xaj*, Vauterin *et al.*, 1995), is one of the most important and widespread diseases of Persian (English) walnut (*Juglans regia* L.). The bacterium causes leaf, twig and nut blight and most commercial cultivars of walnut are susceptible to blight disease (Murlean and Schroth, 1981; Adhikari *et al.*, 1988; Tamponi and Donati, 1990). Walnut blight is very destructive, particularly in locations that experience multiple rain events during the growing season. Disease can cause a yield loss which might exceed 50% in a severe blight year if left untreated (Olson *et al.*, 2002). The extent of disease severity depends on the frequency and amount of rainfall, the time of leafing (early or late) of the cultivar and recent history of disease occurrence in the orchard (Olson *et al.*, 1997). The traditional way of controlling blight disease is by application of copper containing bactericides. They may be applied from one to ten times depending up on the length of the rainy season (Polito *et al.*, 2002). Frequent use of copper over years has contributed to the development of copper tolerance among populations of the walnut blight pathogen (Lee *et al.*, 1993). Bacteria tolerant of copper are present in walnut orchards throughout northern California (Lee *et al.*, 1994). Copper resistant strains of *Xaj* have also been isolated from walnut orchards located in southwestern and southeastern areas of France (Garden *et al.*, 1993).

1.2 Taxonomy

Taxonomic Position

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Xanthomonadales

Family: Xanthomonadaceae

Pierce (1901) gave the first technical description of the disease and named its causal organism *Pseudomonas juglandis*. The name was then changed to *Bacterium juglandis* (Pierce) Smith in 1905. In 1930, a committee of the society of American bacteriologists reclassified it as *Phytomonas juglandis* (Pierce) Bergey *et al.*, and in 1939 it was named *Xanthomonas juglandis* (Pierce) Dowson. With the introduction of the pathovar concept to plant pathology, Dye (1978) proposed the name *Xanthomonas*

campestris pathovar *juglandis* (Pierce) Dye, which was officially accepted according to international standards for naming pathovars of phytopathogenic bacteria (Dye *et al.*, 1980). Vauterin brought the pathogen within a new species, *Xanthomonas arboricola* pv *juglandis*, which is the last proposal (Vauterin *et al.*, 1995; Vauterin *et al.*, 2000; Rademaker *et al.*, 2005). This name has been adopted by most European researchers, although the name *Xanthomonas campestris* pv *juglandis* is still being used by American authors. The name *Xanthomonas arboricola* pv *juglandis*, abbreviated to *Xaj*, is used throughout this thesis.

1.3 Causal organism

Xaj is a rod shaped, gram-negative bacterium, 0.3-0.5 × 0.5-3.0 μm in size, which is motile by means of a polar flagellum (Miller and Bollen, 1946; Lindow, 2002). It produces distinctive yellow colonies on most standard media such as nutrient agar and potato dextrose agar (Teviotdale *et al.*, 1985; Lindow, 2002). Optimum growth *in vitro* occurs at 28-32°C, with the maximum temperature for growth being 37°C and the minimum temperature at 5-7 °C (Miller and Bollen, 1946). *Xaj* does not form spores or a capsule (Adhikari *et al.*, 1988).

The ability to hydrolyse starch in Brilliant cresyl blue starch media (Murlean and Schroth, 1981) and to metabolise quinic acid to produce gallic acid in succinate quinate media (Lee *et al.*, 1992) are characters that have been associated with *Xaj*. Both media appear to be semi-selective for the walnut blight bacterium.

1.4 Host range

Xaj only infects species of *Juglans* (Teviotdale *et al.*, 1985; Lindow 2002). Disease occurs naturally on *Juglans regia* (Persian (English) walnut), on leaves of *J. hindsii* (Northern California black walnut) and rarely on Paradox hybrids (crosses between *J. californica* or *J. hindsii* and *J. regia*). Other hosts reported to be affected by the disease are *J. nigra* (Eastern black walnut), *J. ailantifolia* (Japanese walnut), *J. californica* (Southern California black walnut), *J. cinerea* (Butternut) and *J. sieboldiana* (Japanese walnuts) (Rudolph, 1933; Miller and Bollen, 1946; Elliot, 1951). Symptoms caused by *Xaj* were also artificially induced on *J. manshurica*, during analysis of the host resistance of various *Juglans* species (Belisario *et al.*, 1999). Although associated with a single host genus, the pathogen has a high level of genomic diversity (Lortei *et al.*, 2001; Scortichini *et al.*,

2001).

1.5 Symptoms

The blight pathogen attacks almost all above ground parts of the tree that are green and succulent, although fruit infection accounts for the major economic loss. Necrosis can occur in catkins, female flowers, leaves, nuts and green shoots. The plant parts become more resistant to bacterial attack as they become lignified. In nursery plants, *Xaj* causes formation of cankers on stem and apical buds resulting in malformation and alterations in growth (Belisario *et al.,* 1996).

The disease affects all tissues of the leaves, including parenchyma, midrib, lateral veins, veinlets, rachises and petioles. The symptoms initially appear as dark brown to black irregular spots, a few millimetres in diameter with yellowish green perimeters. These spots may extend to form large necrotic areas. This may lead to malformation and deformity of the leaves as the symptomless, healthy tissue around the necrotic tissue continues to grow (Olson *et al.,* 1976; Lindow, 2002). Leaves infected early in the season are twisted in appearance whereas no distortion occurs on completely grown leaves infected later in the season. Walnut blight does not usually cause defoliation, although defoliation may occur in cases of severe damage (Miller and Bollen, 1946; Teviotdale *et al.,* 1985).

Shoot infection occurs when shoots are young and succulent. The symptom begins from the tip of the shoot, which may lead to death of the entire tip up to a few centimetres in length. Normally symptoms appear in the bark as small lesions of 2.5 cm or more in length, and may sometimes girdle the shoot. Infection may even extend to pith forming cankers (Lindow, 2002). Yellowish bacterial exudate sometimes oozes out of diseased tissues and stems (Teviotdale *et al.,* 1985; Belisario and Zonia, 1995).

Infected male florets become necrotic and deform the expanding catkin. The pathogen spreads from one floret to another internally via the rachis and may contaminate pollen (Ark, 1944; Teviotdale *et al.,* 1985).

The disease exhibits variable symptoms on fruit depending upon the stage of flower or fruit development. The fruit react to bacterial attack by the formation of parietal or apical necrosis often leading to fruit drop before maturity (Garcin *et al.*, 2001). Rain during pollination in the early flowering stage may cause blossom-end infection of young fruit. On the stigma, blight appears as a rapidly enlarging black spot that can sometimes spread over the entire fruit. Many affected young nuts drop from the affected trees (Goss *et*

al., 1961).

When fruit is infected at the early post-blossom stage, the ovary is often invaded by the bacterium causing blackening and withering. The symptoms are usually not visible, but the nut inside the fruit is often damaged.

On partially grown nuts, lesions appear as water-soaked spots. These spots darken and enlarge rapidly to a few centimetres in diameter, extending to over half of the fruit, resulting in black sunken areas on the walnut hull (Olson *et al.,* 1976). Infection before shell hardening results in shrivelled kernels. After shell hardening, the kernel may not shrivel from infection but it is often discoloured (Miller and Bollen, 1946; Olson *et al.*, 1976; Lindow, 2002).

1.6 History and geographical distribution

Xaj cannot live or reproduce for more than a short period in the soil (Rudolph 1933; Miller and Bollen 1946). Given the host specificity of *Xaj* as described above, the disease most likely spreads in the presence of host, possibly through infected nursery stock (Smith, 1921; Rudolph, 1933; Miller and Bollen, 1946).

France is thought to be the location from which the disease spread widely to the other parts of the world. The reason behind this assumption is that the walnut industry in France is a very old one and many of the walnut plantings made during the late 1870's around the world were from nuts and scions imported from France (Rudolph, 1933). In the USA, walnut blight was first observed in 1890 in Los Angeles, California, on trees that came from a nursery in Orange County. The nursery trees most likely originated from France, but this origin cannot be confirmed. Soon thereafter, the disease started appearing in other areas of Orange County. The disease became more destructive and started spreading to other parts of California. Newton B. Pierce was assigned to study the disease by the United States Department of Agriculture. In 1896, Pierce described its bacterial origin. Around 1900, the disease became so destructive that the Californian walnut growers association offered rewards of $20,000 for a suitable means of combating it (Rudolph, 1933). Smith (1907) observed characteristic lesions on scions imported into California from France. He also observed that new shoots from these scions were diseased and no other new shoots other than from French scions developed blight symptoms indicating the imported scions from France to be the source of disease.

The first report of walnut blight from France was by Wormald and Hamond (1931) in England, who proved that the disease occurred in France by conducting inoculation

experiments with *Xaj* isolated from France. After these cultural studies, it was concluded that the walnut blight organism from France was the same species as bacterial isolates from England and California.

The disease was reported from many other counties in the subsequent years, as described in Table 1.1.

Table 1.1 First report of walnut blight in countries other than the United states of America, listed in chronological order.

Country	Author	Year of first report
[a]New Zealand	Boucher, W.A.	1900
[a] Russia	Anonymous	1908
[a] Canada	Gussow, H.T.	1911
[a]Tasmania, Australia	Rodway, L.	1912
[a] Mexico	Smith, C.O.	1912
[a]Victoria, Australia	Cole, C.F.	1914
[a] Chile	Camacho, C.	1917
[a] South Africa	Soidge, E.M.	1918
[a] South Australia	Osbor, T.G. and Samuel,G.	1922
[a] Italy	Savastano, L.	1923
[a] Holland	Van Poeteren, N.	1923
[a] Switzerland	Muller-Thurgau, H. and Osterwalder, A.	1927
[a] England	Wormald, H.	1927
[b]Romania	Veresciaghin, B. *et al.*	1940
[b]West Indies	Waterson, J.M.	1943
[c]India	Adhikari *et al.*	1988

[a]Author and year cited in Rudolph (1933)

[b]Author and year cited in Miller and Bollen (1946)

[c]Adhikari *et al.* (1988)

C.F. Cole, who reported walnut blight in Victoria (Australia), considered that "Mr Brittlebank, plant pathologist" of the Victorian Department of Agriculture, observed this disease in Australia as early as 1888 (Rudolph, 1933), which would have been before the

first report of blight disease in California.

1.6.1 Distribution

Walnut blight is widespread, occuring in Argentina, Austria, Australia, Bermuda, Bulgaria, Canada, China, Chile, Denmark, France, Germany, Great Britain, Greece Holland, India, Iran, Iraq, Israel, Italy, Lebanon, Mexico, Nethèrlands, New Zealand, Poland, Portugal, Romania, Russia, South Africa, Switzerland, Uruguay, the United States of America, West Indies and Zimbabwe (CABI, 1987; Lindow 2002).

1.6.1.1 Distribution in Australia

Walnut blight occurs in all Australian states according to reports listed as follows.

- New South Wales: (Anon, 1938; Bradbury, 1986; CABI/EPPO, 2001; EPPO, 2002)
- Queensland: (Bradbury, 1986; CABI/EPPO, 2001; EPPO, 2002)
- South Australia: (Osborn and Samuel, 1922; Bradbury, 1986; CABI/EPPO, 2001; EPPO, 2002)
- Tasmania: (Rodway, 1912; Dowson, 1932; Bradbury, 1986; CABI/EPPO, 2001; EPPO, 2002)
- Victoria: (Cole, 1914; Adam & Pugsley, 1934; Bradbury, 1986; CABI/EPPO, 2001; EPPO, 2002)
- Western Australia: (Goss & Doepel, 1961; Bradbury, 1986; CABI/EPPO, 2001; EPPO, 2002)

1.7 Economic importance

1.7.1 USA

Smith (1912, cited in Rudolph, 1933), estimated a yield loss due to disease of 50% in California in 1912. In some areas of California, losses up to 80% have been observed in years with favourable weather conditions for disease development (Rudolph, 1933). In northern California, severe epidemics of walnut blight in 1998 caused substantial yield loss (Adaskaveg, 2003). Weather conditions were unfavourable for disease development in subsequent years, 1999 to 2002, which led to a decrease in disease incidence, despite the presence of *Xaj* in dormant buds (Adaskaveg, 2003).

1.7.2 Europe

In France, yield loss of up to 80% has been observed in years with extended spring rains that favour blight disease (Garden *et al.*, 1993). Walnut blight has been responsible

for crop losses up to 50% in Portugal, from fruit falling prematurely and from internal damage to fruits (Martins, 1997).

1.7.3 Australia

Freeman (1958) reported blight disease to be a constant source of yield loss in Victorian walnut groves. Goss *et al.* (1961) indicated that in weather conditions favouring disease, walnut blight caused severe yield losses in susceptible varieties in Western Australia. At some sites in Tasmania, walnut blight has been responsible for more than 50% loss of the walnut crop. In 2004, a loss of this magnitude was estimated to be worth Au$20,000/ha (Hills and Lang, 2004).

1.8 Disease cycle and epidemiology

1.8.1 Survival and sources of inoculum

Xaj has been isolated from symptomless walnut buds during dormancy, twigs, leaves, catkins, nuts, decomposing leaves, mummified nuts, plant debris and from weeds under walnut trees. Major sources of primary inoculum for walnut blight disease are thought to be overwintering *Xaj* in dormant buds and catkins, plus blighted and cankered branches from previous seasons (Esterio and Latorre, 1982; Murlean and Schroth, 1982). Dormant buds appear to be infected more frequently than catkins (Murlean and Schroth, 1982). Epiphytic populations of *Xaj* up to 5.9 x 10^5 colony forming units (cfu) per bud and internal populations in buds up to 1.4 x10^6 cfu per bud have been reported (Murlean and Schroth, 1982). Populations of the bacterium may vary among buds from undetectable to 1×10^7 cells per bud (Lindow, 2002). A survey conducted on eight commercial varieties representing early, middle and late blooming varieties has shown that the dormant buds and catkins of all the varieties tested were infested to some extent with *Xaj* (Murlean *et al.*, 1980).

The presence of a large epiphytic population of *Xaj* on fruits and leaves (1×10^5 to 1×10^6 cfu) was correlated positively to the probability of disease incidence. Epiphytic populations of *Xaj* can act as an immediate source of inoculum under favourable environmental conditions (Murlean and Schroth, 1982; Lindow, 2002). There have been no studies on the survival of *Xaj* as an epiphyte on non hosts.

Xaj has been recovered from plant refuse (semi-decomposed leaves and debris), although the bacterium was most likely associated with plant debris rather than colonising the refuse as a saprophyte (Esterio and Latorre, 1982). The pathogen has also been

recovered from symptomless weeds throughout the growing season, indicating that the bacterium can colonize and survive on weeds (Esterio and Latorre, 1982). Weeds might act as a non host for bacterial survival in the absence of a host or might favour disease spread from one orchard to another by transport on farm equipment after intra-row cultivation. The significance of weeds and plant refuse on disease spread is yet to be clearly established.

1.8.2 Disease cycle

The disease cycle is illustrated in Figure 1.1. Bacteria (*Xaj*) overwintering in healthy dormant buds and catkins infect the leaves early in the growing season (Murlean *et al.*, 1980). Infected leaves and inoculum from infected catkins contribute to early season fruit infection. Many of the young fruits drop soon after infection. The infected leaves and infected fruits remaining in the tree contribute inoculum for spread of the disease to developing buds and catkins throughout the season. Once the bacterium is established in the dormant buds and catkins, the pathogen can survive through the winter until the next growing season when susceptible walnut tissues are produced (Murlean *et al.*, 1980).

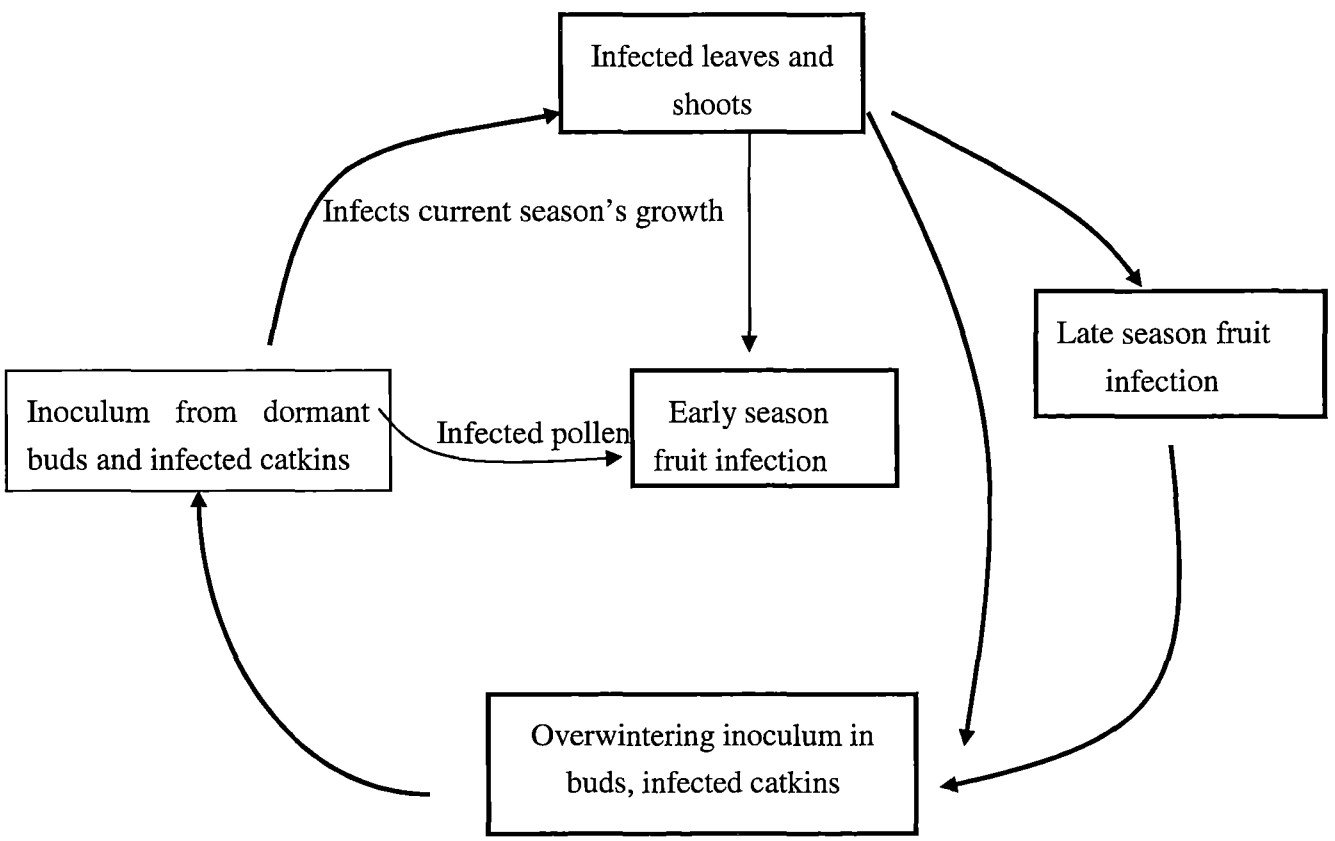


Figure 1.1 Disease cycle of Xanthomonas *arboricola* pv *juglandis*

1.8.3 Mode of infection and disease spread

Xaj enters the host tissue through stomata and other natural openings. Wounds and abrasions also allow entry of the pathogen (Rudolph, 1933). Two factors that are essential for successful establishment of disease are the presence of free water and succulent tissue (Miller and Bollen, 1946). Forms of free water include rain, fog, dew, and sprinkler irrigation, and all may facilitate infection of susceptible walnut tissue (Teviotdale, 1985; Lindow, 2002). Spread of the disease is mainly by wind and rain. Water in the form of rain droplets falling on the infected tissue spreads the bacteria to the current season's growth.

Contaminated pollen carried by wind might be a source of infection of pistillate flowers or leaves (Ark, 1944; Olson *et al.*, 1976). Ark (1944) revealed the importance of infected catkins in the overwintering of *Xaj* and the potential for contaminated pollen to disseminate the bacterium. However, the role of infected catkins in disease development in Portugal was considered overestimated by Martins *et al.* (2001). Experiments were conducted near Coimbra in Portugal to analyse the effects of different schedules of copper sprays for the control of walnut blight on cultivar Lara (Gonclaves *et al.,* 2001). From this experiment, Martins *et al.* (2001) studied the relationship between external necrosis and kernel destruction of blight affected nuts of walnut. They found that kernel destruction was not correlated to external necrosis, whether it was necrosis around the pistil or necrosis over the rest of the fruit. Their studies also indicated that there was no correlation between pistillar necrosis and fruit drop. This result indicated that contaminated pollen grains were not a major source of disease spread and crop loss (Martins *et al.,* 2001). There is no clear evidence for the role that catkins play in disease spread even though they provide a site for over wintering *Xaj*.

Inoculum carried by mites, insects and humans are other sources of disease spread (Rudolph, 1943).

1.8.4 Environmental conditions favouring disease

The severity and commercial impact of walnut blight is determined by the interaction of weather conditions and host factors such as plant age, infection site, tissue succulence and cultivar susceptibility (Belisario *et al.,* 1995). There is a high probability of severe crop loss in years with continuous spring rains, dew and/or high humidity (Belisario *et al.*, 1997).

The effect of wetness duration and temperature on disease expression on flowers

and fruit of walnut plants, was studied by Miller and Bollen (1946) for over 12 years. Experiments were conducted both under field and green house conditions. In field studies, nuts of different developmental stages were inoculated with *Xaj* under rainy and dry conditions. In the greenhouse, plants were inoculated by spraying water suspensions of *Xaj* on uninjured parts and incubated in a humidity chamber for infection at various temperatures within the range of 4°C to 35°C. After inoculation, the plants were moved to a green house maintained at 22 °C. Adequate supply of moisture was provided in the greenhouse for infection. The experiment demonstrated that moisture was a prerequisite for infection both under field and green house conditions. Miller and Bollen (1946) demonstrated that a wetness period of 5 min was sufficient for infection of very young fruit tissue when the stomata were open and the tissue water congested. Mature fruit required a longer wetness period for infection. Miller and Bollen (1946) explained this finding based on observations of a lower amount of water in the tissues, presumably as a result of greater tissue lignification, and smaller stomatal openings.

Under field conditions, infection can occur over a wide range of temperature. Green house studies showed that infection occurs between 4 to 30° C for leaves and between 5 to 27 °C for fruit. The incubation period, or the time from infection to symptom expression, ranged from 10 to 15 days (Miller and Bollen, 1946).

1.9 Management of the disease

"In any disease management program, dependence on any single chemical over years can potentially lead to loss of efficacy of the treatment due to development of resistance among the population of pathogen to that chemical."

Adaskaveg *et al.* (1999)

A vast amount of research has been conducted on the control of *Xaj* since 1900. Pierce (Rudolph, 1933) began exploring ways of managing walnut blight, beginning with the removal of diseased twigs from trees, which proved ineffective. Later he applied Bordeaux mixture to walnut trees and observed suppression of blight disease. From 1930 to 1945, Miller and Bollen (1946) conducted a series of experiments with 30 different copper compounds and found that Bordeaux mixture was the most effective chemical in controlling blight disease. Since 1945, the management of walnut blight has been based on copper containing bactericides. Research on other management strategies including

breeding for resistance, cultural practices and biological agents have failed to deliver a viable alternative to copper containing bactericides.

Copper-containing bactericides were the first biocides used for control of plant diseases, and are registered on many crops. Their general mode of action and their usage on crops for years has led to development of resistant populations of bacteria and fungi (Anderson *et al.*, 1991). Moreover, heavy application of copper for years can result in accumulation of copper in soil which can have a negative impact on the environment and may alter the plants ability to metabolise nitrogen. In walnut trees, heavy application of copper is considered to disturb tree performance, including production of necrotic fruits (Radix *et al.*, 1998). Hence, research on an integrated approach of disease management is needed which is environment friendly and that decreases the potential for resistant populations of bacteria from developing.

1.9.1 Current methods of chemical control

Control of the disease is commonly based upon application of copper containing bactericides that prevent new infection. Copper compounds are applied up to 10 times per growing season, depending on the frequency of the rain (Teviotdale *et al.*, 1985; Polito, 2002). Compounds like cupric hydroxide, other fixed coppers or Bordeaux mixture, which contains copper sulphate, are commonly recommended for control of walnut blight in almost all walnut growing areas. Copper hydroxide (Kocide®) is one of the most effective copper compounds for control of walnut blight in France and the USA, but it's effectiveness was observed to be less than Bordeaux mixture in years experiencing continuous rains (Ginibre, 2001). The possible reason for this observation may be that Kocide® is less resistant to being washed from plant surfaces than Bordeaux mixture (Ginibre, 2001).

The number of applications and timing of treatment plays a key role in achieving optimum walnut blight control (Olson *et al.* 1976). Copper sprays should be applied prior to rainfall for maximum protection (Herkert 1988, Olson 2002). The total number of applications required depends on the disease history of the orchard and climatic conditions, such as the intensity and frequency of rainfall. Orchards with a history of disease probably need more protective sprays applied early in the season because disease onset might be earlier when initial inoculum is high during wet periods. However, research on disease epidemiology is required to understand the effect of a high level of inoculum carried over

the winter. Of greater concern is the rapid emergence of susceptible leaves that are unprotected by copper, given that this fungicide only protects against infection at the site that it is applied (Olson *et al.*, 1976). Good coverage of newly emerging tissues gives more protection against disease spread (Teviotdale *et al.*, 1985). Miller and Bollen (1946) considered three initial sprays as essential in Oregon, USA: (1) at bud break (2) at full female flower bloom and (3) at fruit set. A fourth application was then recommended 2-4 weeks after the third spray.

Intensive application of copper sprays over years for blight control has resulted in the development of populations of *Xaj* that are resistant to copper. Copper resistant strains have been described in California and France (Lee *et al.,* 1994; Garden *et al.*, 1993) and the management of walnut blight has become a major challenge in central and northern California. Heavy use of copper-based sprays can also result in accumulation of copper in the soil to levels that have a negative impact on the soil biology and future land use (Radix *et al.*, 1994; Garcin *et al.*, 2001).

1.9.1.2 Management in the USA

Walnut growers in California routinely apply 4.5 kg/ha of metallic copper per application for economic control of walnut blight, which has lead to walnut being ranked ninth for crops that use copper in California (McNeil, 2004; Adaskaveg *et al.*, 2005). Application of copper for more than 40 years has led to the development of copper-resistance among populations of walnut blight organism in northern California (Lee *et al.* 1993). It is possible that such copper- resistant strains of *Xaj* may not be killed by standard copper applications under field conditions.

Development of copper resistant strains of *Xaj* (Lee *et al.*, 1994; Garden *et al.* 1993) and/or lack of protection during environmental conditions conducive for disease development may be possible reasons for failures in disease control, despite adoption of recommended bactericide spray schedules (Olson *et al.*, 1997). Copper sprays conjugated with other chemicals to enhance the copper toxicity and for reducing copper inputs are being evaluated. Examples of chemicals used to conjugate copper include EBDC (ethylene bis dithiocarbamate) compounds, e.g. maneb, mancozeb, iron or zinc materials such as copper–manex (eg. Mankocide®, copper-iron or zinc-Bordeaux).

Free copper ions present in copper sprays inhibit bacterial growth. Copper containing bactericides generally have low water solubilities and can be easily mobilized

by carbon dioxide in rain water, microbial exudates and by solubilising agents in exudates from plant surfaces. These copper ions may be either in free form or chelated by organic compounds to form soluble complexes, and in the latter form they lose their toxicity towards bacteria (Menkissoglu and Lindow, 1991; Lee *et al.*, 1993).

Copper in combination with EBDCs have reportedly increased copper toxicity with effective control of copper tolerant strains of *X.c. vesicatoria* and *Pseudomonas syringae* pv *tomato*. This combination is now used for effective control of copper resistant strains of *Xaj* in California (Buchner *et al.*, 2001). Increase in toxicity of copper with addition of EBDCs may be due to a chelating ability that may prevent copper ions from complexing with other organic substances, transporting the cation to a copper susceptible site within the cells and hence increasing the availability of free copper ion. EBDCs are also considered to modify cell permeability and may cause disruption of cellular transport mechanisms, which allows lethal amounts of cupric ion into bacterial cell (Anderson *et al.*, 1991; Lee *et al.*, 1993).

Lee *et al.* (1993) showed that iron enhanced the effect of copper when applied to copper resistant strains of *Xaj in vitro* and on walnut leaves. Addition of ferric chloride to cupric hydroxide increased the availability of Cu^{2+} on leaf surfaces by reducing pH and through cation exchange between Fe^{3+} and Cu^{2+}. The addition of HCl decreased pH but did not increase the availability of Cu^{2+} ions on leaves. While studying effects of various formulations of copper on copper sensitive and copper resistant strains of *Pseudomonas syringae* pv *syringae*, it was found that addition of ferric chloride to cupric hydroxide increased Cu^{2+} concentration to 40-fold that of cupric hydroxide alone (Scheck and Pscheidt, 1998). Buchner *et al.* (2001) found that copper hydroxide mixed with $FeCl_3/MgSO_4$ and the adjuvant CS7 controlled walnut blight in field trials, but the mixture was not significantly ($P = 0.05$) more effective than standard copper treatments.

Zinc-containing compounds were found to be effective for management of walnut blight. Miller and Bollen (1946) indicated that mixtures of zinc sulphate and calcium hydroxide were effective for management of walnut blight, but found copper to be more effective in control than the zinc formulation. Zinc formulations alone or in combination with copper have been effective in controlling copper resistant strains of *Xaj* (Burnham, 1995, cited in Adaskaveg *et al.*, 1999). Furthermore, copper-maneb plus zinc (Kocide101®-Manex®) controlled the disease effectively in locations where copper resistant populations occurred (Olson *et al.*, 1993, cited in Adaskaveg *et al.*, 1999). This

result was confirmed in trials conducted in 1994-1995 and indicated that rotation of zinc containing compounds with copper formulations to be an effective treatment for blight control. Conversely, Buchner *et al.* (2001) found that blight control with zinc compounds was not statistically separated from untreated trees. Although zinc treatments were effective in some cases, they were phytotoxic to walnut tissues when applied at higher rates. Phytotoxicity was reduced by the addition of lime to the tank containing zinc sulphate (Zinc-Bordeaux); however, tank mixing of $ZnSO_4$ with lime was difficult and sprayer nozzles became clogged. This practical problem has limited the usage of zinc compounds for effective control of blight disease.

Copper plus Manex® improved disease control significantly when compared with the standard copper treatment (Buchner *et al.*, 2001). Several new bactericides were evaluated for control of walnut blight including DBPNA (a water treatment biocide), an organic chemical biocide (DOW-01A), an antibiotic (Starner), a premixed Bordeaux–maneb mixture, compounds for systemic acquired resistance (eg actigard, milsana), peroxyacetic acid (e.g., Zerotol), biopesticides (e.g., Serenade organic), new formulations of copper (e.g., Bioacumen) and copper-silver compounds (MagnaBon). Many of these compounds including Serenade, Bioacumen, Magnamen and DOW-01A suppressed walnut blight and researchers continue to evaluate new products. Starner® is currently registered in Japan for many bacterial diseases including those caused by *Xanthomonas* species (Adaskaveg *et al.*, 1999, 2000, 2002). However, no antibiotics appear to be registered for use against plant bacterial diseases in Australia and registration of an antibiotic for walnut blight control may be perceived by some as a retrograde step.

Streptomycin is an antibiotic used to control fire blight of pears in the USA and it was first tested for controlling walnut blight by Ark (1955). The wettable form of streptomycin was effective in reducing blight disease (Ark, 1955) but it is not registered for use on walnut because it's application leads to increased fruit drop (Buchner *et al.*, 2001; Polito *et al.*, 2001). The mechanism by which streptomycin caused fruit drop during the walnut bloom was investigated by Polito *et al.* (2001). They recommended that streptomycin, if it were to be used for blight control, should not be applied in the period coinciding with pistillate bloom and pistillate flower receptivity.

Given that *Xaj* overwinters primarily in dormant buds, copper hydroxide (Kocide) has been applied during dormancy to reduce inoculum, but this treatment did not improve walnut blight control. While it is possible to reduce the epiphytic (external) populations of

Xaj on buds, internal inoculum that is protected from copper is sufficient to initiate disease under suitable environmental conditions (Murlean and Schroth, 1981).

1.9.1.3 Management in Europe

In France, different copper compounds (sulphate, hydroxide or oxide) were tested alone or supplemented with an EBDC material, boron or iron when applied to the cultivar Franquette. Copper in combination with an EBDC compound (Manex) proved to be the most effective material tested (Ginbre, 2001).

In Portugal different compounds were tested for their efficacy in controlling walnut blight, including Bordeaux mixture. Kocide® was found to be the effective treatment when compared with the others tested.

In Europe, a standard schedule of seven applications of copper is usually followed for blight control. Ninot *et al.* (2002) evaluated a reduced spray schedule where the last four applications were omitted. The reduced schedule was just as effective as the standard schedule, indicating that in the year the trial was conducted, the extra copper sprays were only contributing to the accumulation of copper in the soil. Hence development of a suitable disease forecasting system for walnut blight should enable timing of sprays according to pathogen activity, while reducing the amount of copper applied per hectare each season. Such a forecasting system, called Xanthocast™, has been developed in California and is available to walnut growers free of cost. Xanthocast™ is described in more detail below.

Although copper is important in the management of walnut blight, it has been found to interfere with some physiological processes during walnut fruit development, whose mechanism has still not been explained or investigated thoroughly. Experiments were conducted to assess the effects of different copper spray schedules in cv Lara. The treatments did not affect nut yield, but nut shape and volume at harvest differed significantly among treatments, indicating the interference of copper in the physiology of fruit development (Gonclaves *et al.,* 2001). Copper sprays used against blight may also alter the nature of soil affecting its permeability and this in turn may cause imbalances in polyphenolic contents of walnut tissue. This factor might be one of the reasons for crop losses despite the benefit of copper sprays for disease control (Radix *et al.,* 1998). Factors which may influence the severity of necrosis, such as the physiochemical composition of soil, contents of individual elements in leaves, application of chemical fertilisers and the number of copper treatments have been investigated (Garcin and Duchnese, 2001).

However, this work has not yet provided clues for further research.

1.9.1.4 Management in Australia

As in Europe and the USA, walnut blight is managed in most of walnut growing areas of Australia by application of protective copper-based bactericides. Goss and Doepel (1961) evaluated Bordeaux mixture in Western Australia and found that disease incidence was reduced when spray timing was appropriate. Another approach to disease management in Australia and elsewhere is planting varieties that are moderately susceptible to walnut blight, like Franquette.

Walnut blight is managed in Tasmania by multiple applications of copper to protect susceptible tissue. Research from 2002-2004 has focused on identifying the most effective type of copper compounds and their timing in relation to pathogen activity (J. Hills, Agronico Pty Ltd, personal communication). Mankocide®, a mixture of copper hydroxide and mancozeb, was the most effective of all products tested. In the 2002-2003 season, Mankocide® at a rate of 0.5 kg/ha was applied 10 times at weekly intervals starting from 5% bud burst. This treatment resulted in 12% of diseased nuts at harvest, in contrast to the untreated control that had 29% of blighted nuts. An organosilicone penetrant, Pulse, when added to a mixture of Penncozeb plus Kocide for the first four sprays after terminal bud burst was found to provide greater control of the disease, while applying this mixture for first two applications (one at bud burst and other a week later) had no effect when compared with the untreated control.

The timing of bactericide application appears to be critical for effective control of walnut blight in Tasmania. Bordeaux mixture, at (10kg $CuSO_4$+ 10kg $CaCO_3$)/ha, when applied three weeks after 5% bud burst had more blighted nuts (32%) when compared with Bordeaux mixture applied initially at bud burst or two weeks after bud burst (12% and 14% blighted nuts, respectively). Among non-copper based compounds, an anti-microbial material under development (7287) suppressed walnut blight, in that 27% of nuts were diseased when compared with 44% diseased nuts observed in untreated control plots (Lang and Evans, 2005).

More research is required to understand disease progression and environmental factors favouring the development of walnut blight so that protective bactericides can be applied according to pathogen activity.

1.9.2 Control of blight in walnut nurseries

Blight disease has been a serious problem in Italian walnut nurseries. Belisario and Zonia (1995) found that, under natural conditions, an inter-row spacing of 1.4 m gave better control than 0.7 m spacing, with 1 to 4 % of blighted plants compared to 4 to 12% of blighted plants. Results were confirmed by inoculating plants to create a high inoculum pressure. Hence, identifying appropriate cultural conditions that reduce plant succulence and the duration of water films on canopies can help control the disease successfully. Foliage flush stage was found to be the most vulnerable stage to infection by *Xaj* in nurseries. Two sprays of Bordeaux mixture, one at the beginning and one at the end of vegetative stage, were recommended for heavily infested nurseries (Belisario and Zonia, 1995).

1.9.3 Forecasting walnut blight

The aim of disease forecasting is to use weather to predict when a pathogen is active by identifying either discrete infection periods or an increase in disease severity. Leaf wetness and temperature are two important parameters that influence the development of new infections of walnut blight in the growing season. Researchers in California have developed Xanthocast™, which is a 7-day cumulative index that is based on host phenology, temperature, and leaf wetness (Olson and Buchner, 2001; Adaskaveg *et al.,* 2002). This forecast model utilizes wetness period and temperature for calculating the risk of disease based on current ambient conditions measured by a network of weather stations throughout California. This tool can be used for providing more efficient disease control. The model was found to reduce the number of bactericide application compared to calendar-based applications, and disease control was found to be similar in both cases. In dry years, zero to one application has been forecasted whereas two to four applications were forecast in wet years. A reduction of 1.5 applications was achieved in Butte and Tehama, California, where three sprays were recommended by Xanthocast™ compared to 4.5 sprays applied on a calendar basis. The model can be accessed by walnut growers in California at no cost for support in making decisions about spray timing (Olson and Buchner, 2001; Adaskaveg *et al.,* 2003). Xanthocast™ is being evaluated in walnut orchards in Tasmania (M. Lang, Agronico Pty Ltd, personal communication).

1.9.4 Breeding for resistance

Research on breeding for blight-resistant walnut cultivars began in the early 1900's, although there has never been a blight-resistant cultivar suitable for commercial

production. The important traits that are of agronomical interest in walnut breeding are lateral fruit bearing, good nut size and maximum nuts per cluster. However, lateral fruit bearing types are susceptible to walnut blight because of their early leafing habit. Few studies were conducted to select types with lateral fruiting and late leafing, probably because there is a negative relationship between these two characters (Akca *et al.,* 2001; Germain, 1990). In 1978 at Bordeaux in France, a cross made between cultivars Franquette and Lara gave a late maturing variety called Fernor (corporate author, 1997). This variety was the first to combine late leafing, lateral fruiting, tolerance of bacterial blight and good nut and kernel quality. Fernor is likely to be useful for breeding blight resistant cultivars in the future. Farris (1994) reported results of breeding *J. regia* for late leafing. A late leafing variety Alpha was produced which was then crossed with line 609 to produce progeny that had leaves that emerged later by 7-14 days than Alpha. Farris (1994) then retained two selections with lateral and precocious fruiting, late leafing, resistant to walnut blight and nuts of good size and shape. Although these two selections have many desirable characters, their status of further development and commercialisation is unknown.

Research on disease susceptibility can be used when breeding walnut varieties. The susceptibility of *Juglans* species and *Juglans regia* varieties was summarised by Belisario *et al.* (1997). Seedlings of *Juglans* species were either sprayed with a bacterial (*Xaj*) suspension or bacterial suspensions were injected into the leaves on *J.regia* varieties. Observations indicated that *J. nigra* and *J. sieboldiana* were highly resistant and *J. regia* was the species most susceptible to infection by *Xaj*. Varieties Franquette and Hartley were resistant to walnut blight, whereas Malzia was moderately susceptible while Payne, Serr and Sorrento were highly susceptible to disease (Belisario *et al.,* 1997a, b). However, Tamponi and Donati (1990) made careful observations on 200 nuts at random on each cultivar and found that the variety Sorrento was the most tolerant in tests of pathogenicity. Furthermore, these researchers observed that Californian selections (Vina, Serr, Payne, Gustine, Midland, Tehama, Hartley, Chico) and Hungarian selection (A 117) of *J. regia* were rated as highly susceptible to walnut blight whereas French cultivars (Franquette) were rated as moderately susceptible and Italian cultivar (Sorrento) was one of the most tolerant to walnut blight. An explanation for the difference in ranking among cultivars for blight susceptibility between studies might be the use of different strains of *Xaj* in pathogenicity testing and/or the plant type or plant part (whole plant or detached plant part) used for assays.

Woeste and Mcgranahan (1992) inoculated leaves and nuts of walnut germplasm

with *Xaj* to assess the levels of blight resistance. Genotype PI 159568 had severe leaf infection whereas leaves of cultivars Adams and Chandler were given a low disease rating. Nut drop caused by blight disease was very low in Adams, Payne, PI 18256 and Sinensis 5, although nut quality was much higher in PI 18256 and Sinensis 5 when compared with nut quality of other three cultivars.

Wild populations of *Juglans regia* seedlings in Spain that appeared to be resistant to blight disease were selected and planted together with five commercial cultivars (Adams-10, Amigo, Chandler, Franquette and Vina) in a field condition located in an environment favourable to disease development (Aleta *et al.*, 2001). To study blight susceptibility, immature fruits were collected after nut set and were inoculated with *Xaj* under controlled environmental conditions. Field studies indicated that Adam-10 was highly tolerant and Amigo and Vina were highly susceptible to blight (Aleta *et al.*, 2001). A local selection MBLu-20 showed tolerance on all plant organs analysed. Laboratory studies of immature fruits of all these cultivars and clones revealed that nine of the local selections were more tolerant of blight disease than the other lines monitored. Selection MBPo-3 had almost no damage to immature fruits whereas Chandler and Franquette were susceptible varieties with more damage to immature fruits. Under field conditions, the level of blight susceptibility was much lower on leaves and mature fruits of Franquette and Chandler. Hence, genotype and plant organ being affected plays an important role in blight susceptibility.

Genetic engineering, the transfer of selected genes into specific genotypes, potentially is a useful tool for plant breeders in improving varieties of perennial crops where conventional plant breeding is a time-consuming process. In walnut, gene transfer using somatic embryo culture has been tried for traits such as resistance to codling moth or nematodes and also for improving the ability of plant cuttings to form roots from *Agrobacterium rhizogenes* (rootability). There do not appear to be any reports of genetic engineering in walnut for blight resistance. Difficulty in regenerating plants from matured somatic tissues, inefficiencies in the regeneration process and the limited number of useful genes available are the main drawbacks of genetic engineering in walnut (Leslie *et al.*, 1997).

A study was conducted to analyse the amount of phenolic contents on young walnut fruits of six walnut cultivars that differ in their susceptibility and time of bud breaking. Varieties that were late or very late leafing with low susceptibility to blight (Elit, Franquette, Fernor) had higher contents of vanillic acid, catechin and myricetin, but lower

in chlorogenic acid compared to early leafing and more susceptible cultivars (Cisco, Hartley, Champion). A thorough study on phenolics might be very useful for identifying genes responsible for production of these phenolic substances and can be used in the future to produce a walnut genotype that is blight tolerant.

1.9.5 Biological and cultural control

Biological control agents have not yet been deployed for the management of walnut blight. Research on bacteriophages which infect *Xaj* has progressed to the selection of strains for further development (McNeil *et al.*, 2001; M. Walter, HortResearch, New Zealand, personal communication). Bacteriophages are viruses that infect unicellular organisms and have considerable host specificity. Bacteriophages have potential to control bacterial diseases by specifically infecting and destroying the pathogen causing the disease.

Cultural control methods, such as fertilisation, irrigation, cultivation or canopy management have been given little attention in relation to integrated management of walnut blight. Removal of infected tissues does not appear to provide significant control as the bacteria overwinter in dormant buds (Lindow 2002).

1.10 Bactericide resistance

Copper sprays like copper hydroxide, Bordeaux mixture and various formulations of copper have been used to control bacterial diseases for years. Copper resistance is observed among both saprophytic and phytopathogenic bacteria. *Xanthomonas campestris* pv. *vesicatoria*, *Xaj*, *Pseudomonas syringea* pv. *tomato*, *Pseudomonas syringea* pv *syringea*, pathogens of pepper, walnut, tomato and cherry, respectively, are examples of phytopathogenic species where copper resistance has been documented (Macro and Stall, 1986; Bender and Cooksey, 1986; Lee *et al.*, 1993; Garden *et al.*, 1993; Sundin *et al.*, 1989). Copper resistance in a phytopathogenic bacterium is characterised by a quantitative increase in tolerance to copper (10-50×) when compared with sensitive strains of the bacterium. Copper resistance in a particular bacterial strain is confirmed when a mutation in the gene responsible for the resistant phenotype is characterised (J. Vanneste, HortResearch, New Zealand, personal communication). Study in this field began to expand when the presence of copper resistant strains in the field led to noticeable failures in the disease control by application of copper (Menkissoglu and Lindow, 1991).

Bacterial leaf spot of tomato and pepper is caused by *Xanthomonas campestris* pv.

vesicatoria (*Xcv*). Macro and Stall (1983) first reported copper resistance in *Xcv* in Florida. Development of copper tolerance is widespread among populations of *Xcv* in several geographical locations. Copper-tolerant strains of *Xcv* have also been reported in Australia (Martin and Hamilton, 2004). The level of copper tolerance varies for different phytopathogenic bacteria and also methods used for screening sensitivity of strains towards copper vary, as described in Chapter 5. Several authors have used a threshold concentration of copper for identifying bacterial strains that are considered resistant or tolerant to copper as tabulated below.

Table 1.2 List of available figures on threshold levels of copper ion concentration, at or above which growth of various strains of phytopathogenic bacteria are considered copper tolerant.

Name of organism	Threshold level of Cu^{2+} (μg/ml)	Authors
Xcv	32	Macro and Stall (1983)
Pseudomonas syringae pv syringae	32	Sudin *et al.* (1989)
Pseudomonas syringae	20	Anderson *et al.* (1991)
Pseudomonas syringae	20	Scheck *et al.* (1996)
Xaj	30	Lee *et al.* (1993)
Xaj	32	Garden *et al.* (1993)
Xaj	20	Scortichini *et al.* (2001)
Xcv	64	Martin *et al.* (2004)

1.10.1 Genetics and molecular basis of copper resistance

The genetic basis of bactericide resistance has been studied since the mid-1980s. Resistance involves the inactivation of the bactericide or prevention of its entry into the cell. The development of bactericide resistance in bacterial communities does not require independent evolution of resistance by each stain or species, but rather, the community can evolve resistance by exchanging genetic information between strains, pathovars, species and genera (Cooksey, 1990). For example, homologous, plasmid borne copper–resistance genes (cop) have been found in *Xcv* isolated from plants in California and *P. syringae* pv *tomato*. Since cop homologs have not been found in other *Xanthomonas* strains, *Xcv* from California have acquired the copper resistant genes from *P. syringae* pv *tomato* or other species carrying cop genes. This supports the above statement that plant pathogens exchange genetic information for their successful survival as plant pathogens. Strains of a

bacterium that are resistant to copper or their genes are designated with the symbol Cu^r.

Voloudakis *et al.* (1993) has shown that there is homology between pCOP2, which carries Cu^r genes of *P. syringae* pv *tomato* and DNA of Cu^r and Cu^s of Xanthomonad strains. Lee *et al.* (1994) confirmed the above results indicating that *Xaj* shared homology with Cu^r genes of *P. syringae* pv. *tomato, P. syringae* pv. *syringae* and *Xcv.*

Different types of plasmid encoded resistance to copper have been demonstrated in phytopathogenic and other bacteria. Stall *et al.* (1986) found that strains of *Xcv* from Florida contained Cu^r genes born on the large plasmid, pXvCu. The plasmids were self-transmissible, polymorphic, variable in profiles of DNA digested with restriction enzymes and generally 200 kilobases (kb) or larger. Being self transmissible, their role in copper resistance can be assessed through conjugation experiments between copper resistant and sensitive strains (Stall *et al.*, 1986; Bender *et al.,* 1990). Cu^r strains of *Xcv* from Oklahoma contained plasmid, pXV10A, that shared a similar nucleotide sequence to pXvCu but there was no sequence homology with IS476, an insertion sequence present on pXvCu. Also plasmid pXV10A was successfully transferred to eight *Xanthomonas campestris* pathovars while there was no sign of the plasmid being transferred to other phytopathogenic genera (Bender *et al.*, 1990). In contrast, Cu^r genes in *Xcv* strains isolated from tomato plants and seeds in California are located on a plasmid that is not self transmissible, nearly 100 kb in size and does not have strong homology with pXvCu, but has homology with Cu^r genes in *Pseudomonas syringae* pv *tomato* (Cooksey *et al.*, 1990). In strains of *P. syringea* pv *tomato*, Cu^r genes were present on a 35 kb plasmid, pPT23D, which has never been shown to be self transmissible (Sudin *et al.,* 1989).

Copper resistant strains of *Xaj* from northern California were investigated for the genetic basis of copper resistance. Hybridization analysis revealed that Cu^r genes were located on a 4.9-kb ciaI fragment on the chromosome and shared a nucleotide sequence similarity with copper resistant genes from *Xanthomonas campestris* pv. *vesicatoria*, *Pseudmonas syringea* pv. *tomato* and *Pseudmonas syringea* pv *syringe.* Plasmid encoded copper resistance was not detected in Cu^r strains and mating with copper sensitive strains did not result in copper resistant conjugants. However, there is the possibility that Cu^r genes may be located on a high molecular weight nonmobilizable plasmid, which may have been sheared during DNA extraction (Lee *et al.*, 1994).

A similar study was conducted for determining the genetic basis of copper resistant strains of *Xaj* in France. Ten strains were analysed for their resistance to different antibiotics and heavy metals including copper. Strains found to be copper resistant were

then studied for their association with conjugative plasmids. It was found that three Cu^r strains were associated with large, self-transmissible plasmids varying in size. The plasmids were designated pXj Cu As, for those isolated from strains CFBP 1022 and Cu 16-2 and pXj Cu As Cd, isolated from strain W 19-2. The strain resistant to copper was also resistant to arsenic and one strain (w 19-1) was resistant to copper, arsenic and cadmium (Garden and Germain, 1993).

1.11 Summary and research objectives

In summary, walnut blight, caused by *Xanthomons arboricola* pv *juglandis*, is widespread and has become a major concern for the walnut industry worldwide, including Australia. The disease has been responsible for significant yield losses of more than 50%, especially in early leafing varieties of walnut in Tasmania (Lang and Evans, 2005). Walnut blight is controlled by multiple applications of copper containing bactericides, yet a satisfactory level of disease control under Tasmanian conditions has yet to be achieved (Hills and Lang, 2004). The possible reasons for poor disease control may be (a) application of ineffective bactericides (b) poor spray timing in relation to weather and crop stage and/or (c) the presence of copper tolerant populations of *Xaj*.

The main aim of this study was to estimate the range of copper sensitivity in populations of *Xaj* from Tasmania, by collecting isolates of *Xaj* from walnut fruit on a broad geographical scale. Within this overall aim, a specific objective was to confirm the identity of each bacterial isolate and relationships among them by characterising isolates in terms of their phenotype on semi-selective media, fatty acid profile and rep-PCR genotype.

General materials and methods

2.1 Origin of bacterial isolates

Webster Ltd, a large scale commercial producer of walnuts, manages 650 hectares of walnut orchards in Tasmania. The four main walnut growing regions are the East Coast, Coal River Valley, Central Coast and the Tamar Valley. The East Coast orchards are sited at Swansea and Cranbrook, the Coal River Valley orchards near Richmond and Penna, the Central Coast orchards between Devonport and Burnie, and the Tamar Valley orchards are near Rowella. The productions system for walnuts from these four regions is similar.

2.1.1 Sampling strategy

Walnut fruit with symptoms of bacterial blight were collected from four walnut production regions in Tasmania (Table 2.1). The symptoms were dark water-soaked sunken spots, brown to blackish in colour, from a few millimetres diameter to lesions that extended to over half of the fruit surface area. Diseased fruit were also collected from walnut trees in home gardens that had not been exposed to copper sprays, as indicated by the current landowners.

Table 2.1 Regions of Tasmania and the number of orchards in each region where diseased fruit were sampled.

Region	No. of orchards
East Coast	4
Coal River Valley	2
North West	4
Tamar Valley	2

Four trees were randomly selected within each orchard. From each tree a minimum of five symptomatic fruit were collected and placed together into one plastic bag per tree. Secateurs were rinsed with 100% ethanol between each tree sampled. The fruit in four plastic bags per orchard was stored in a cool container and transported directly to the laboratory. In the laboratory the samples were stored at 4°C and isolation was attempted within two days of sampling.

2.2 Isolation and culture maintenance

Each diseased fruit was swabbed with 70% ethanol and allowed to dry in a laminar air flow cabinet for 10 min. A piece of diseased fruit skin (hull) of approximately 3-4 mm^2 was aseptically excised from the margin of the necrotic lesion and suspended in 2 ml of sterile distilled water in a sterile microfuge tube. The suspended tissue was then agitated in water for 1 min on the flat bed adapter (Mo Bio Laboratories Inc., USA) of a Vortex Genie 2 set on maximum speed (Scientific Industries Inc, New York, USA). The suspension was allowed to rest for 15-20 min to allow bacteria to stream from the diseased tissue into the water. A loop full of the suspension was streaked onto nutrient agar (Murlean and Schroth, 1981; Du Plessis and Vander Westhuizen, 1995) and incubated at 28°C for 48 h. This isolation procedure was based partially upon the method described by Du Plessis and Westhuizen (1995). After 48 h, single bacterial colonies with yellow pigmentation were collected and restreaked onto nutrient agar to ensure purity. The recovered isolates were stored at -80°C in Microbank™ tubes according to the manufacturer's instructions (Pro-Lab Diagnostics, Texas, USA).

Glucose Yeast Extract Calcium Carbonate Agar (GYCA) was used as a general growth and maintenance medium (Fahy and Hayward 1983). The medium contained the following (g/L): glucose, 5; yeast extract, 5; $CaCO_3$, 40; and agar, 15. Calcium carbonate reduces the death rate of cells (Fahy and Hayward 1983) and was dispersed thoroughly in the medium immediately before pouring to ensure close contact with the surface of the agar.

Table 2.2 lists the origin of the 64 strains of bacteria that were isolated in this study, including five strains from various home gardens.

Table 2.2 Origin of bacterial isolates from walnut fruit with symptoms of bacterial blight.

Isolate	Orchard or garden no.	Latitude, Longitude	Region	Variety	Date isolated in 2005
Commercial orchards					
US 01 05	NW-1	41°10'45"S, 146°16'41"E	North West	Vina	2 Feb.
US 02 05	NW-1	41°10'45"S, 146°16'41"E	North West	Franquette	2 Feb.
US 04 05	TV-1	41°11'39"S, 146°55'29"E	Tamar Valley	Serr	17 Feb.
US 05 05	TV-1	41°11'39"S, 146°55'29"E	Tamar Valley	Serr	17 Feb.
US 06 05	TV-1	41°11'39"S, 146°55'29"E	Tamar Valley	Serr	17 Feb.
US 07 05	TV-1	41°11'39"S, 146°55'29"E	Tamar Valley	Serr	17 Feb.
US 08 05	TV-2	41°10'49"S, 146°55'00"E	Tamar Valley	Chandler	17 Feb.
US 09 05	TV-2	41°10'49"S, 146°55'00"E	Tamar Valley	Chandler	17 Feb.
US 10 05	TV-2	41°10'49"S, 146°55'00"E	Tamar Valley	Franquette	17 Feb.
US 11 05	TV-2	41°10'49"S, 146°55'00"E	Tamar Valley	Franquette	17 Feb.
US 12 05	TV-2	41°10'49"S, 146°55'00"E	Tamar Valley	Franquette	17 Feb.
US 13 05	TV-2	41°10'49"S, 146°55'00"E	Tamar Valley	Chandler	17 Feb.
US 14 05	TV-2	41°10'49"S, 146°55'00"E	Tamar Valley	Chandler	17 Feb.
US 15 05	TV-2	41°10'49"S, 146°55'00"E	Tamar Valley	Chandler	17 Feb.
US 17 05	NW-1	41°10'45"S, 146°16'41"E	North West	Vina	18 Feb.

US 18 05	NW-1	41°10'45"S, 146°16'41"E	North West	Vina	18 Feb.
US 19 05	NW-1	41°10'45"S, 146°16'41"E	North West	Vina	18 Feb.
US 20 05	NW-1	41°10'45"S, 146°16'41"E	North West	Vina	18 Feb.
US 22 05	NW-1	41°10'45"S, 146°16'41"E	North West	Vina	18 Feb.
US 23 05	NW-1	41°10'45"S, 146°16'41"E	North West	Franquette	18 Feb.
US 26 05	NW-2	41°05'24"S, 146°03'00"E	North West	Vina	1 Mar.
US 28 05	NW-2	41°05'24"S, 146°03'00"E	North West	Vina	1 Mar.
US 29 05	NW-2	41°05'24"S, 146°03'00"E	North West	Vina	1 Mar.
US 30 05	NW-3	41°05'57"S, 145°59'28"E	North West	Chandler	1 Mar.
US 31 05	NW-3	41°05'57"S, 145°59'28"E	North West	Chandler	1 Mar.
US 32 05	NW-3	41°05'57"S, 145°59'28"E	North West	Chandler	1 Mar.
US 39 05	NW-2	41°05'24"S, 146°03'00"E	North West	Vina	2 Mar.
US 40 05	NW-2	41°05'24"S, 146°03'00"E	North West	Vina	2 Mar.
US 41 05	NW-2	41°05'24"S, 146°03'00"E	North West	Vina	2 Mar.
US 42 05	NW-2	41°05'24"S, 146°03'00"E	North West	Vina	2 Mar.
US 43 05	NW-2	41°05'24"S, 146°03'00"E	North West	Vina	2 Mar.
US 44 05	NW-2	41°05'24"S, 146°03'00"E	North West	Vina	17 Mar.
US 45 05	EC-1	42°03'55"S, 148°03'04"E	East Coast	Vina	17 Mar.
US 46 05	EC-1	42°03'55"S, 148°03'04"E	East Coast	Vina	17 Mar.
US 47 05	EC-1	42°03'55"S, 148°03'04"E	East Coast	Vina	17 Mar.

US 48 05	EC-1	42°03'55"S, 148°03'04"E	East Coast	Vina	17 Mar.
US 49 05	EC-1	42°03'55"S, 148°03'04"E	East Coast	Vina	17 Mar.
US 50 05	EC-2	42°03'41"S, 148°03'24"E	East Coast	Serr	17 Mar.
US 51 05	EC-2	42°03'41"S, 148°03'24"E	East Coast	Serr	17 Mar.
US 52 05	EC-2	42°03'41"S, 148°03'24"E	East Coast	Serr	17 Mar.
US 53 05	EC-2	42°03'41"S, 148°03'24"E	East Coast	Serr	17 Mar.
US 54 05	EC-2	42°03'41"S, 148°03'24"E	East Coast	Serr	17 Mar.
US 56 05	EC-3	41°59'06"S, 148°04'54"E	East Coast	Vina	18 Mar.
US 57 05	EC-3	41°59'06"S, 148°04'54"E	East Coast	Vina	18 Mar.
US 58 05	EC-3	41°59'06"S, 148°04'54"E	East Coast	Vina	18 Mar.
US 59 05	EC-4	41°59'47"S, 148°04'50"E	East Coast	Serr	18 Mar.
US 60 05	EC-4	41°59'47"S, 148°04'50"E	East Coast	Serr	18 Mar.
US 62 05	EC-4	41°59'47"S, 148°04'50"E	East Coast	Serr	18 Mar.
US 63 05	CV-1	42°47'06"S, 147°28'06"E	Coal River Valley	Vina	24 Mar.
US 64 05	CV-1	42°47'06"S, 147°28'06"E	Coal River Valley	Vina	24 Mar.
US 65 05	CV-1	42°47'06"S, 147°28'06"E	Coal River Valley	Vina	24 Mar.
US 66 05	CV-1	42°47'06"S, 147°28'06"E	Coal River Valley	Vina	24 Mar.
US 67 05	CV-1	42°47'06"S, 147°28'06"E	Coal River Valley	Vina	24 Mar.
US 71 05	CV-2	42°42'24"S, 147°26'59"E	Coal River Valley	Chandler	24 Mar.
US 72 05	CV-2	42°42'24"S, 147°26'59"E	Coal River Valley	Chandler	24 Mar.

US 73 05	CV-2	42°42'24"S, 147°26'59"E	Coal River Valley	Chandler	24 Mar.
US 74 05	CV-2	42°42'24"S, 147°26'59"E	Coal River Valley	Chandler	24 Mar.
US 75 05	CV-2	42°42'24"S, 147°26'59"E	Coal River Valley	Chandler	24 Mar.
US 76 05	CV-2	42°42'24"S, 147°26'59"E	Coal River Valley	Chandler	24 Mar.
Home gardens					
US 03 05	HH-1	42°53'S, 147°18'E	West Hobart	unknown	2 Feb.
US 16 05	HTV-1	41°23'57"S, 146°55'55"E	Tamar Valley	unknown	17 Feb.
US 24 05	HNW-1	41°05'24"S, 146°03'00"E	North West	unknown	22 Feb.
US 25 05	HNW-1	41°05'24"S, 146°03'00"E	North West	unknown	22 Feb.
US 35 05	HNW-1	41°16'03"S, 146°12'09"E	North West	unknown	1 Mar.

Phenotypic characterisation

3.1 Introduction

A first step in identifying a bacterium isolated from diseased tissue is to describe its phenotype, beginning with colony morphology. The yellow colonies of *Xanthomonas* species usually produce copious amounts of extracellular polysaccharide, the source of "xanthan gum", on media containing glucose (Schaad *et al.* 2001). GYCA medium, described in Chapter 2, is a suitable medium for observing colony colour and mucoid growth of *Xanthomonas* species (Schaad *et al.* 2001). Given that many saprophytic bacteria isolated from plants are yellow in colour, the use of semi-selective media favours the growth of the putative causal organism and can allow differentiation from other species based on nutrition. Two semi-selective media used previously to characterise *X. arboricola* pv *juglandis* (*Xaj*) are Brilliant Cresyl Blue Starch (BS) medium and Succinate Quinate (SQ) medium. The property of hydrolysing starch in BS media (Murlean and Schroth, 1981) and ability to metabolise quinic acid to produce gallic acid, protocatechuic acid, pyrogallol and several other compounds in SQ media (Lee *et al.*, 1992) are two phenotypic characters associated with *Xaj*.

BS media was developed by Murlean and Schroth (1981) to study the epidemiology of *Xaj* because of the medium's ability to eliminate many of the microorganisms observed on more general growth media. Murlean and Schroth (1981) used BS media to quantify the nature and extent of walnut bud and catkin infestation by *Xaj* with regard to the overwintering of the bacterium.

SQ media was developed by Lee *et al.* (1992) based on the capacity of pathovars of *Xanthomonas campestris* to metabolize quinate. The test was conducted on five species of *Xanthomonas* and 77 pathovars of *Xanthomonas campestris* including 100 strains of *Xaj*. Results indicated that only four pathovars of *X. campestris* belonging to DNA homology group 6 were positive for the test with exception of *X. c.* pathovar *celebensis*, which belonged to DNA homology group 5. Strains tested were able to metabolize quinate, but could not utilise quinate as a sole carbon source for their growth.

Once basic phenotypic characters have been described, the three major techniques for identifying phytopathogenic bacteria are biochemical tests, fatty acid profiling and DNA sequencing. There are general biochemical tests for the genus *Xanthomonas* (Schaad *et al.* 2001) but there do not appear to be a range of tests specifically for the walnut blight bacterium. Fatty acid profiling is based on a characteristic of bacteria possessing a unique

complement of cellular fatty acids. Fatty acids between nine and 20 carbons in length can be used to characterise genera and species of bacteria, especially Gram negative organisms (Sasser, 2006). A technique applied routinely for bacterial identification is Gas Chromatograph Analysis of Fatty Acids Methyl Esters (GC-FAME, Sasser 2006). DNA-based technology typically uses the sequence of the 16S rRNA or other genes which are highly conserved at the species level but differences at the subspecies and strain level are not shown. Separation of bacterial isolates at the subspecies level has been achieved by sequencing several housekeeping genes or by sequence analysis of the 16S to 23S rRNA internal transcribed spacer (for example, refers to Barak and Gilbertson 2002). Other PCR-based methods for discrimination at the sub-specific level are reviewed by Louws *et al.* (1999). Application of DNA sequencing for species identification was beyond the scope of this project.

The objective of the research was to determine the phenotype of 64 strains of bacteria described in Chapter 2 by determining colony metabolism and growth on semi-selective media. Results of GC-FAME analysis of a sub-set of these isolates, by Dorothy Noble, NSW Department of Primary Industries, are reported concurrently for interpretation of phenotypes observed.

3.2 Materials and Methods

3.2.1 Metabolism of bacterial isolates on BS and SQ media

All 67 bacterial strains isolated in this study were streaked onto SQ and BS media in 9-cm diameter Petri plates, incubated at 28° C and scored as described below.

SQ media contained the following (g/L): succinic acid, 10; quinic acid, 5; K_2HPO_4, 1.5; $(NH_4)_2SO_4$, 1.0 and agar, 15. The medium was adjusted to pH; 7.2-7.5 with 10 N NaOH and autoclaved at 120 °C for 20 min. After sterilisation, 7.5 ml of sterile 20% $MgSO_4$ was added when the medium had cooled to 50°C. Colony morphology and reaction with the medium was scored after incubation at 28° C for 4-5 days. The diffusion of a deep green colour around the bacterial colonies was considered a positive reaction for ability of the bacteria to metabolize quinate. However, the intensity of colour development varied among isolates and so the reaction with the medium was categorised as follows:

Positive reaction with a deep green colour: +++

Positive with a mid-green colour: ++

Positive with a pale green or yellow colour: +

Negative reaction (no green colour): -

BS media contained the following (g/L): potato starch, 10; $K_2HPO_4.3H_2O$, 3.0; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 2.0; L-methionine, 0.25; nicotinic acid, 0.25; L-glutamate, 0.25; brilliant blue cresyl, 0.01; methylene green, 0.01 and agar, 15. The medium was adjusted to pH 6.8-7.0 and autoclaved at 120°C for 20 min. The reaction of the bacterial isolate with the medium was scored after incubation at 28°C for 6-7 days. Greenish blue colonies surrounded by a distinct opaque zone where the medium has changed colour due to starch hydrolysis was considered a positive reaction. Some bacterial strains produced a small opaque zone and so the following scoring system was applied:

Positive reaction with a distinct opaque zone: ++

Positive reaction with a small opaque zone: +

Negative reaction: -

The growth of each bacterial strain on both SQ and BS media was characterised qualitatively in terms of the number and size of colonies as strong, moderate or weak.

3.2.2 Colony morphology on GYCA media

As described previously, GYCA medium was used as a general growth and maintenance medium. It was noted during routine transfers that isolates identified as a *Xanthomonas* species by GC-FAME produced shiny mucoid yellowish bacterial colonies on GYCA. A subset of 22 strains isolated was tested for this character. After incubation at 28°C for 48 h, colony form, texture and colour was noted, especially the presence or absence of yellow mucoid bacterial colonies.

3.2.3 GC-FAME

A subset of 45 of the 64 isolates was selected based on their reaction to BS and SQ media and analysed by GC-FAME. This analysis was conducted by Ms Dorothy Noble, Senior Technical Officer, NSW Department of Primary Industries, according to procedures of the Sherlock® Microbial Identification System Version 4.5 (Anon 2005). In summary, fatty acids were cleaved from lipids by saponification and then methylated to produce methyl esters for analysis. The methyl esters were extracted and applied to the column of the gas chromatograph. The elution time for each methyl ester was converted to Equivalent (carbon) Chain Length (ECL) for bacterial fatty acid naming. The ECL value was derived as a function

of its elution time in relation to the elution times of a known series of straight chain fatty acids. In the Sherlock® Microbial Identification System (Anon 2005), analysis of an unknown sample results in an automatic comparison of the composition of an unknown strain to a database containing profiles of more than 100,000 strains of over 1,500 bacterial species from culture collections around the world (Sasser 2006). For common taxa, analyses for 20 or more strains of a species or subspecies are present in the database (Sasser 2006). Identification is based on comparison with the profile library; therefore, sample identification is not definitive. The similarity index (SI, Anon 2005), a numerical value between 0.000 and 1.000, indicates how closely the bacterium compares to fatty acid profiles in the library collection. The algorithm for the SI is not specified by the Sherlock® MIS (Anon 2005); however, analysis assumes that species of microorganisms have a normal distribution for their fatty acid profiles and that the mean of the population for fatty acid percentages characterises the group. Therefore, the similarity index is not a "probability", but an expression of the relative distance of the unknown sample to the population mean. Bacterial identifications are ranked in order based on similarity indices. With this in mind, samples with a SI of 0.600 or higher and with a separation of 0.100 between the first and second choice were considered good library comparisons.

3.3 Results

Table 3.1 lists the phenotypic characters observed on the semi-selective SQ and BS media of 67 strains of bacteria isolated from blight lesions on walnut fruit in Tasmania. Figures 3.1 and 3.2 illustrate the typical maximum reaction observed on the respective media. Fifty two of the 67 bacterial isolates produced a dark green colour in the SQ medium indicating their ability to metabolise quinate. Seven of the 67 bacterial strains (US 16 05, US 23 05, US 30 05, US 40 05, US 60 05, US 74 05, US 76 05) were rated as '++' producing a moderate green colour rather than a dark green colour on SQ media. Five of the 67 bacterial strains, (US 08 05, US 42 05, US 58 05, US 59 05, US 66 05) were rated as '+' producing a mild green or bright yellow colour. The remaining three bacterial isolates, US 04 05, US 35 05 and US 63 05, were not able to metabolize quinate. In general, the growth of all strains was strong on SQ media except for three isolates which grew weakly (US 04 05, US 56 05, US 63 05). Two of these three isolates also had a negative reaction on SQ media.

Table 3.1 Colony metabolism, growth and morphology on semi-selective media for 67 isolates of bacteria from blight lesions on walnut fruit from various locations in Tasmania. SQ = Succinate Quinate medium and BS = Brilliant Cresyl Blue Starch medium. The putative identity of bacterial isolates is presented in Table 3.3. Isolate codes in italics represent isolates that were not identified by GC-FAME. Isolates in bold text were non–Xanthomonads based on GC-FAME analysis and the genus identity is listed underneath the isolate code.

Isolate	**Colony metabolism & growth on SQ media**		**Colony metabolism & growth on BS media**	
	Reaction[a]	**Growth**	**Reaction[b]**	**Growth**
US 01 05	+++	strong	++	moderate
US 02 05	+++	strong	++	moderate
US 03 05	+++	strong	+	moderate
Pseudomonas sp.				
US 04 05	-	weak	-	weak
US 05 05	+++	strong	++	moderate
US 06 05	+++	moderate	++	moderate
Serratia sp.				
US 07 05	+++	strong	++	strong
US 08 05	+	strong	++	strong
Pseudomonas sp.				
US 09 05	+++	strong	++	strong
US 10 05	+++	strong	++	moderate
US 11 05	+++	strong	++	strong
US 12 05	+++	strong	++	strong
US 13 05	+++	strong	++	moderate
US 14 05	+++	strong	++	strong
US 15 05	+++	strong	++	moderate
US 16 05	++	strong	++	moderate
US 17 05	+++	strong	++	moderate
Serrratia sp.				
US 18 05	+++	strong	++	moderate
US 19 05	+++	strong	++	strong
US 20 05	+++	strong	++	moderate

US 22 05	+++	strong	++	strong
US 23 05	++	strong	+	moderate
US 24 05	+++	strong	++	strong
US 25 05	+++	strong	++	moderate
US 26 05	+++	strong	++	moderate
US 28 05	+++	strong	+	moderate
US 29 05	+++	strong	++	moderate
US 30 05	++	strong	++	moderate
US 31 05	+++	strong	++	moderate
US 32 05	+++	strong	+	weak
Erwinia sp.				
US 33 05	+++	strong	++	weak
US 35 05	-	strong	++	strong
US 39 05	+++	moderate	++	weak
US 40 05	++	moderate	++	weak
US 41 05	+++	strong	++	weak
US 42 05	+	strong	++	moderate
US 43 05	+++	strong	++	weak
US 44 05	+++	strong	++	weak
US 45 05	+++	strong	+	strong
Pantoea sp.				
US 46 05	+++	strong	+	strong
US 47 05	+++	strong	++	moderate
US 48 05	+++	strong	++	strong
US 49 05	+++	strong	++	weak
US 50 05	+++	strong	++	weak
US 51 05	+++	strong	++	moderate
US 52 05	+++	strong	++	strong
US 53 05	+++	strong	++	strong
US 54 05	+++	strong	++	moderate
US 56 05	+++	weak	++	weak
US 57 05	+++	strong	++	moderate
US 58 05	+	moderate	+	weak

Erwinia sp.				
US 59 05	+	strong	+	weak
US 60 05	++	strong	++	strong
US 61 05	+++	strong	++	strong
US 62 05	+++	strong	++	strong
US 63 05	-	weak	-	weak
US 64 05	+++	strong	++	strong
US 65 05	+++	strong	++	strong
US 66 05	+	strong	++	moderate
US 67 05	+++	strong	++	moderate
US 68 05	+++	moderate	++	strong
US 71 05	+++	strong	++	strong
US 72 05	+++	moderate	++	strong
US 73 05	+++	strong	++	strong
US 74 05	++	moderate	++	strong
US 75 05	+++	strong	++	moderate
US 76 05	++	strong	++	strong

[a]+++ deep green; ++ mid green; + pale green or yellow; - no colour development

[b]++ distinct opaque zone; + small opaque zone; - no opaque zone

Figure 3.1 Illustration of the maximum reaction on BS media with greenish blue colonies forming clear opaque zones

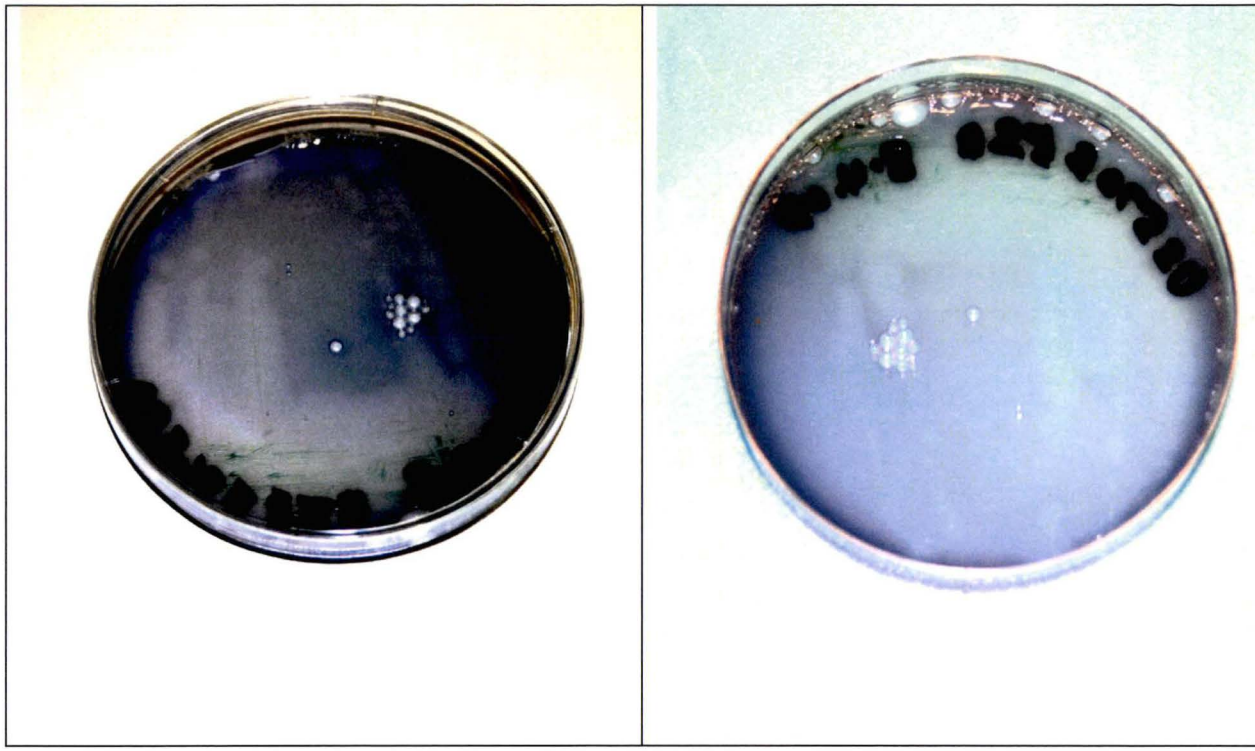
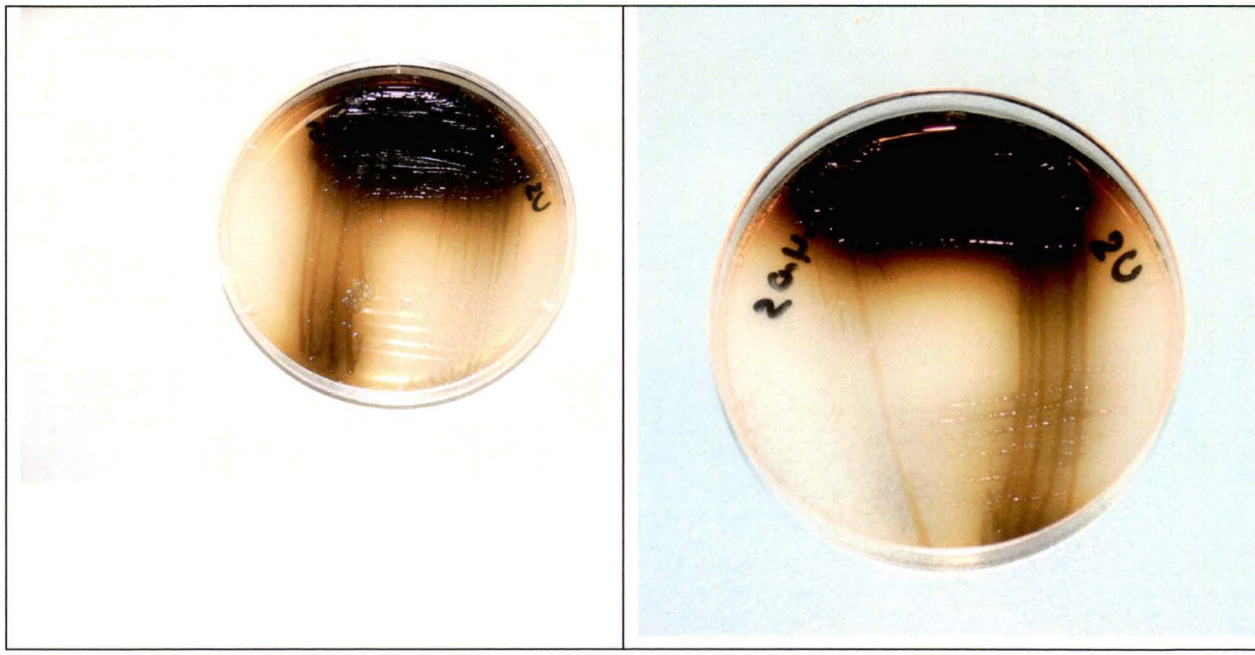


Figure 3.2 Illustration of the maximum reaction on SQ media with diffusion of green colour around bacterial colonies



On BS media, 57 of 67 strains were rated '++', producing clear opaque zones which is an indication of their ability to hydrolyze starch. Although 57 strains showed a positive reaction, almost 41 of 67 strains had poor to moderate growth on BS media. Eight strains (US 03 05, US 08 05, US 23 05, US 28 05, US 32 05, US 45 05, US 46 05, US 58 05, US 59 05) showed a poor response to BS media and were rated '+' for producing small opaque zones. Two of the 67 strains (US 04 05, US 63 05) were rated '-', and did not form a pale opaque zone around bacterial colonies indicating their inability to hydrolyze starch.

All 22 strains of bacteria inoculated on GYCA media grew well (Table 3.2). Sixteen of those strains produced mucoid yellowish colonies (Table 3.2) and 15 of these 16 strains were identified by GC-FAME as *Xanthomonas* species (Table 3.3). The non-Xanthomonad strain that produced mucoid yellow colonies on GYCA was US 17 05 (putatively *Serratia plymuthica*, Table 3.3). The other six strains were non-Xanthomonads and produced orange or bright yellow colonies that were not mucoid. An example of the difference in colony morphology is illustrated in Figure 3.3.

Figure 3.3 Example of the difference in colony morphology (incubated at 28° C for 48 h) between (a) *Serratia liquefaciens* (US 06 05) and (b) *Xanthomonas arboricola* (US 10 05) on GYCA media. The yellow colonies of isolate US 10 05 have a distinct mucoid appearance.

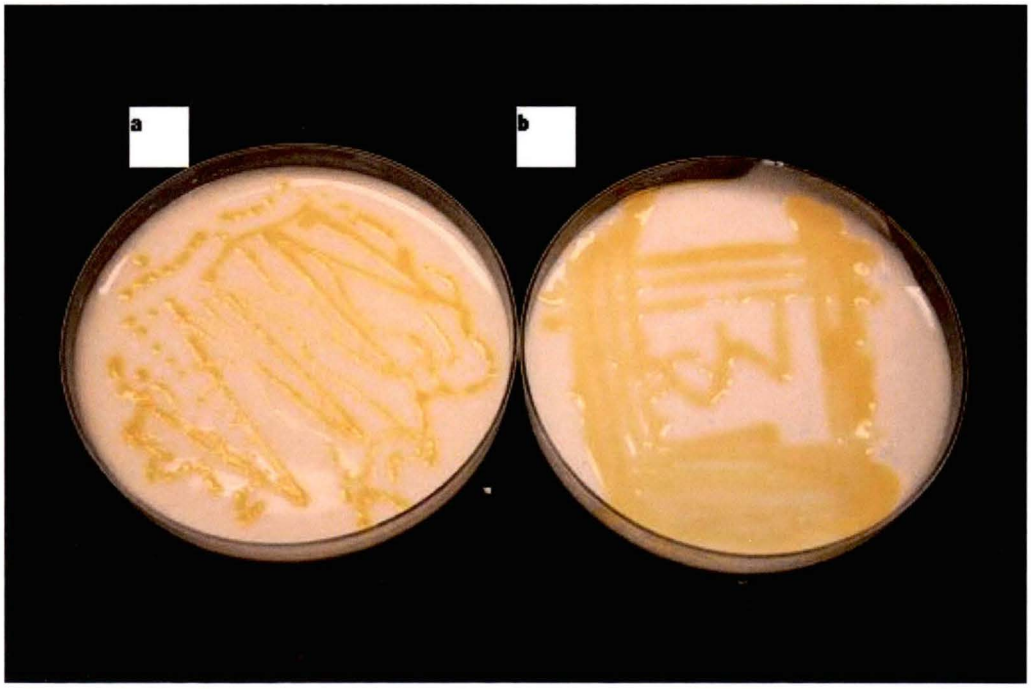


Table 3.2 Colony morphology on GYCA medium for a subset of 22 bacterial isolates from blight lesions on walnut fruit from various locations in Tasmania.

Isolate	Presence of mucoid yellow growth on GYCA
US 01 05	Yes
US 02 05	Yes
US 03 05	No
US 04 05	Yes
US 05 05	Yes
US 06 05	No
US 07 05	Yes
US 08 05	No
US 10 05	Yes
US 16 05	Yes
US 17 05	Yes
US 23 05	Yes
US 32 05	No
US 35 05	Yes
US 44 05	Yes
US 45 05	No
US 50 05	Yes
US 53 05	Yes
US 56 05	Yes
US 58 05	No
US 63 05	Yes
US 66 05	Yes

Of the 45 bacterial isolates analysed by GC-FAME, only one isolate (US 08 05) that appeared to be a non-Xanthomonad had a similarity index (SI) of less than 0.6 to profiles in the database (Table 3.3). Thirteen isolates were considered a good comparison to the Sherlock database: 11 isolates were putatively named *X. arboricola* pv *pruni*, one isolate was putatively named *X. arboricola* pv *juglandis* (US 56 05) and the remaining two isolates were non-Xanthomonads (US 03 05 *Pseudomonas stutzeri* and US 58 05 *Erwinia rhapontici*). A further nine isolates (excluding US 10 05 Aug. 2006) had their top two SIs separated by less than 0.100 (one of the putative names in each case was *X. arboricola* pv. *pruni* or *X. arboricola* pv *juglandis*). For isolates that had three or more similar values of SI (separated by less than 0.100), 15 isolates (excluding US 23 05 Aug. 2006) had either *X. arboricola* pv. *pruni* or *X.*

arboricola pv *juglandis* in the listed identifications, one isolate was listed as another pathotype of *X. arboricola* (US 33 05) and one isolate had *Xanthomonas* species other than *X. arboricola* in the list (US 22 05). The remaining four isolates with multiple names and values of similarity index (SI) separated by less than 0.100 appeared to be non-Xanthomonads (US 06 05, US 1705, US 32 05 and US 45 05). Isolates US 10 05 and US 23 05 were analysed on two separate occasions and were putatively identified as *X. arboricola* each time. In summary, the name *X. arboricola* was associated with varying indices of similarity to 37 of the 45 isolates analysed by GC-FAME. One isolate had *Xanthomonas* species other than *X. arboricola* in the list and the genera associated with the remaining seven isolates included *Pseudomonas*, *Serratia*, *Erwinia* and *Pantoea*. Five of these seven strains reacted positively to the SQ test and three of them gave a positive reaction on BS media. One strain, US 35 05, named *X. arboricola*, reacted negatively to the SQ test, but gave a positive reaction on BS media.

Table 3.3 Putative identification of bacterial isolates according to GC-FAME using Sherlock Version 4.5. The analysis conducted by Dorothy Noble, NSW Department of Primary Industries. Bacterial identifications are listed in order of similarity index for values within 0.100 of the highest similarity index. A single listing (in bold text) represents identification where the difference in SI to the next name on the list was more than 0.100. The star (*) represents a change to the library entry since the previous version of the library. Isolates US 10 05 nad US 23 05 were analysed on two separate occasions as indicated by the dates.

Isolate	Putative identification	Similarity Index
US 01 05	**Xanthomonas arboricola pruni**	0.844
US 02 05	**Xanthomonas arboricola pruni**	0.783
US 03 05	**Pseudomonas stutzeri (includes P. perfectomarina)**	0.664
US 05 05	Xanthomonas arboricola juglandis	0.799
	Xanthomonas arboricola pruni	0.790
US 06 05	Serratia liquefaciens*	0.757
	Serratia plymuthica*	0.709
	Erwinia rhapontici	0.696
	Rhanella aquatilis*	0.680
	Alcaligenes piechaudii*	0.677
	Hafnia alvei*	0.671
US 08 05	Pseudomonas aeruginosa*	0.460
	Pseudomonas stutzeri* (includes P. perfectomarina)	0.368
US 09 05	Xanthomonas arboricola pruni	0.842
	Xanthomonas arboricola celebensis-GC subgroup A	0.808
US 10 05 Nov. 2005	**Xanthomonas arboricola pruni**	0.835
US 10 05 Aug. 2006	Xanthomonas arboricola pruni	0.846
	Xanthomonas arboricola celebensis-GC subgroup A	0.772
US 11 05	Xanthomonas axonopodis vitians	0.717
	Xanthomonas axonopodis manihotis	0.674
	Xanthomonas arboricola pruni	0.661
	Xanthomonas fragariae (48 hr)	0.649
	Xanthomonas axonopodis alfalfae	0.642
	Xanthomonas arboricola celebensis-GC subgroup A	0.619
US 12 05	Xanthomonas arboricola celebensis-GC subgroup A	0.799
	Xanthomonas arboricola pruni	0.759
	Xanthomonas axonopodis vitians	0.730
US 13 05	Xanthomonas axonopodis vitians	0.721
	Xanthomonas arboricola celebensis-GC subgroup A	0.717
	Xanthomonas arboricola corylina	0.669
	Xanthomonas arboicola pruni	0.623

US 14 05	Xanthomonas arboricola pruni	0.791
	Xanthomonas hortorum pelargonii	0.705
US 15 05	Xanthomonas arboricola celebensis-GC subgroup A	0.781
	Xanthomonas axonopodis vitians	0.732
	Xanthomonas arboricola pruni	0.719
	Xanthomonas arboricola corylina	0.699
US 16 05	Xanthomonas arboricola juglandis	0.758
	Xanthomonas arboricola pruni	0.739
	Xanthomonas arboricola corylina	0.658
US 17 05	Serratia plymuthica*	0.702
	Enterobacter agglomerans – GC subgroup I (see also	0.671
	Pantoea)	0.662
	Serratia liquefaciens*	0.651
	Rhanella aquatilis*	0.641
	Erwinia rhapontici*	0.641
	Hafnia alvei*	0.628
	Yersinia psuedotuberculosus*	
US 19 05	Xanthomonas axonopodis vitians	0.680
	Xanthomonas arboricola celebensis-GC subgroup A	0.672
	Xanthomonas arboricola corylina	0.643
	Xanthomonas arboricola pruni	0.638
	Xanthomonas axonopodis alfalfae	0.610
	Xanthomonas axonopodis manihotis	0.599
	Xanthomonas fragariae (48 hr)	0.592
US 20 05	Xanthomonas arboricola celebensis-GC subgroup A	0.721
	Xanthomonas arboricola pruni	0.712
	Xanthomonas arboricola corylina	0.704
	Xanthomonas axonopodis vitians	0.698
US 22 05	Xanthomonas fragariae (48hr)	0.672
	Xanthomonas axonopodis manihotis	0.627
	Xanthomonas axonopodis vitians	0.619
	Xanthomonas axonopodis alfalfae	0.604
	Xanthomonas axonopodis carotae	0.599
	Xanthomonas axonopodis phaseoli	0.574
US 23 05	Xanthomonas arboricola juglandis	0.798
Nov. 2005	Xanthomonas arboricola pruni	0.769
US 23 05	Xanthomonas arboricola pruni	0.768
Aug. 2006	Xanthomonas arboricola celebensis-GC subgroup A	0.763
	Xanthomonas axonopodis vitians	0.751
	Xanthomonas axonopodis manihotis	0.679

US 25 05	**Xanthomonas arboricola pruni**	0.856
US 26 05	Xanthomonas arboricola pruni	0.756
	Xanthomonas arboricola celebensis-GC subgroup A	0.748
	Xanthomonas axonopodis vitians	0.696
	Xanthomonas arboricola corylina	0.691
US 28 05	**Xanthomonas arboricola pruni**	0.839
US 31 05	Xanthomonas axonopodis alfalfae	0.625
	Xanthomonas axonopodis vitians	0.617
	Xanthomonas fragariae (48 hr)	0.614
	Xanthomonas arboricola corylina	0.611
	Xanthomonas axonopodis manihotis	0.605
	Xanthomonas arboricola pruni	0.595
	Xanthomonas axonopodis phaseoli	0.578
	Xanthomonas arboricola celebensis-GC subgroup A	0.573
US 32 05	Erwinia rhapontici	0.765
	Hafnia alvei*	0.750
	Yersinia pseudotuberculosis*	0.678
	Serratia fonticola	0.676
US 33 05	Xanthomonas fragariae (48hr)	0.615
	Xanthomonas axonopodis vitians	0.572
	Xanthomonas axonopodis carotae	0.556
	Xanthomonas axonopodis manihotis	0.553
	Xanthomonas axonopodis alfalfae	0.546
	Xanthomonas arboricola corylina	0.538
	Xanthomonas arboricola celebensis-GC subgroup A	0.537
	Xanthomonas axonopodis phaseoli	0.526
US 35 05	Xanthomonas arboricola pruni	0.798
	Xanthomonas arboricola celebensis-GC subgroup A	0.711
	Xanthomonas axonopodis vitians	0.700
US 39 05	Xanthomonas arboricola celebensis-GC subgroup A	0.743
	Xanthomonas arboricola pruni	0.723
	Xanthomonas axonopodis vitians	0.699
	Xanthomonas arboricola corylina	0.673
US 41 05	Xanthomonas arboricola celebensis-GC subgroup A	0.743
	Xanthomonas arboricola pruni	0.733
	Xanthomonas axonopodis vitians	0.723
	Xanthomonas arboricola corylina	0.691
US 43 05	Xanthomonas arboricola celebensis-GC subgroup A	0.729
	Xanthomonas arboricola corylina	0.728
	Xanthomonas axonopodis vitians	0.712
	Xanthomonas arboricola pruni	0.690
US 44 05	Xanthomonas arboricola pruni	0.836

	Xanthomonas arboricola celebensis-GC subgroup A	0.737
US 45 05	Pantoea agglomerans*	0.833
	Erwinia rhapontici	0.820
	Serratia liquefaciens*	0.805
	Serratia grimesii	0.748
US 47 05	Xanthomonas arboricola celebensis-GC subgroup A	0.820
	Xanthomonas arboricola pruni	0.769
US 48 05	Xanthomonas arboricola pruni	0.834
	Xanothomonas axonopodis dieffenbachiae	0.792
US 49 05	Xanthomonas arboricola pruni	0.783
	Xanthomonas arboricola celebensis-GC subgroup A	0.754
	Xanthomonas arboricola corylina	0.743
	Xanthomonas hortorum pelargonii	0.713
US 50 05	Xanthomonas arboricola pruni	0.827
	Xanthomonas arboricola celebensis-GC subgroup A	0.747
US 53 05	**Xanthomonas arboricola pruni**	0.817
US 56 05	**Xanthomonas arboricola juglandis**	0.760
US 58 05	**Erwinia rhapontici**	0.875
US 62 05	**Xanthomonas arboricola pruni**	0.832
US 63 05	Xanthomonas arboricola pruni	0.808
	Xanthomonas arboricola juglandis	0.758
US 64 05	Xanthomonas arboricola pruni	0.808
	Xanthomonas arboricola celebensis-GC subgroup A	0.789
	Xanthomonas arboricola corylina	0.733
US 65 05	**Xanthomonas arboricola pruni**	0.825
US 71 05	**Xanthomonas arboricola pruni**	0.850
US 74 05	**Xanthomonas arboricola pruni**	0.827
US 75 05	Xanthomonas arboricola juglandis	0.730
	Xanthomonas hortorum pelargonii	0.716
	Xanthomonas arboricola pruni	0.702
	Xanothomonas axonopodis dieffenbachiae	0.680

3.4 Discussion

The results of GC-FAME were interpreted with the knowledge that the matching of an unknown fatty acid profile to ones in the Sherlock data base was only as good as the collection of typed strains in the database. The frequent isolation of *Xanthomonas arboricola* from blight lesions on walnut fruit confirms previous findings that this species is associated with blight symptoms. A sub-optimal technique for surface sterilisation might explain the relatively infrequent isolation of common epiphytic bacteria such as *Erwinia* and *Pantoea* (J. Vanneste, HortResearch NZ, personal communication). The apparent and frequent isolation of pathovar *pruni* relative to *juglandis* should be treated with caution because G-C FAME may be unreliable at discriminating *X. arboricola* at the pathovar level. Yang *et al.* (1993) analysed the whole cell fatty acid content of nearly one thousand strains of the genus *Xanthomonas* by gas-liquid chromatography and identified 21 different fatty acids within *Xaj*. Of the 31 major clusters identified by Yang *et al.* (1993), *Xaj* was included in the second largest cluster with *X. campestris* pathovars *aberrans, armoraciae, barbareae, campestris, carotae, cassiae, celebensis, corylina, eucalypti, guizotiae, incanae, manihotis, papavericola, phaseoli, poinsetticola, pruni, raphani, vesicatoria, vitians* and *zinniae.* In the G-C FAME analyses reported here, pathovars *carotae, celebensis, corylina, manihotis, phaseoli* and *vitians* were often in the same list as *pruni,* whereas *juglandis* was in the same list as *pruni* and/or *corylina. X. arboricola* pathovars *juglandis, corylina* and *pruni* appear to be closely related with an average DNA homology of 89% (Vauterin *et al.* 1995).

Xanthomonads recognized as distinct plant pathogens have often been designated as pathovars in *Xanthomonas campestris*, including the causal organism of walnut blight, *X. campestris* pv. *juglandis* (Pierce 1901) Dye (Dye *et al.* 1980). The term "pathovar" was proposed in 1978 as a temporary means of preserving the names of bacterial plant pathogens that could not easily be differentiated from all other species (Schaad *et al.* 2001). Using DNA homology and phenotypic data, Vauterin *et al.* (1995) proposed fourteen new *Xanthomonas* species including *X. arboricola* pv. *juglandis* for the walnut blight pathogen. According to Schaad *et al.* (2001), the pathovar epithet remains a temporary solution to naming plant pathogens until phylogenetic studies can be used to determine species more precisely. Based on the results of G-C FAME, analysis of DNA for investigating genetic relatedness among isolates of *Xanthomonas* from walnut blight lesions is warranted.

The results supported previous findings that BS and SQ media were semi-selective for the isolation of *Xanthomonas arboricola.* Bacterial isolates that were not Xanthomonads gave a positive reaction on both media and two isolates of *Xanthomonas,* putatively *X. arboricola*

(US 35 05, US 63 05), did not appear to metabolise quinate on SQ media. This result was somewhat contrary to the findings of Lee *et al.* (1992) who found that all 100 isolates of *Xaj* from different locations could metabolise quinate on SQ media. Isolate US 63 05 grew weakly on both SQ and BS media, which may have explained its lack of reaction on both media. The genetic relationship between isolate US 35 05 and other isolates named *X. arboricola* should be investigated further. Although quinate metabolism is generally stable among *Xaj,* this phenotypic property is shared by three other members of DNA homology group 6; namely *X.c. celebensis, X.c. corylina* and *X.c. pruni.* Most strains of DNA homology group 5, *X.c. carotae,* also metabolise quinate and one strain of *X.c. fragariae* has also produced a positive result to the SQ test (Lee *et al.*, 1992). Fluorescent pseudomonads (eg. *P.s. syringea*) utilized quinate for growth and produced a yellow colour change on SQ media (Lee *et al.*, 1992). In this study few non -Xanthomonads isolated from walnut blight lesions were able to metabolise quinate, for example, species of *Erwinia* (US 58 05) and *Pseudomonas* (US 08 05) and the results were consistent with the findings of Lee and Chou (2003) and Lee *et al.* (1992). An explanation for why quinate is metabolized but is still not being utilized as a source for growth is yet to be established. Furthermore, the range of phytopathogenic and saprophytic bacteria that can metabolise quinate is unknown.

A number of pathovars of *X. campestris* can be recovered well on BS medium, including *juglandis, malvacearum, phaseoli, pruni* and *vesicatoria. P. syringea* is frequently observed as a contaminant, although it cannot hydrolyse starch (Murlean and Schroth, 1981). Strains US 03 05 and US 08 05 from Tasmania were Pseudomonads and hydrolysed starch to a small extent, but their growth on BS medium was weak. BS medium was found to be ineffective for isolation and diagnosis of *Xaj* in South Africa (Du Plessis and Van Der Westhuizen, 1995), possibly because of an absence of hydrolytic activity (Scortichini *et al.* 2001). In general, growth of *Xaj* was moderate on BS media compared to SQ, GYCA and Nutrient agar (NA). Given the relatively slow rate of growth of Xanthomonads on BS media reported here, it was concluded that the BS medium was less useful than other media in selecting *Xanthonomonas* sp. from walnut blight lesions.

All bacterial isolates grew well on GYCA and it appeared to be a useful semi-selective medium for the isolation of *Xanthomonads* from walnut blight lesions. Scortichini *et al.* (2001) used GYCA for isolation of *Xaj* based on its characteristic mucoid growth on this medium. GYCA media has also been used to isolate *X.a* pv *fragariea* which also produced a mucoid bacterial growth (Scortichini and Rossi, 2002). In this study, all 15 strains of *Xanthomonas* and one of seven non-Xanthomonads tested produced a mucoid growth on

GYCA media. Hence, mucoid growth on GYCA may provide an efficient preliminary screening tool to separate non Xanthomonds from Xanthomonads. SQ and BS media may be used for further characterisation of mucoid colonies, although analysis by GC-FAME, DNA sequencing and/or DNA genotyping appears to be necessary for determining the identity of the isolate.

Genetic characterisation

4.1 Introduction

The limitations of phenotypic methods and GC-FAME in identifying the cause of bacterial blight of walnut were highlighted in Chapter 3. Semi-selective media aided isolation of *Xanthomonas* species at a high frequency and the results of GC-FAME provided evidence that *Xanthomonas arboricola* (*Xa*) was isolated frequently. However, GC-FAME did not appear to define variation of *Xa* at the pathovar or sub-specific level with confidence. DNA based methods can assist in the identification and classification of bacteria at various levels of taxonomic resolution. Genera and species can be assigned using DNA-DNA hybridisation methods (Vauterin and Swings, 1997) and bacterial phylogeny is often based on 16S rRNA sequence analysis (for example, Nesme *et al.*, 1995; Lee *et al.*, 1997). The polymerase chain reaction (PCR) has become the basic tool in generating molecular phylogenies.

DNA fingerprinting or genotyping is used for characterising genetic variation within and among species and for supporting taxonomic treatment. Repetitive sequence based PCR (rep-PCR) fingerprinting is a DNA amplification technique that uses primers that are complementary to highly conserved, repetitive DNA sequences present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria (Lupski and Weinstock 1992; Versalovic *et al.* 1994; Louws *et al.* 1996). The method, collectively known as rep-PCR, allows differentiation to the level of species, subspecies and strain and appears to be rapid, reproducible and highly discriminatory (for example, Louws *et al.* 1994). Three families of repetitive sequences have been identified: the 35-40 base pair (bp) repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX element (Versalovic *et al.* 1991, 1994). Oligonucleotide primers have been designed to prime DNA synthesis outward from the inverted repeats in REP and ERIC, and from the boxA subunit of BOX in the PCR (Versalovic *et al.* 1994). The method is referred to as REP-PCR, ERIC-PCR or BOX-PCR genomic fingerprinting, and the term rep-PCR is used to describe PCR genomic fingerprinting in a collective sense.

Louws *et al.* (1996) utilized REP-, ERIC- and BOX- PCR to generate genomic fingerprints of diverse *Xanthomonas* and *Pseudomonas* strains to differentiate them at pathovar level, and to characterise strains that were previously not distinguished by other phenotypic characteristics. For example, *Xanthomonas oryzae* pv *oryzae* and pv *oryzocola* are

closely related to each other based on studies of various characters, but were easily distinguished by rep-PCR. Similarly, *Pseudomonas syringae* pv *syringae*, an economically insignificant pathogen of tomato and *P. syringae* pv *tomato*, an economically significant pathogen produced fingerprint profiles that clearly distinguished the two pathovars. In addition, differences among other pathovars of *Xanthomonas* and *Pseudomonas* and dissimilarities between strains within a pathovar were studied by utilizing all three primer sets. The distribution of REP, ERIC and BOX sequences was found to be a true reflection of genomic structure.

Repetitive sequence PCR was first applied to study genetic variation in *Xanthomonas arboricola* pv. *juglandis* (*Xaj*) by Scortichini *et al.* (2001). Genetic diversity and three genetic groups were observed among 61 isolates of *Xaj* from Italy, Greece, Iran, France, Spain, Portugal, Hungary, Romania, The Netherlands, United Kingdom, Iran, USA (California) and New Zealand. Although each walnut region sampled appeared to have a different *Xaj* population, the overall genetic similarity of *Xaj* strains was high with two genetic groups being approximately 85% similar and the third genetic group being 78% similar to the other two groups. The isolates from New Zealand and the USA clustered with the isolates from European countries. Xanthomonads used as outgroups showed the following similarities to *Xaj*: *X. campestris* (*Xc*) pv *campestris* 43%, *Xc* pv *phaseoli* 40% and *X. vesicatoria* 39%, demonstrating the ability of rep-PCR to discriminate *Xaj* from these closely related phytopathogenic bacteria.

The aims of this research were to develop a rep-PCR protocol for bacteria isolated from lesions on walnut fruit and to characterise, in a preliminary manner, genetic variation among bacterial isolates from Tasmania in relation to their identification by GC-FAME.

4.2 Materials and Methods

4.2.1 Extraction of DNA from isolates of *Xanthomonas* from walnut fruit

Nucleic acids were extracted from each bacterial isolate cultured on Tryptone Soya Agar (Oxoid), for 24-48 h. This culture medium with its low level of carbohydrate was selected for minimising the amount of extra-cellular polysaccharide produced by the bacterium. Bacterial cells were removed from the medium by scraping the equivalent of one loop full. These cells were suspended in 1 ml of resuspension buffer (RB, 150 mM NaCl, 50 mM EDTA pH 8, and 50 mM Tris.HCl pH 8.0) to wash off excess polysaccharide coating in the bacterial cells. After centrifugation at 16,249 x ***g*** (r_{av} 8.6 cm) for 10 min, the upper aqueous phase (*ca* 400 µl) was removed and the pellet of cells washed once more in RB

buffer. The pellet of cells was suspended in 500 µl 0.1 M EDTA pH 8 containing 0.1 M NaCl and the cells lysed at room temperature by the addition of 50 µl 20% SDS. When a thick mucous-like suspension was observed, 55 µl of 5 M sodium perchlorate was added and mixed by inversion to precipitate proteins. Nucleic acids were extracted with an equal volume (*ca* 600 µl) chloroform-isoamyl alcohol (24:1 v/v) for 10 min. After centrifugation at 16,249 x ***g*** (r_{av} 8.6 cm) for 20 min, the upper aqueous phase (*ca* 400 µl) was removed to a new tube. DNA was recovered and purified using the UltraClean™15 DNA Purification Kit (Mo Bio Laboratories, Inc.), according to the manufacturer's instructions but with the exception that the ULTRA WASH step was repeated. DNA was resuspended in 20 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8) at 4°C. DNA preparations (3 µl) were separated by electrophoresis on a 1% TAE agarose gel at 60 V alongside 500 ng of a λ-*Hin*dIII DNA quantitative standard. TAE buffer contained 40 mM Tris, 20 mM sodium acetate and 1 mM EDTA, pH 7.8. After electrophoresis, the gel was stained for 30 min with ethidium bromide (1 $\mu g.ml^{-1}$ in TAE buffer). Nucleic acids were visualised under UV light, and the concentration of the DNA estimated by relating the intensity of bands of the genomic DNA to the intensity of bands provided by the quantitative standard. The remaining DNA preparation was stored at -20°C until needed.

4.2.2 Repetitive sequence based PCR (rep-PCR) fingerprinting

Repetitive sequence based PCR fingerprinting was conducted according to the method summarised by Burdman *et al.* (2005) and elaborated here. Primers REP1R-I and REP2-I (for repetitive extragenic palindrome-PCR; REP-PCR), ERIC 1R and ERIC 2 (for enterobacterial repetitive intergenic consensus-PCR; ERIC-PCR) and BOXA1R (for BOX element-PCR; BOX-PCR) as described by Versalovic *et al.* (1991 and 1994), were supplied by Sheree McCammon, University of Tasmania. The primer sequences are presented in Table 4.1.

Table 4.1 Sequences of primers used in REP PCR, reproduced from Rademaker *et al.* (2004).

Primer	Sequence	**Reference**
BOX A1R	5′-CATCggCAAggCgACgCTgACg-3′	Versalovic *et al.* 1994
ERIC 1R	5′-ATgTAAgCTCCTggggATTCAC-3′	Versalovic *et al.* 1991
ERIC 2	5′-AAgTAAgTgACTggggTgAgCg-3′	Versalovic *et al.* 1991
REP 1R	5′-IIIICgICgICATCIggC-3′	Versalovic *et al.* 1991
REP 2I	5′-ICgICTTATCIggCCTAC-3′	Versalovic *et al.* 1991

The PCR mixtures were prepared in a final volume of 25 µl in a 0.5 ml thin-walled PCR tube and contained 20 ng of template DNA, 2.5 µl 10x buffer (Qiagen Pty Ltd, Australia), 2.5 mM $MgCl_2$, 0.16 mg/ml bovine serum albumin (BSA), 0.2 mM of each dNTP (Bioline, Australia), 5 pmols of each primer (10 pmols for BOX A1R) and 1 U of Taq Polymerase (Qiagen Pty Ltd, Australia). Negative controls were PCR mixtures without template. Amplifications were performed in an Eppendorf Mastercycler Gradient PCR machine with an initial denaturation cycle of 7 min at 95°C, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 40, 42 or 53°C for REP-, ERIC- and BOX-PCR, respectively, and elongation at 65°C for 8 min. A final extension step was performed at 65°C for 16 min and the amplified DNA fragments maintained at 4°C for immediate use or stored at -20°C. Samples of 12 µl from each reaction were run in 20 cm-long 1.5% agarose gels in TAE buffer for 12 h at 25 V. A 5 µl sample of a 1 Kb (kilobase) DNA molecular weight ladder (Amresco Inc., USA) was loaded each of two wells located in the centre or near the edge of the gel. The gels were stained with 0.5 µg ethidium bromide/ml of TAE buffer and photographed under UV light.

4.2.3 Data analyses

Banding patterns for each isolate were scored using 1 for presence and 0 for absence of a band. All bands that stained intensely were scored, whereas bands of lower intensity were scored if the amplicon was clearly visible as a discrete band. It was assumed that bands of the same size were homologous.

Cluster analyses were conducted to form groups of isolates with highly similar fingerprints in such a way that the fingerprints in different groups were as dissimilar as possible. Pairwise comparisons were made between all isolates using the algorithms by Nei and Li (1979). The resulting distance matrix was used to construct a dendrogram generated by the unweighted pair-group method, using arithmetic averages (UPGMA, Sneath and Sokal 1973) and the TREECON program (Van de Peer and De Wachter, 1994). Isolate US 03 05, a *Pseudomonas* sp. and genetically distinct from isolates of *Xanthomonas* sp., was included in all analyses. Statistical significance of branches of trees was tested using the bootstrap technique (Felsenstein, 1985) with 1000 replicates within the TREECON program.

The genetic relatedness among DNA fingerprints was also determined using multivariate statistics. Genetic relatedness was visualised using non-metric multidimensional scaling (NMDS), an ordination analysis that is useful for recovering non-hierarchical patterns

of genetic variation (Lessa, 1990). The stress value, associated with NMDS, indicates how well the distribution of points on the plot matches the actual distances between observations (individuals). Stress values < 0.2 correspond to meaningful representation of the data with little chance of misrepresentation, whereas values > 0.3 generally indicate poor representation (Clarke and Warwick, 2001). NMDS plots were generated from Euclidian distance measures among pairwise comparisons of unique DNA fingerprints.

4.3 Results

Genetic variation was observed among isolates of *Xanthomonas arboricola* (*Xa*) from walnut fruit from various locations in Tasmania. PCR fingerprinting of isolates of *Xa* using ERIC primers resulted in 4 to 8 bands, between 0.5 and 8 kilobasepairs (kbp), that were easy to score (Figs 4.1 and 4.2). Amplification of bacterial DNA with REP and BOX primers was generally less robust, with some faint fingerprints or DNA that was not amplified for particular samples (Figs 4.3 and 4.4).

The rep-PCR technique appeared to be reproducible because *Xa* isolates US1005, US2305, US3505, US4405, US5005 and US5605 produced identical ERIC fingerprints when separate PCR reactions from the same DNA isolation were performed on different days (Figs 4.1 and 4.2). Amplification of the same DNA sample in separate BOX-PCRs also produced the same DNA fingerprint; for example, lanes 10 and 12 for isolate US3505 in Fig. 4.4. Furthermore, some isolates of *Xa* produced identical fingerprints when only major bands were scored. For example, the following pairs of isolates were identical for bands that stained intensely using ERIC PCR (Fig. 4.2): US6505 and US0505 (lanes 15 and 19), US5605 and US6305 (lanes 8 and 14), and US2305 and US6205 (lanes 3 and 13).

Only two of 16 isolates of *Xa* (US2505 and US2805) assayed using ERIC PCR produced the same DNA fingerprint (Fig 4.1). Both isolates were from the same general location, although isolate US2505 was from a walnut tree in a home garden and isolate US2805 was from the nearby commercial orchard. The genetic separation of these two isolates by REP or BOX PCR was inconclusive because the banding pattern for US2805 amplified weakly (Figs 4.3 and 4.4). The ERIC DNA fingerprint of US2505 and US2805 was almost identical to *Xa* isolate US1005 from the Tamar Valley.

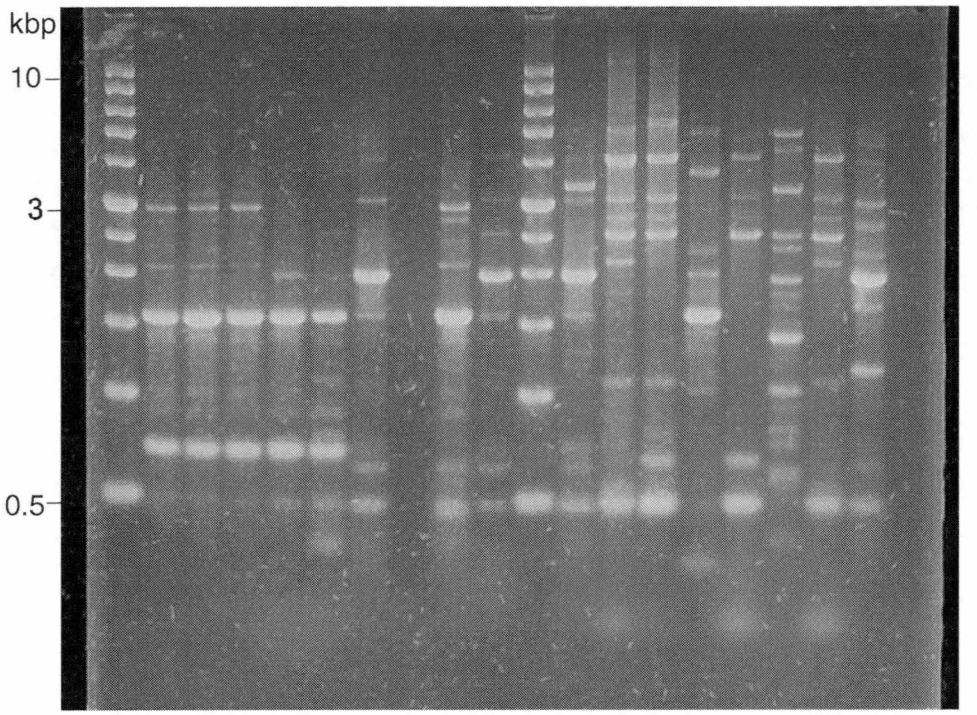


Figure 4.1 Agarose gel electrophoresis of DNA from various bacterial isolates amplified using ERIC-PCR. Xa refers to *Xanthomonas arboricola* according to putative identification by GC-FAME (Chapter 3). The size of fragments in kilobase pairs (kpb) is indicated. Lanes from left to right: Lane 1, 5 µl 1 Kb DNA ladder; Lane 2, Xa isolate US1005; Lane 3, Xa isolate US2505; Lane 4, Xa isolate US2805; Lane 5, Xa isolate US5305; Lane 6, Xa isolate US2305; Lane 7, Xa isolate US5005; Lane 8, Xa isolate US1605 (no amplification); Lane 9, Xa isolate US3505; Lane 10, Xa isolate US4405; Lane 11, 5 µl 1 Kb DNA ladder; Lane 12, Xa isolate US5605; Lane 13, *Erwinia rhapontici* isolate US3205; Lane 14, *Pseudomonas aeruginosa* isolate US5805; Lane 15, *Pseudomonas aeruginosa* isolate US0805; Lane 16, *Pantoea agglomerans* isolate US4505; Lane 17, *Pseudomonas stuzeri* isolate US0305; Lane 18, *Serratia liquefaciens* isolate US0605; Lane 19, undetermined isolate US0705; Lane 20, no DNA control.

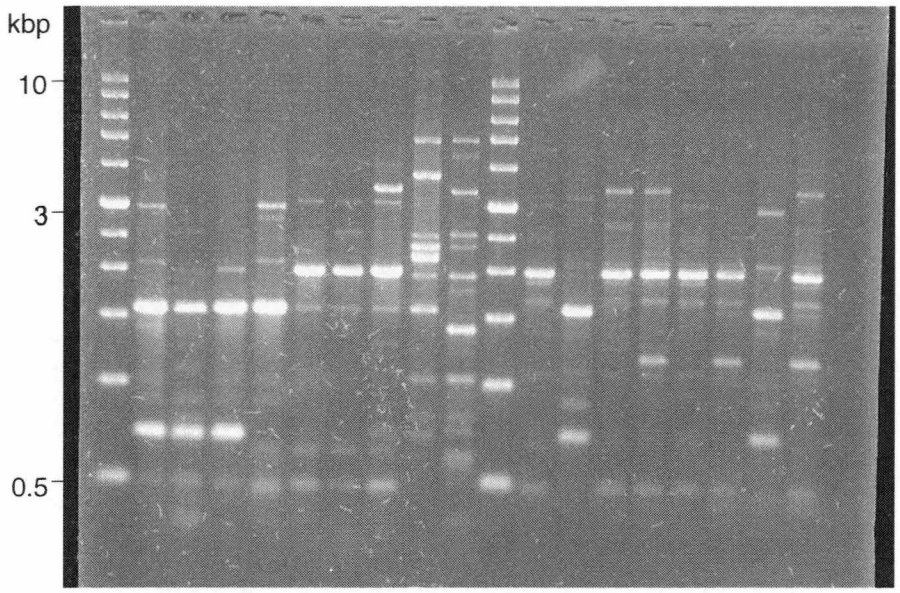


Figure 4.2 Agarose gel electrophoresis of DNA from various bacterial isolates amplified using ERIC-PCR. Xa refers to *Xanthomonas arboricola* according to putative identification by GC-FAME (Chapter 3). The size of fragments in kilobase pairs (kpb) is indicated. Lanes from left to right: Lane 1, 5 µl 1 Kb DNA ladder; Lane 2, Xa isolate US1005; Lane 3, Xa isolate US2305; Lane 4, Xa isolate US5305; Lane 5, Xa isolate US3505; Lane 6, Xa isolate US5005; Lane 7, Xa isolate US4405; Lane 8, Xa isolate US5605; Lane 9, Xa isolate US7105; Lane 10, *Pseudomonas stuzeri* isolate US0305; Lane 11, 5 µl 1 Kb DNA ladder; Lane 12, Xa isolate US7405; Lane 13, Xa isolate US6205; Lane 14, Xa isolate US6305; Lane 15, Xa isolate US6505; Lane 16, Xa isolate US6405; Lane 17, Xa isolate US0105; Lane 18, Xa isolate US0205; Lane 19, Xa isolate US0505; Lane 20, no DNA control.

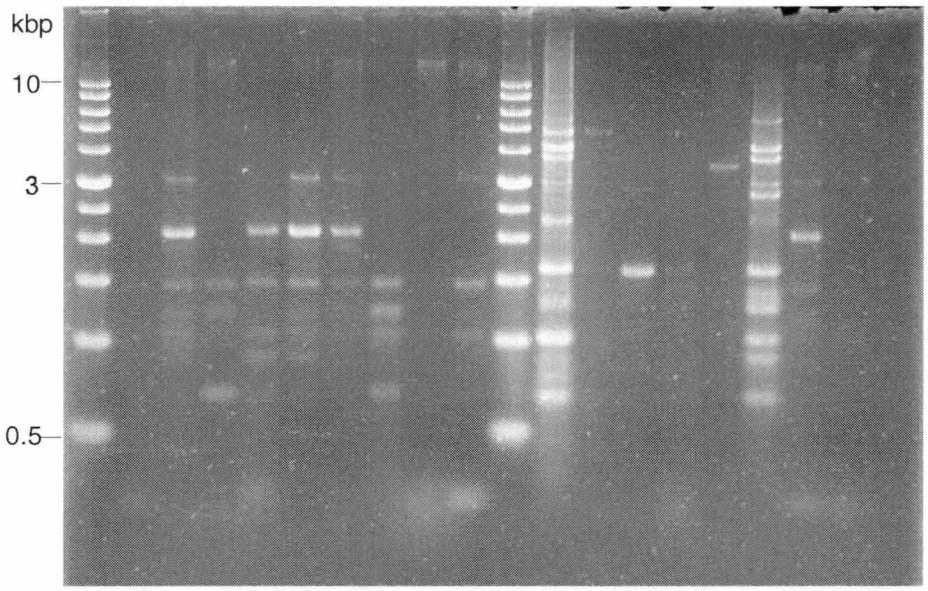


Figure 4.3 Agarose gel electrophoresis of DNA from various bacterial isolates amplified using REP-PCR. Xa refers to *Xanthomonas arboricola* according to putative identification by GC-FAME (Chapter 3). The size of fragments in kilobase pairs (kpb) is indicated. Lanes from left to right: Lane 1, 5 µl 1 Kb DNA ladder; Lane 2, Xa isolate US0505 (no amplification); Lane 3, Xa isolate US1005; Lane 4, Xa isolate US1605; Lane 5, Xa isolate US2305; Lane 6, Xa isolate US2505; Lane 7, Xa isolate US2805; Lane 8, Xa isolate US3505; Lane 9, Xa isolate US4405 (no amplification); Lane 10, Xa isolate US5005; Lane 11, 5 µl 1 Kb DNA ladder; Lane 12, *Erwinia rhapontici* isolate US3205; Lane 13, *Serratia liquefaciens* isolate US0605; Lane 14, *Pseudomonas aeruginosa* isolate US0805; Lane 15, *Pantoea agglomerans* isolate US4505; Lane 16, *Pseudomonas stuzeri* isolate US0305; Lane 17, *Pseudomonas aeruginosa* isolate US5805; Lane 18, Xa isolate US5305; Lane 19, Xa isolate US5605 (no amplification); Lane 20, no DNA control.

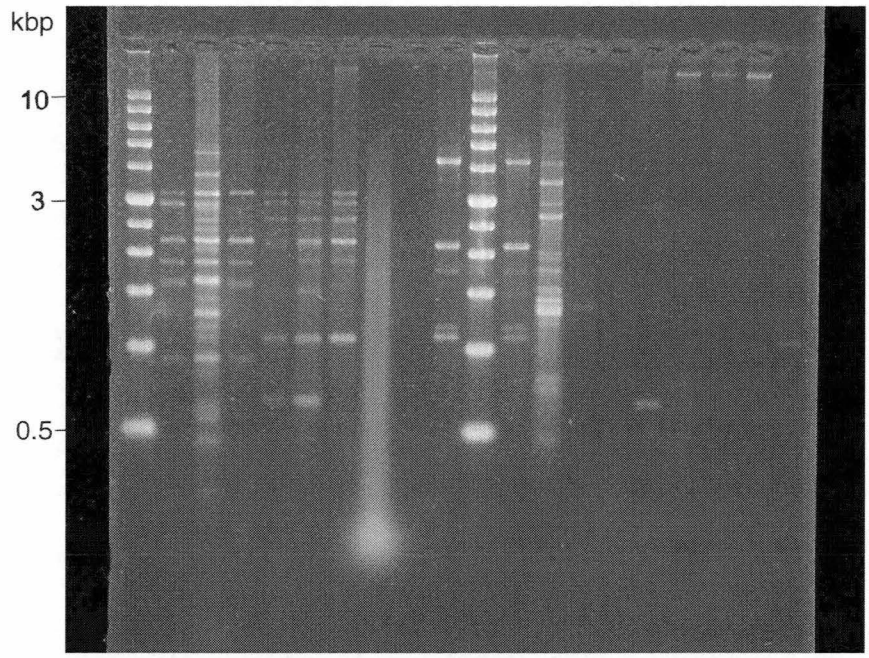


Figure 4.4 Agarose gel electrophoresis of DNA from various bacterial isolates amplified using BOX-PCR. Xa refers to *Xanthomonas arboricola* according to putative identification by GC-FAME (Chapter 3). The size of fragments in kilobase pairs (kpb) is indicated. Lanes from left to right: Lane 1, 5 µl 1 Kb DNA ladder; Lane 2, Xa isolate US1005; Lane 3, Xa isolate US2505; Lane 4, Xa isolate US2805; Lane 5, Xa isolate US7405; Lane 6, undetermined isolate US2605; Lane 7, undetermined isolate US1205; Lane 8, Xa isolate US2305 (no discernable fingerprint); Lane 9, Xa isolate US5305 (no amplification); Lane 10, Xa isolate US3505 (sample A); Lane 11, 5 µl 1 Kb DNA ladder; Lane 12, Xa isolate US3505 (sample B); Lane 13, Xa isolate US1605; Lane 14, *Pseudomonas aeruginosa* isolate US0805; Lane 15, *Pantoea agglomerans* isolate US4505 (no amplification); Lane 16, Xa isolate US4405; Lane 17, Xa isolate US5605 (no amplification); Lane 18, undetermined isolate US0705 (no amplification); Lane 19, *Pseudomonas aeruginosa* isolate US5805 (no amplification); Lane 20, *Pseudomonas stutzeri* isolate US0305.

Table 4.2 Tentative allocation of isolates of *Xanthomonas arboricola* from symptomatic walnut fruit in Tasmania to one of two putative genetic groups according to ERIC PCR. Genetic group 1 has an intensely staining band at approximately 1.5 kbp and genetic group 2 has an intensely staining band at approximately 2 kbp (Fig. 4.2).

Isolate	Region of Tasmania	Putative genetic group
US0205	North West	1
US1005	Tamar Valley	1
US2305	North West	1
US2505	North West	1
US2805	North West	1
US3505	North West	1
US5305	East Coast	1
US6205	East Coast	1
US0105	North West	2
US0505	Tamar Valley	2
US4405	North West	2
US5005	East Coast	2
US5605	East Coast	2
US6305	Coal River Valley	2
US6405	Coal River Valley	2
US6505	Coal River Valley	2
US7405	Coal River Valley	2
US7105	Coal River Valley	neither 1 or 2

Visual comparison of DNA fingerprints generated using ERIC PCR (Fig. 4.2) revealed that isolates of Xa could be separated into two groups, based on the presence of an intensely staining band at approximately 1.5 kbp (group 1) or 2 kbp (group 2, Fig. 4.2). These putative genetic groups are presented in Table 4.2. The exception to this general observation was Xa isolate US7105, which had bands that stained weakly to moderately at both 1.5 kbp and 2 kbp (Fig. 4.2).

DNA from non-Xanthomonads appeared to produce distinctly different DNA fingerprints to those for Xa across all repetitive sequence based PCRs (Figs 4.1 to 4.4). This observation was supported, albeit weakly, by genetic distance as determined by cluster

analysis or multivariate statistics (Figs 4.5 to 4.13). *Pseudomonas stuzeri* isolate US0305 was included in all data analyses and was separated genetically from all isolates of Xa analysed using ERIC and REP-PCR (Fig. 4.11). The exception was Xa isolate US7105, which shared some DNA bands with US0305 when analysed by ERIC-PCR (Figs 4.7 and 4.8). The separation of non Xanthomonads US0305 and US 08 05 from isolates of Xa using BOX-PCR was less evident (Figs 4.12 and 4.13).

Eight isolates of Xa analysed by REP-PCR formed a cohesive genetic group in the dendrogram, supported by a bootstrap percentage of 78% (Fig. 4.9). When six of these isolates were analysed by combining data from REP and ERIC-PCR, the bootstrap percentage increased to 96%, again providing statistical support for the genetic group (Fig. 4.11). Using the combined data set, the genetic distance between any two of these six isolates was greater than 0.4.

There was weak statistical support for genetic sub-structuring of Xa within dendrograms for individual repetitive sequences (Figs 4.7, 4.9 and 4.12), as indicated by bootstrap values at major nodes of less than 50%. Subsets of Xa isolates US1005, US2305, US2505, US2805 and US5305, from the Tamar Valley, North West or East Coast, clustered together in various dendrograms (Figs 4.5, 4.7, 4.9, 4.12). Xa isolates US4405, US5005 and US5605, from the North West or East Coast, also clustered together when analysed by ERIC PCR (Figs 4.5 and 4.7)

MDS did not reveal distinct genetic groups among isolates of Xa for ERIC, REP or BOX-PCR (Figures 4.3, 4.6 and 4.10). Like the results presented in the dendrograms, isolates US1005, US2505, US2805 and US5305 appeared in the lower-right sector of the ordination plot for REP PCR (Fig. 4.10). These isolates were clustered in the first ERIC analysis (Fig. 4.6) and isolates US1005 and US5305 appeared in the upper-left of the ordination plot for the second ERIC analysis (Fig. 4.8), with isolates US0205, US2305, US3505, US4405 and US6205 (Fig. 4.8).

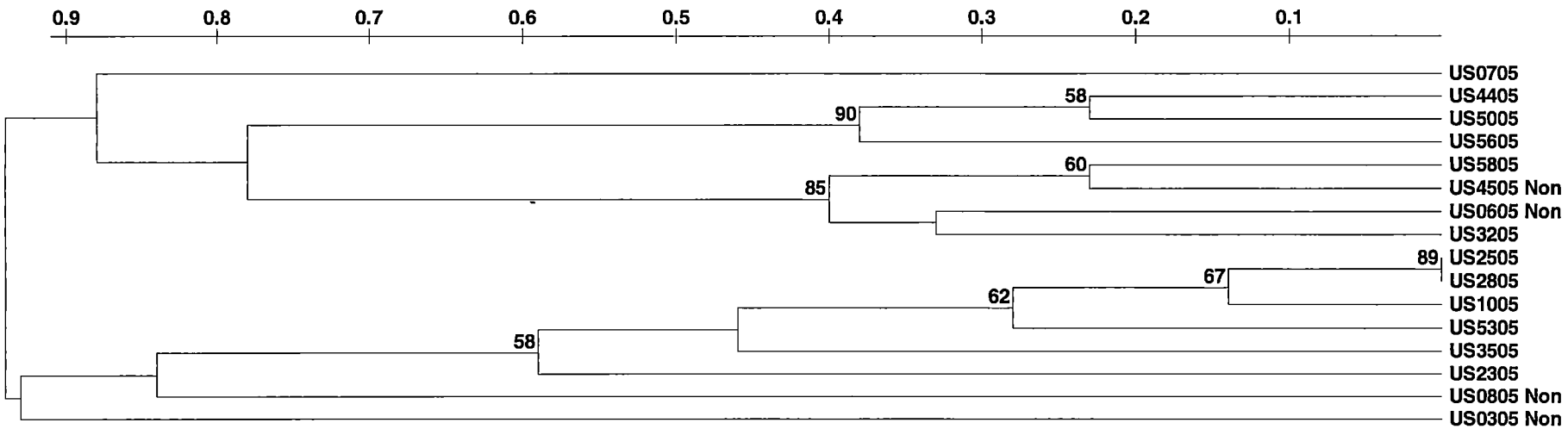


Figure 4.5 Dendrogram of genetic distance among 16 DNA fingerprints generated by ERIC-PCR using a total of 50 loci and UPGMA with 1000 bootstrap replicates. Bootstrap values, expressed as a percentage, are presented at the nodes of branches where the value is greater than 50%. Isolate number is indicated on the right hand side of the dendrogram. Isolates US0305, US0605, US0805, US3205, US4505 and US5805 were non-Xanthomonads (Non). Isolate US0705 was not determined by GC-FAME. The other ten isolates were *Xanthomonas arboricola.*

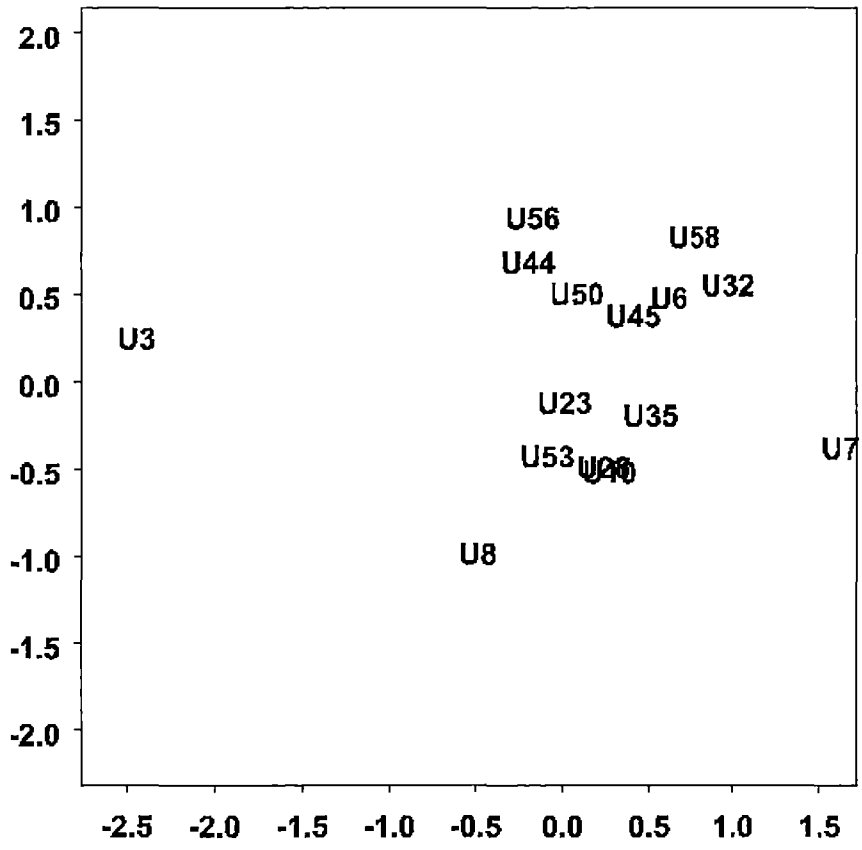


Figure 4.6 Genetic relationship among 16 DNA fingerprints generated by ERIC-PCR using a total of 50 loci, as visualised by a NMDS plot with 2D Stress = 0.0749. Each code represents a bacterial isolate from walnut fruit expressing symptoms of bacterial blight. Isolates U3 (US0305), U6 (US0605), U8 (US0805), U32 (US3205), U45 (US4505) and U58 (US5805) were non-Xanthomonads. Isolate US0705 was not determined by GC-FAME. The other ten isolates were *Xanthomonas arboricola*.

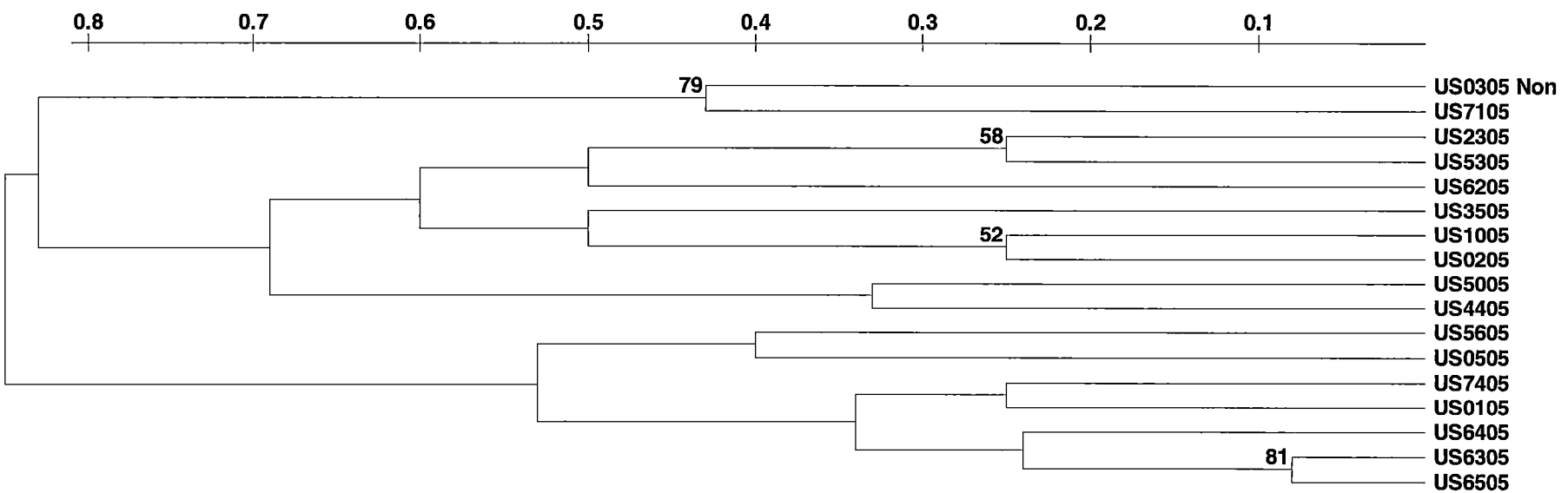


Figure 4.7 Dendrogram of genetic distance among 17 unique DNA fingerprints generated by ERIC-PCR using a total of 33 loci and UPGMA with 1000 bootstrap replicates. Bootstrap values, expressed as a percentage, are presented at the nodes of branches where the value is greater than 50%. Isolate number is indicated on the right hand side of the dendrogram. Isolate US0305 (Non, for non-Xanthomonad) was *Pseudomonas stuzeri*. The remaining 16 isolates were *Xanthomonas arboricola.*

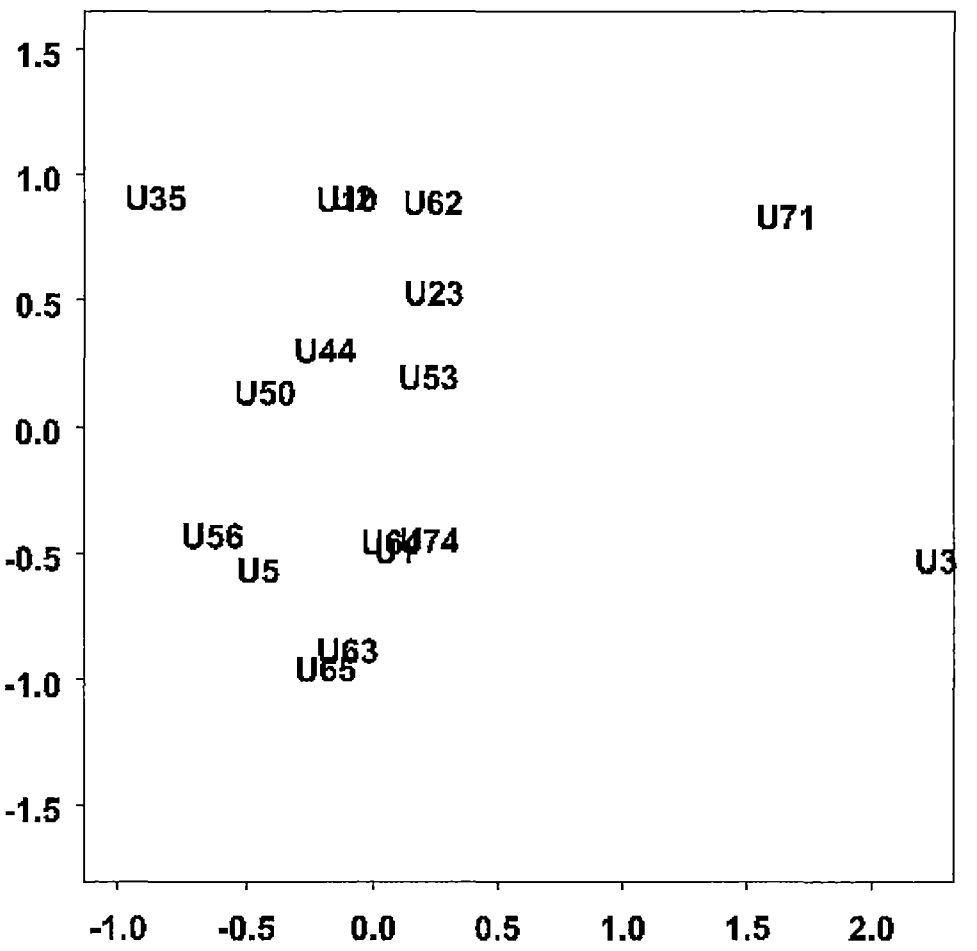


Figure 4.8 Genetic relationship among 17 unique DNA fingerprints generated by ERIC-PCR using a total of 33 loci, as visualised by a NMDS plot with 2D Stress = 0.0581. Each code represents a bacterial isolate from walnut fruit expressing symptoms of bacterial blight. Isolate U3 was isolate US0305 of *Pseudomonas stuzeri*. The remaining 16 isolates were *Xanthomonas arboricola*.

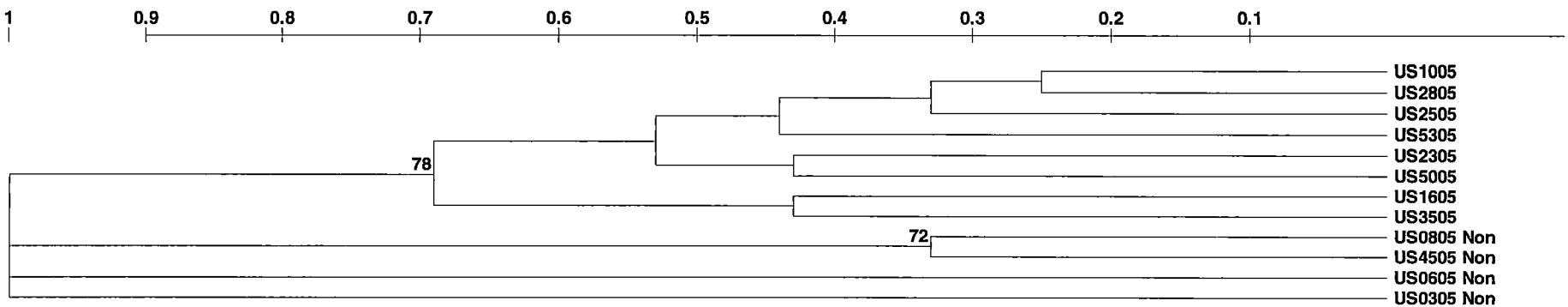


Figure 4.9 Dendrogram of genetic distance among 12 unique DNA fingerprints generated by REP-PCR using a total of 29 loci and UPGMA with 1000 bootstrap replicates. Bootstrap values, expressed as a percentage, are presented at the nodes of branches where the value is greater than 50%. Isolate number is indicated on the right hand side of the dendrogram. Isolates US0305, US0605, US0805 and US4505 were non-Xanthomonads (Non). The other eight isolates were *Xanthomonas arboricola.*

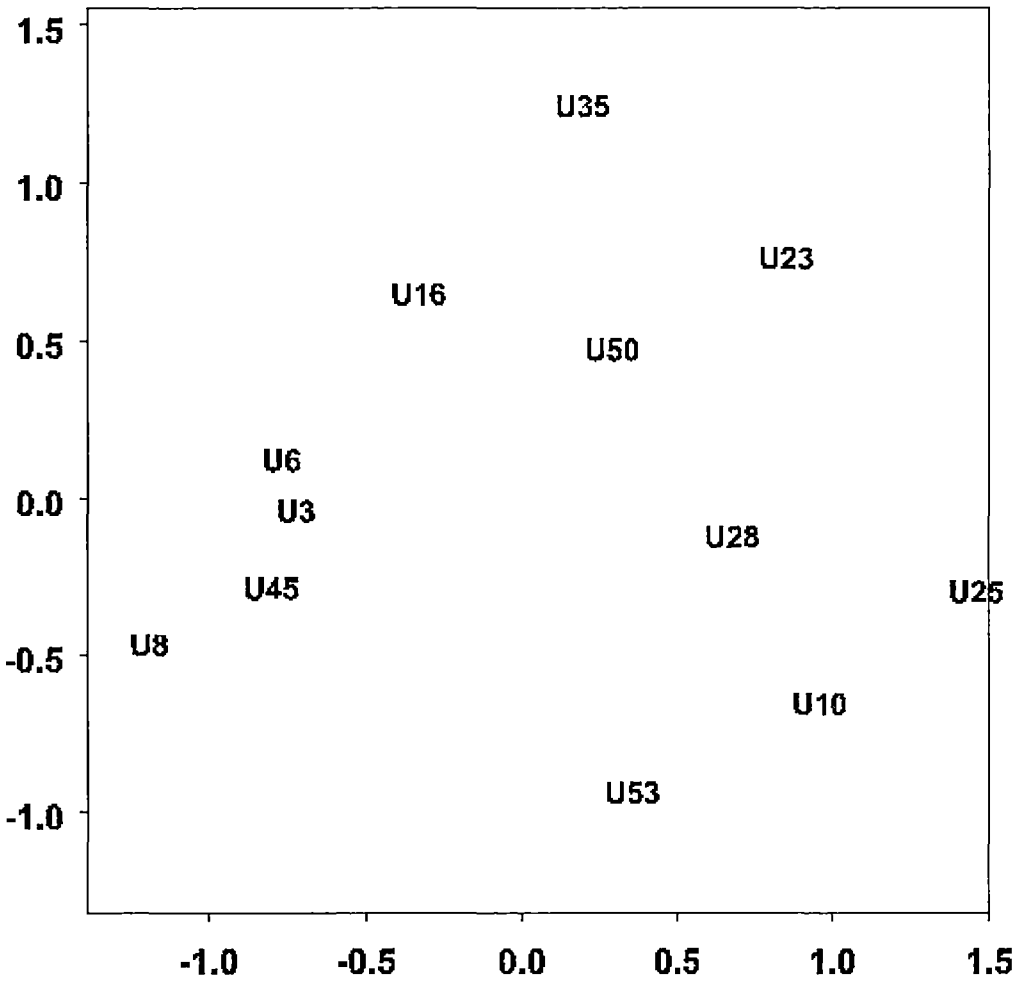


Figure 4.10 Genetic relationship among 12 unique DNA fingerprints generated by REP-PCR using a total of 29 loci as visualised by a NMDS plot with 2D Stress = 0.0608. Each code represents a bacterial isolate from walnut fruit expressing symptoms of bacterial blight. Isolates U3, U6, U8 and U45 represent the non-Xanthomonad strains US0305, US0605, US0805 and US4505, respectively. The other eight isolates were *Xanthomonas arboricola.*

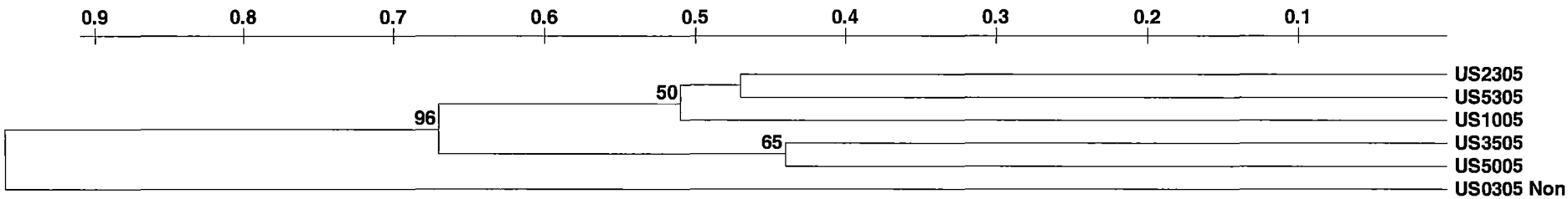


Figure 4.11 Dendrogram of genetic distance among six unique DNA fingerprints generated by combining data from ERIC and REP-PCR, using a total of 32 loci and UPGMA with 1000 bootstrap replicates. Bootstrap values, expressed as a percentage, are presented at the nodes of branches where the value is greater than 50%. Isolate number is indicated on the right hand side of the dendrogram. Isolate US0305 (Non, for non-Xanthomonad) was *Pseudomonas stuzeri*. The other five isolates were *Xanthomonas arboricola*.

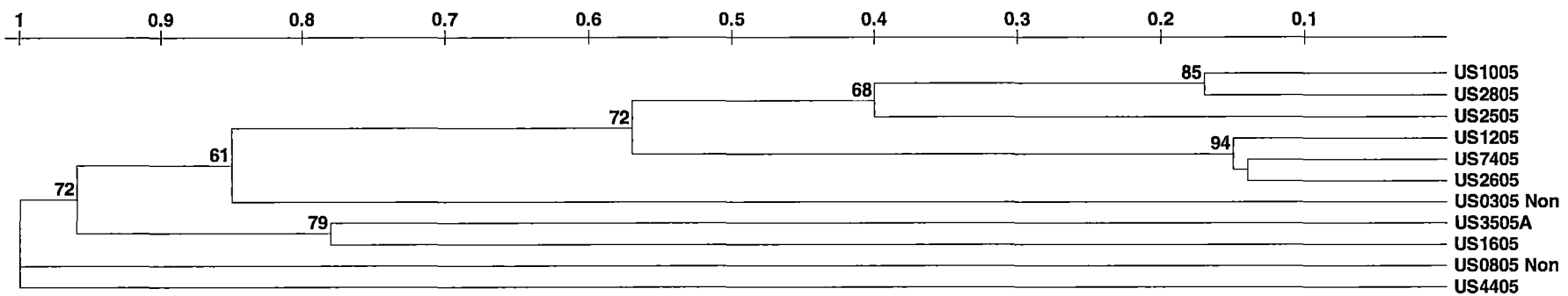


Figure 4.12 Dendrogram of genetic distance among 11 unique DNA fingerprints generated by BOX-PCR using UPGMA with 1000 bootstrap replicates. Bootstrap values, expressed as a percentage, are presented at the nodes of branches where the value is greater than 50%. Isolate number is indicated on the right hand side of the dendrogram. Isolates US0305 and US0805 were the non-Xanthomondads (Non) *Pseudomonas stutzeri* and *Pseudomonas aeruginosa*, respectively. The other nine isolates were *Xanthomonas arboricola*, except for US2605 and US1205, which were not identified by GC-FAME.

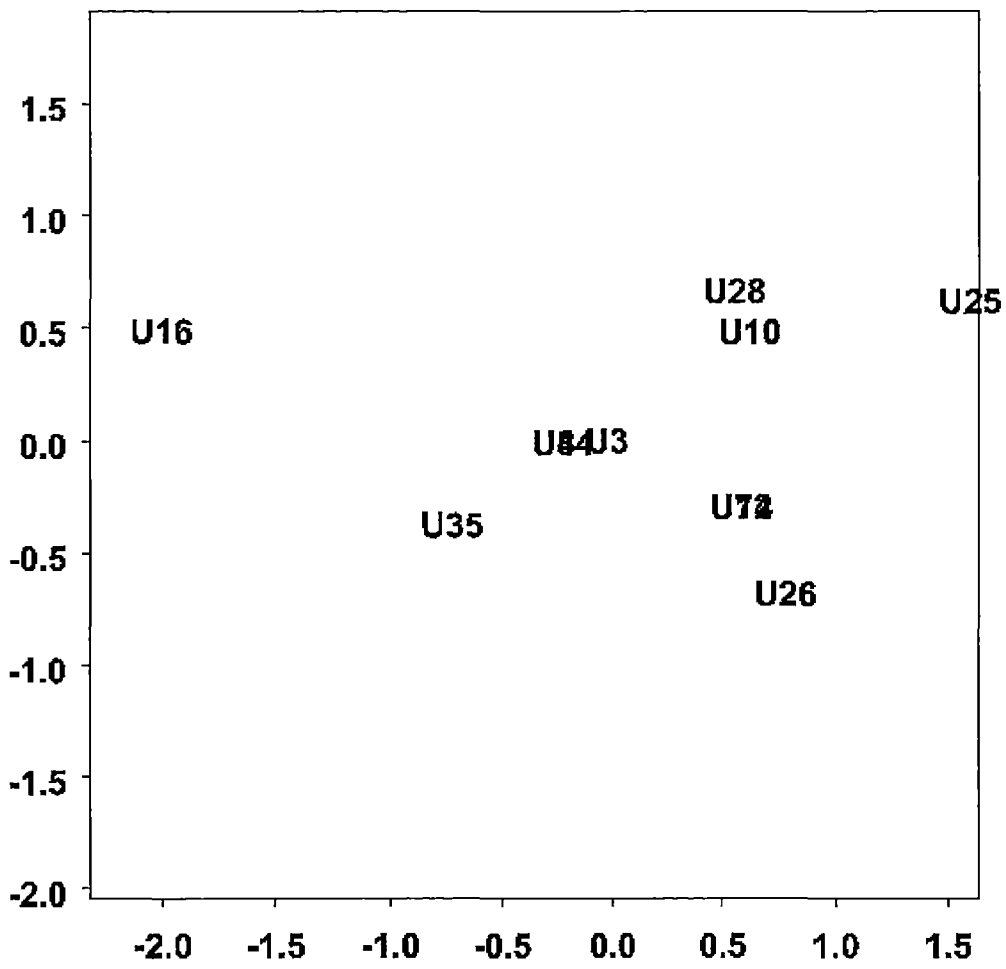


Figure 4.13 Genetic relationship among 11 unique DNA fingerprints generated by BOX-PCR as visualised by a NMDS plot with 2D Stress = 0.0223. Each code represents a bacterial isolate from walnut fruit expressing symptoms of bacterial blight: US0305 (U3, *Pseudomonas stutzeri*), US0805 (U8, *Pseudomonas aeruginosa*), US1005 (U10, *Xanthomonas arboricola* (Xa), US1205 (U12, undetermined), US1605 (U16, Xa), US2505 (U25, Xa), US2605 (undetermined, U26), US2805 (U28, Xa), US3505 (U35, Xa), US4405 (U44, Xa) and US7405 (U74, Xa).

4.4 Discussion

This is the first report of genetic variation among isolates of *Xanthomonas arboricola* (*Xa*) from walnuts in Tasmania and Australia. The rep-PCR protocol adopted for this study appeared to be reproducible; however, PCRs conducted in the future should be performed in triplicate to confirm that the assay conditions have been optimised. This preliminary study builds on the findings of Scortichini *et al.* (2001) by identifying additional populations of *Xa* that display genetic variation. Using ERIC-PCR, isolates of *Xa* in this study could generally be separated into two groups that were not related to their geographic origin, based on the presence or absence of one of two intensely staining bands. However, there was only weak statistical support for genetic sub-structuring of *Xa* across the whole data set. The capacity of rep-PCR to distinguish isolates of *Xa* from non-Xanthomonads isolated from symptomatic walnut fruit was also apparent, although non-Xanthomonads sometimes clustered with isolates of *Xa* for individual repetitive sequences.

Cluster analyses of *Xa* isolated in Tasmania were not directly comparable to the dendrogram reported by Scortichini *et al.* (2001), because different measures of genetic distance or similarity were used in UPGMA. However, Xa from Tasmania appeared to form a cohesive genetic group when compared with non-Xanthomonads. Further study of genetic variation of *Xa* in Tasmania should include other Xanthomonad bacteria to determine how well the technique distinguishes closely related species and to confirm the findings of Scortichini *et al.* (2001) in this regard.

Results of all rep PCR studies to date emphasise the ability of this technique in discriminating closely related phytopathogenic bacteria and its usefulness as a rapid and discriminatory technique to determine genetic diversity and for clarifying taxonomic treatment (Louws *et al.*, 1999; Rademaker *et al.*, 2000). Scortichini and Rossi (2002) utilised rep PCR to study genetic relationships among 26 strains of *Xathomonas arboricola* pv *fragiarae* (*Xaf*) and among pathovars of *X. arboricola* pv *corylina* (*Xac*), *Xaj* and *X. arboricola* pv *pruni* (*Xap*). Within strains of *Xaf*, rep PCR produced distinct fingerprint profiles, although overall similarities were very high. Each pathovar produced distinct genomic profiles, and *Xac* and *Xaj* were found to be closely related to each other. This finding was consistent with the results of GC-FAME presented in Chapter 3, where *Xac* was often listed with *Xaj* or *Xa* pv *pruni* in potential matches to the fatty acid profiles in Sherlock® Microbial Identification System (Anon 2005).

Application of more than one diagnostic technique, including DNA tools, provides strong evidence for the association of one or more phytopathogenic bacteria with a

particular disease and allows targeted selection of bacterial isolates to be screened in studies of pathogenicity and for satisfying Koch's postulates (Agrios 1997). Facelli *et al.* (2005) studied the cause of pistachio dieback in Australia and confirmed an association of *Xanthomonas transluces* with the disease using GC FAME and SDS PAGE techniques. Rep PCR was then applied to isolates of *Xanthomonas transluces* from Pistachio, which were found to be present in two genetic groups. Isolates of *Xa* from Tasmania that have different rep-PCR profiles can now be selected for studies of pathogenicity in walnut that include other species of Xanthomonas and non-Xanthomonad for determining which isolates cause walnut blight symptoms.

Genetic variation in phytopathogenic bacteria is to be expected given that plants and their associated microflora must adapt to a changing environment. Walnut trees were introduced to Tasmania by early European settlers. Trees and associated microflora that survived introduction would have adapted to their new location. Presumably the walnut blight pathogen was introduced at early settlement and populations have evolved and moved onto trees planted in recent decades as the walnut industry expanded.

Results of this preliminary study should be validated further, by analysing a larger number of the same isolates for each repetitive sequence and combining the data set for cluster and MDS analyses. A hierarchical sampling strategy (McDonald 1987) should be employed to examine the organisational level at which genetic variation should be studied, including among plant organs, within trees, within and among orchards, and among growing regions and seasons. For example, fine scale genetic variation in *Xa* was identified by Scortichini *et al.* (2001), who found genetic variants among isolates of *Xa* from the same walnut leaf. The presence of fine scale genetic variation in *Xa* in Tasmanian walnut orchards needs to be confirmed. This information would enable genetic variation among populations to be related to variation in pathogenicity, if it occurs. Ultimately, this information might dictate disease management, if variable bacterial populations respond differently to a particular control measure.

Copper sensitivity

5.1 Introduction

Walnut blight has been responsible for losses of more than 50% of walnut yield in Tasmania (Lang and Evans, 2005). Blight in Tasmania is typically controlled with copper sprays, with up to 10 applications from bud burst to pistillate bloom. This control measure is sometimes ineffective under Tasmanian conditions. Unless effective control measures are identified, blight disease could threaten the viability of the walnut industry in Tasmania (Lang and Evans, 2005). As mentioned earlier in this thesis, development of *Xaj* populations tolerant of copper at concentrations present in commercial bactericides may be one of the possible reasons for failure in disease control in Tasmania. Frequent use of copper has contributed to the development of copper resistance among populations of the walnut blight pathogen throughout northern California and in walnut orchards in France (Garden *et al.*, 1993; Lee *et al.*, 1993).

Copper-containing compounds, including cupric hydroxide, Bordeaux mixture, and various formulations of basic copper sulfate, have been applied to horticultural crops extensively for the control of bacterial diseases. Copper resistance has most likely been present for many years among plant pathogenic bacteria. Bacterial strains resistant to copper were reported as early as 1968 (Marco and Stall, 1983). The concept of bactericide resistance has been studied in detail only since the mid 1980's (Cooksey, 1990). For example, copper resistance was first detected in the pathogen *Xanthomanas campestris* pv *vesicatoria*, causal agent of bacterial spot of pepper and tomato in 1983 (Marco and Stall, 1986). In recent years, there have been a number of anecdotal reports of reduced effectiveness of copper sprays for controlling plant bacterial diseases (Lee *et al.*, 1993; Scheck *et al.*, 1996, Bender and Cooksey, 1987). Development of resistance to copper by bacterial populations may be one of the possible reasons for poor disease control.

Microorganisms respond differently to heavy metals such as copper in their environment. Each cell line or strain can be classified broadly into three categories: sensitive, resistant or tolerant of the heavy metal. Resistance and tolerance are related but distinct concepts and have been used by different authors to refer to different things. In this chapter, 'resistance' is defined as the trait of the microbial cell line or strain that allows the toxic effects of the heavy metal to be reduced. 'Tolerance' is a broader concept and is defined here as the ability of the microbial strain to reproduce in the presence of the heavy metal at a specified concentration. Therefore, both terms are applied here depending on

the context.

Copper tolerance among phytopathogeneic bacteria is considered a quantitative increase in tolerance rather than a qualitative increase in tolerance. Strains that are resistant to copper can tolerate copper concentrations 10 to 80 times higher than that tolerated by sensitive strains of same species. In general, increased use of antimicrobial agents has lead to selection of resistant strains of bacteria that may have acquired resistance either through mutation or by acquiring plasmids bearing resistance to copper (Davies and Smith, 1978; Menkissoglu and Lindow, 1991).

It is considered that both eukaryotic and prokaryotic cells require copper for normal growth. Copper is also an essential cofactor for many enzymes involved in respiration (eg, oxygenases and electron transport proteins). However, copper is toxic to the cell above a certain concentration. Copper has the ability to cause the cell to generate free radicals, which can damage DNA and lipid membranes. Bacteria can develop a detoxification system for protection from the toxic concentration of copper while also meeting their nutritional requirements. Genes for detoxification systems are found to be plasmid or chromosomally borne (Nies *et al.*, 1999; Voloudakis *et al.,* 2004).

Different tests for the sensitivity of a bacterial isolate to copper can give different results, depending on the method selected. Casitone yeast extract glycerol (CYEG) medium is considered a metal buffering medium, in which even high concentrations of copper remain constant during bacterial growth. This medium has often been used to assess sensitivity of bacterial isolates to copper ions (Zevenhuizen *et al.,* 1979). For example, Anderson *et al.* (1991) and Scheck *et al.* (1996) analysed copper sensitivity of *Pseudomonas syringae* strains by spreading bacterial suspensions onto CYEG agar medium containing different concentrations of $CuSO_4.5H_2O$. Garden *et al.* (1993), working with copper resistant strains of *Xaj* in France, grew bacteria on yeast extract peptone glucose agar (YPGA) medium, then on CYEG agar medium containing different concentrations of copper. Lee *et al.* (1994) followed the same procedure using CYEG agar medium amended with different concentrations of copper for screening copper resistant strains of *Xaj* in northern California. Unlike with solid medium, screening bacterial strains using CYEG broth cultures amended with different concentrations of copper allows the entire bacterial cell to be exposed to copper (Zevenhuizen *et al.,* 1979; Menkissoglu and Lindow, 1991). This approach was adopted by Martin *et al.* (2004) who quantified copper tolerance in populations of *Xanthomonas campestris* pv. *vesticatoria* from infected peppers grown in Queensland, Australia.

The objective of the research was to characterise the copper sensitivity of *Xaj* isolates sampled on a broad geographical scale in Tasmania. This work contributed baseline information towards identification of populations of *Xaj* in Tasmania that tolerate copper at concentrations applied commercially. If such populations are identified and found to be an important factor in effective management, then alternative strategies for blight control can be developed.

5.2 Materials and Methods

Thirty four strains of *Xanthomonas arboricola (Xa)* from commercial orchards and three isolates of *Xa* from walnut trees growing in home gardens that had never been exposed to copper, were analysed for their copper sensitivity. The 34 isolates from commercial orchards had different historical exposure to copper as illustrated in Table 5.1. As described in the previous chapter, the identity of bacterial isolates was determined by Gas Chromatograph Analysis of Fatty Acids Methyl Esters (GC-FAME), according to procedures of the Sherlock® Microbial Identification System Version 4.5 (Anon 2005).

Table 5.1 Origin of 37 isolates of *Xanthomonas arboricola* assayed for copper sensitivity and history of copper use, described as the total number of growing seasons (i.e. 0, 1-5, >5) that copper has been applied to the foliage of walnut trees at that site of isolate origin.

Isolate	Region	Orchard or garden no.	Variety	No. of seasons copper used
Commercial orchards				
US 01 05	North West	NW-1	Vina	>5
US 02 05	North West	NW-1	Franquette	>5
US 05 05	Tamar Valley	TV-1	Serr	1-5
US 09 05	Tamar Valley	TV-2	Chandler	1-5
US 10 05	Tamar Valley	TV-2	Franquette	1-5
US 11 05	Tamar Valley	TV-2	Franquette	1-5
US 12 05	Tamar Valley	TV-2	Franquette	1-5
US 13 05	Tamar Valley	TV-2	Chandler	1-5

US 14 05	Tamar Valley	TV-2	Chandler	1-5
US 15 05	Tamar Valley	TV-2	Chandler	1-5
US 19 05	North West	NW-1	Vina	>5
US 20 05	North West	NW-1	Vina	>5
[A]US 22 05	North West	NW-1	Vina	>5
US 23 05	North West	NW-1	Franquette	>5
US 26 05	North West	NW-2	Vina	>5
US 31 05	North West	NW-3	Chandler	>5
US 33 05	North West	NW-3	Chandler	>5
US 39 05	North West	NW-2	Vina	>5
US 41 05	North West	NW-2	Vina	>5
US 43 05	North West	NW-2	Vina	>5
US 44 05	North West	NW-2	Vina	>5
US 47 05	East Coast	EC-1	Vina	1-5
US 48 05	East Coast	EC-1	Vina	1-5
US 49 05	East Coast	EC-1	Vina	1-5
US 50 05	East Coast	EC-2	Serr	1-5
US 53 05	East Coast	EC-2	Serr	1-5
US 56 05	East Coast	EC-3	Vina	>5
US 62 05	East Coast	EC-4	Serr	>5
US 63 05	Coal River	CV-1	Vina	>5
US 64 05	Coal River	CV-1	Vina	>5
US 65 05	Coal River	CV-1	Vina	>5
US 71 05	Coal River	CV-2	Chandler	1-5
US 74 05	Coal River	CV-2	Chandler	1-5
US 75 05	Coal River	CV-2	Chandler	1-5
Home gardens				
US 16 05	Tamar Valley	HTV-1	unknown	0
US 25 05	North West	HNW-1	unknown	0
US 35 05	North West	HNW-2	unknown	0

[A]Isolate US 22 05 was *Xanthomonas fragariea* or *axonopodis* according to GC- FAME analysis.

The procedure described by Martin *et al.* (2004), except for a few modifications as specified below, was adopted for analysing copper sensitivity. The CYEG medium used in this research was similar to that described by Zevenhuizen *et al.* (1979) and modified by Anderson *et al.* (1990). A supply of casitone could not be sourced for this study and so Bacto™ peptone (Becton, Dickinson and Company) was used instead (H.L. Martin, Queensland Horticulture Institute, personal communication). Therefore, the medium used in this study was called PYEG (Peptone Yeast Extract Glycerol) media. PYEG broth contained the following (g/l): Bacto™ peptone 1.7 g, yeast extract (Amyl) 0.35 g and glycerol 2.0 g.

Strains of *Xanthomonas arboricola* stored in Microbank™ tubes (Pro-Lab Diagnostics, USA) at -80°C, were recovered according to the manufacture's instructions, streaked on nutrient agar and incubated at 48°C for 72 h. A colony was selected from this culture and inoculated into a flask containing 25 ml of sterile PYEG broth. Each flask was shaken on a platform orbital shaker at 130 rpm for 24 h, after which the optical density at 620 nm of each liquid culture was recorded using a UV/Vis spectrophotometer (Pye Unicam PU 8600, Philips, Cambridge, UK; Du Plessis and Van der Westhuizen, 1994). These measurements indicated the variation in optical density of the bacterial cultures prior to inoculating PYEG broth containing different concentrations of copper sulfate. A 1 ml sample of culture was then inoculated into a flask of PYEG broth amended with $CuSO_4.5H_2O$ at a concentration of 0, 0.1, 0.2, 0.25, 0.3, 0.4, 0.5 or 0.7 mM. The cupric ion is 25% of $CuSO_4.5H_2O$ (Lee *et al.*, 1994) and so the equivalent concentration of Cu^{2+} was 0, 6.36, 12.7, 15.9, 19.1, 25.5, 31.8 or 44.5 µg Cu^{2+}/ml.

The concentrations of copper tested were lower than the dose of copper applied to the plant surface immediately after spray application. For example, the commonly used bactericide, Mankocide®, contains 300 g/kg copper and is applied to walnuts at a concentration up to 500 g/100 L. The concentration of copper in this spray mixture would be 1,500 µg/ml of copper, which is nearly 34 fold higher than the highest copper dose used in this study. However, single bacterial cells would not be uniformly exposed or immersed in this dose of copper, as occurs in liquid culture.

All eight flasks were shaken on a platform orbital shaker at 130 rpm for 48 h at 28°C. The number of colony forming units (cfu) after incubation was determined by creating a dilution series (10^{-1} to 10^{-8} fold dilutions) (Anderson *et al.*, 1991; Martin *et* al., 2004), in sterile phosphate buffer. Nutrient agar plates prepared for colony counts were

allowed to dry in a laminar air-flow cabinet for 30 min to remove any excess moisture before inoculation with diluted culture. For each culture, 0.1 ml of each dilution was spread evenly across a plate of nutrient agar and allowed to dry for 10 min. Cultures were incubated at 28°C for 72 h, after which resulting bacterial colonies were counted. If a bacterial isolate grew at 0.7 mM, then the procedure described above was repeated at higher concentrations of copper; namely, 1.0, 1.1 or 1.2 mM $CuSO_45H_20$.

Nine of 37 strains indicated in Table 4.2, were assayed for copper sensitivity a second time to check the reproducibility of results. These nine strains were selected over the range of copper sensitivity observed in the first assay. Two of the nine strains used in the repeat assay were from home gardens to check their high level of sensitivity to copper as observed in the first assay. One strain was from a commercial orchard and was sensitive to copper according to the first assay and another of the nine strains, US 53 05, had 100% mortality at 0.3 and 0.4 mM copper sulfate in the first assay, but growth of strains was observed at 0.5 mM copper sulfate. The only difference in procedure between the first and second assays was that the brand of yeast extract was changed (from Amyl to Sigma).

5.2.1 Data analyses

Data were evaluated qualitatively to identify the minimum inhibitory concentration (MIC) of copper sulfate, defined here as the lowest concentration of copper sulfate that appeared to reduce multiplication (cfu) of the bacterial strain, when compared with the cfu in the absence of copper sulfate. MIC was a discrete value and equal to one of the concentrations of copper sulfate tested.

Data were also evaluated quantitatively to estimate the minimum lethal concentration (MLC) of copper sulfate and the lethal dose at which 50% of the bacterial cells were killed (LD50) by copper sulfate. The MLC was defined as the lowest concentration that killed the test bacterial strain (a cfu count of zero) and was used to calculate the percentage of strains surviving at each dose of copper sulfate. MLC and LD50 were estimated from a dose response curve generated for each isolate with the x-axis (independent variable) representing the concentration of copper sulfate (mM) and the y-axis (response) representing $Log_{10}(cfu+1)$. Linear regression was used to fit a second order polynomial ($f(x) = y = ax^2+bx+c$) to the response. The minimum lethal concentration (MLC) was estimated as the positive value of x when $y = 0$. For estimation of the LD50, the value of y at $x = 0$ was estimated and back-transformed to cfu, then halved and

transformed according to $Log_{10}((cfu/2)+1)$. This value was then substituted into the model to estimate x. These calculations are detailed in Appendix 1. The value of x when y was at a maximum was estimated as $-(b/2a)$ when the maximum value of y did not occur at $x = 0$.

The median and spread of MIC, MLC and LD50 values within each walnut region was summarised as box plots. The median was shown as a line across the box which stretched from the 25th percentile to the 75th percentile (the middle half of all scores in the distribution). The vertical lines extended to the 5th and 95th percentile, respectively.

5.3 Results

In the first assay of 37 isolates of *Xa,* the optical density (620 nm) of each bacterial culture used to inoculate flasks with different concentrations of copper sulfate ranged from 0.09 (US0505) to 0.202 (US3305), with a median of 0.144. The number of colony forming units (cfu) after incubation of these bacterial isolates in the absence of copper sulfate ranged from 2.4×10^6 (US4305) to 9.5×10^8 cfu (US3105), with a median of 5.4×10^7 cfu (Table 5.2).

The second order polynomial model for copper sensitivity fitted the observed data well for those isolates where the number of cfu increased as the concentration of copper sulfate increased from 0 to 0.1 mM and then declined with increasing copper sulfate concentration (Appendix 2). However, a flat response was observed for a number of isolates at the lower concentrations of copper sulphate, with the number of colonies declined sharply with increasing copper concentration. Estimates of LD50, MIC and MLC presented below were derived from the polynomial model for each isolate.

In the first assay, isolates of bacteria from home gardens were highly sensitive to copper sulfate in that the MIC was ≤ 0.1 mM and the MLC never exceeded 0.2 mM (Table 5.2, Appendix 2). The difference in sensitivity between isolates of *Xa* from home gardens and the isolates from commercial walnut orchards was evident in the plot of percentage strain mortality as a function of the concentration of copper sulfate (Fig. 5.1).

Bacterial isolates from commercial walnut orchards varied in their sensitivity to copper, from highly sensitive to less sensitive (Table 5.2). When the number of cfu was estimated to be at its highest (estimated x at y_{max} in Table 5.2), the concentration of copper sulfate ranged from 0 to 0.16 mM. The spread of MIC values for the first assay, by growing region, is presented in Fig. 5.2. Most isolates had MICs in the range 0.2-0.3 mM. The highest MIC observed was 0.4 mM copper sulfate for isolates US6505 and US7405. Like the home garden isolates, nine *Xa* from commercial orchards had a MIC of ≤ 0.1 mM.

Of all the *Xa* isolates US2605 and US6305 in the first assay grew at 0.5 mM. The highest MLC observed was 0.68 mM copper sulphate for isolate US6305, which was collected in the Coal River Valley. *Xa* isolates from commercial orchards that had a MLC of $\leq$ 0.2 mM were isolates US1005, US4305, US4805, US6205 and US7105. The spread of MLC values for the first assay, by growing region, is presented in Fig. 5.3.

Table 5.2 Parameters and estimates from assays of isolates of *Xanthomonas arboricola* from Tasmania for sensitivity to copper sulfate *in vitro*. Selected isolates were assayed twice where indicated. The abbreviation nd = not determined.

Isolate[A]	**cfu**[C] **at x = 0**	**Estimated x (mM) at y_{max}**	**LD_{50} (mM)**	**MIC (mM)**	**MLC (mM): estimated x at y = 0**
US 01 05 (1)	4.5×10^7 (1.9×10^7)	0.08	0.178	0.30	0.30
US 01 05 (2)	6.7×10^8 (4.0×10^9)	0.00	0.047	0.25	0.682
US 02 05	1.2×10^7 (6.0×10^6)	0.13	0.265	0.25	0.41
US 05 05	1.9×10^7 (9.1×10^6)	0.09	0.185	0.30	0.30
US 09 05	3.6×10^6 (1.1×10^7)	0.10	0.226	0.30	0.50
US 10 05 (1)	1.3×10^7 (nd)	0.00	0.004	<0.10	0.1
US 10 05 (2)	5.7×10^7 (5.7×10^7)	0.05	0.109	0.20	0.20
US 11 05	4.1×10^7 (1.4×10^7)	0.08	0.175	0.25	0.26
US 12 05	8.2×10^8 (7.2×10^8)	0.07	0.158	0.2	0.40
US 13 05	1.3×10^8 (7.8×10^7)	0.07	0.157	0.2	0.30
US 14 05	7.4×10^6 (1.4×10^7)	0.14	0.30	0.3	0.5
US 15 05	5.4×10^7 (1.7×10^8)	0.03	0.038	0.2	0.48
US 19 05	4.5×10^7 (2.1×10^7)	0.08	0.171	0.2	0.30
US 20 05	3.6×10^7 (8.0×10^7)	0.00	0.058	0.3	0.5
US 22 05	8.9×10^7 (2.0×10^7)	0.05	0.130	0.1	0.34

US 23 05 (1)	7.7×10^7 (1.4×10^8)	0.046	0.143	0.3	0.49
US 23 05 (2)	1.4×10^8 (7.7×10^7)	0.075	0.221	0.2	0.71
US 26 05	6.3×10^7 (5.3×10^8)	0.00	0.159	0.25	0.67
US 31 05	9.5×10^8 (3.0×10^8)	0.076	0.174	0.1	0.41
US 33 05	6.2×10^8 (2.5×10^8)	0.11	0.246	0.3	0.52
US 39 05	5.3×10^7 (6.6×10^7)	0.072	0.182	0.2	0.50
US 41 05	1.4×10^7 (9.7×10^6)	0.16	0.329	0.3	0.51
US 43 05	2.4×10^6 (2.4×10^6)	0.046	0.102	0.1	0.20
US 44 05 (1)	1.3×10^8 (5.9×10^7)	0.11	0.232	0.3	0.40
US 44 05 (2)	1.6×10^9 (3.6×10^9)	0.00	0.064	0.2	0.69
US 47 05	5.4×10^7 (5.5×10^7)	0.05	0.135	0.2	0.40
US 48 05	5.4×10^7 (5.4×10^7)	0.00	0.042	0.1	0.20
US 49 05	6.1×10^7 (5.7×10^7)	0.00	0.045	0.1	0.40
US 50 05 (1)	6.5×10^7 (1.5×10^7)	0.13	0.266	0.3	0.40
US 50 05 (2)	8.3×10^7 (2.6×10^7)	0.11	0.239	0.2	0.41
US 53 05 (1)	3.1×10^7 (2.4×10^7)	0.06	0.127	0.2	0.25
US 53 05 (2)	1.4×10^7 (1.4×10^7)	0.07	0.163	0.25	0.40
US 56 05 (1)	8.3×10^8 (2.2×10^8)	0.10	0.205	0.1	0.41
US 56 05 (2)	2.1×10^7 (1.1×10^7)	0.09	0.203	0.25	0.40
US 62 05	1.8×10^8 (1.7×10^8)	0.03	0.079	0.1	0.20
US 63 05	1.1×10^8 (5.7×10^8)	0.00	0.099	0.25	0.68
US 64 05	1.1×10^8 (9.7×10^7)	0.13	0.270	0.3	0.49
US 65 05	8.9×10^6 (2.7×10^6)	0.17	0.353	0.4	0.52
US 71 05	2.2×10^8 (2.2×10^8)	0.00	0.024	0.1	0.20
US 74 05	1.1×10^8 (2.1×10^7)	0.13	0.268	0.4	0.41
US 75 05	4.2×10^8 (1.8×10^8)	0.10	0.230	0.25	0.50

Home garden					
US 16 05 (1)	2.4×10^7 (1.4×10^7)	0.03	0.067	0.1	0.08
US 16 05 (2)	1.1×10^9 (1.1×10^9)	0.03	0.078	0.1	0.20
US 25 05(1)	4.1×10^7 (nd)	0.00	nd	<0.1	nd
US 25 05(2)	2.0×10^8 (2.0×10^8)	0.04	0.092	0.1	0.20
US 35 05	8.6×10^7 (8.6×10^7)	0.00	0.032	0.1	0.20

[A]Numbers in parentheses indicate the first (1) or second (2) assay for copper sensitivity. Isolates without these numbers were assayed once.

[B]The number in parenthesis is the estimated value of y when x = 0 from the quadratic function.

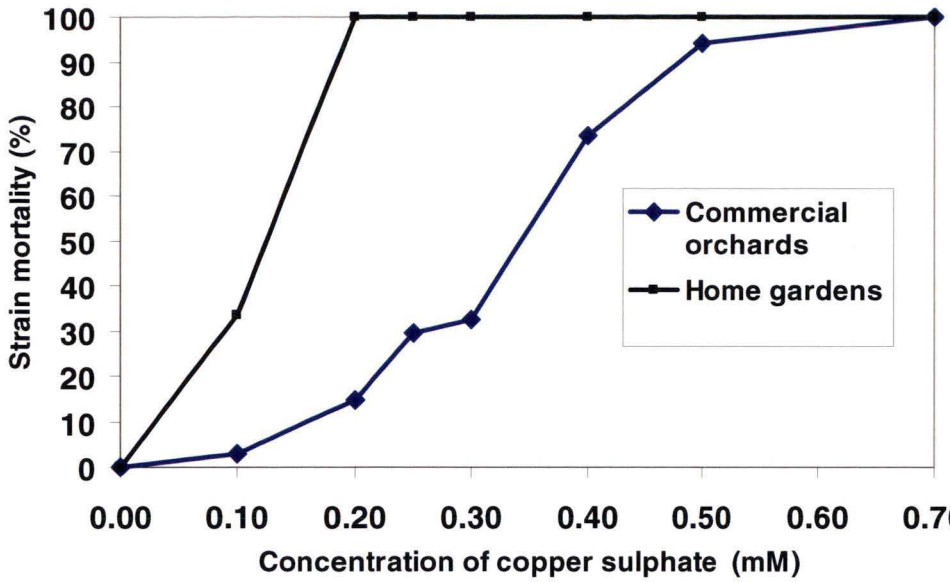


Figure 5.1 Mortality of *Xanthomonas arboricola* strains as a percentage of the total number of strains tested from home gardens (three isolates) or commercial walnut orchards (34 isolates), as a function of copper sulfate concentration.

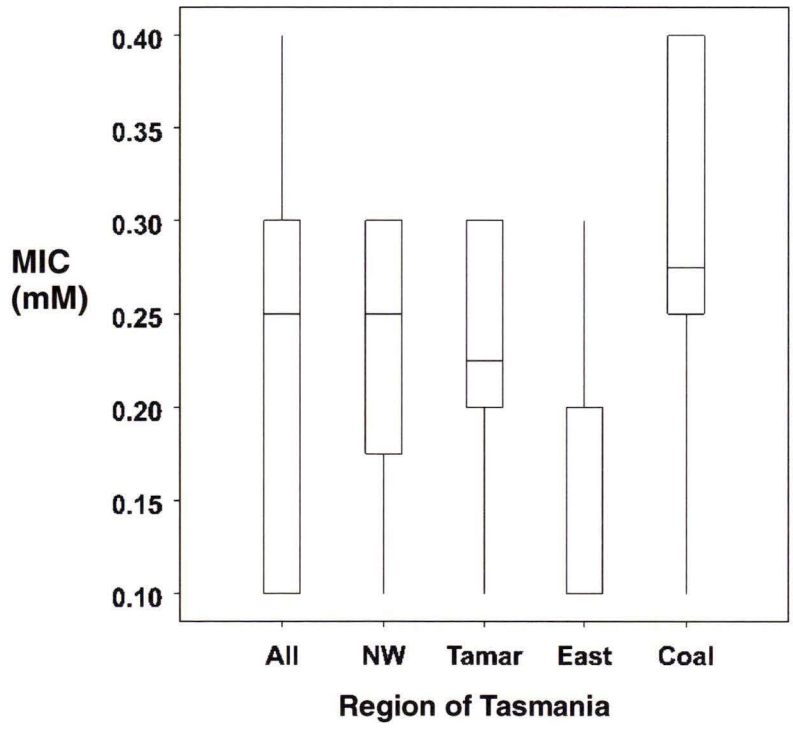


Figure 5.2 Box plot of estimates of MIC for 34 isolates of *Xanthomonas arboricola* collected from commercial walnut orchards, by growing region in Tasmania. The y axis is the MIC or minimum concentration of copper sulfate (mM) below which bacterial growth is not inhibited.

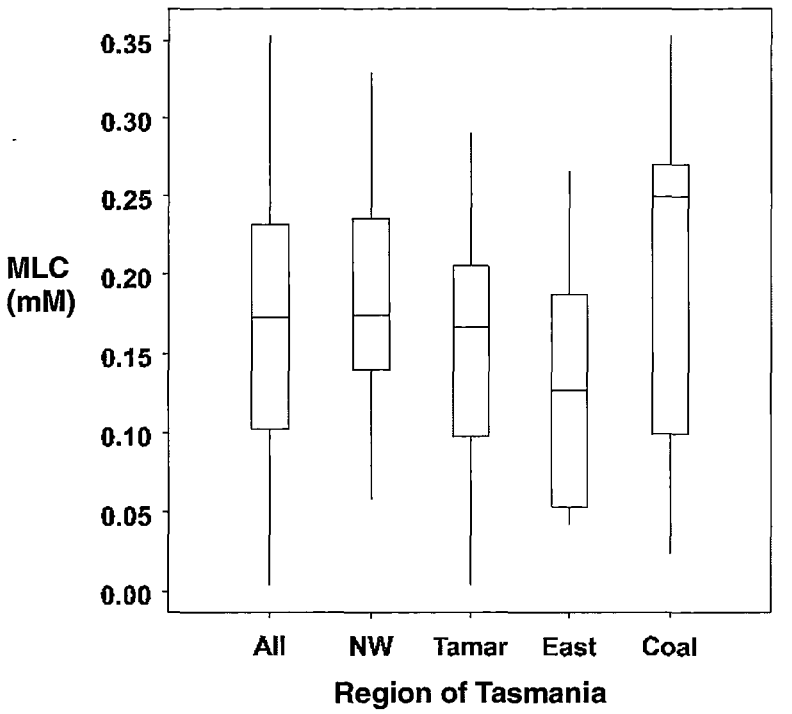


Figure 5.3. Box plot of estimates of MLC for 34 isolates of *Xa* collected from commercial walnut orchards, by growing region in Tasmania. The y axis is the MLC or the minimumconcentration of copper sulfate (mM) that killed the bacterial strain.

Table 5.3 Number of isolates of *Xanthomonas arboricola* from commercial walnut orchards in each range of estimated LD_{50} according to region sampled.

Region	LD_{50} 0-0.10	LD_{50} 0.11-0.20	LD_{50} 0.21-0.3	LD_{50} >0.3	Total
North West	2	6	4	1	13
Tamar Valley	2	4	2	0	8
East Coast	3	2	2	0	7
Coal River Valley	2	0	3	1	6
All regions	9	12	11	2	34

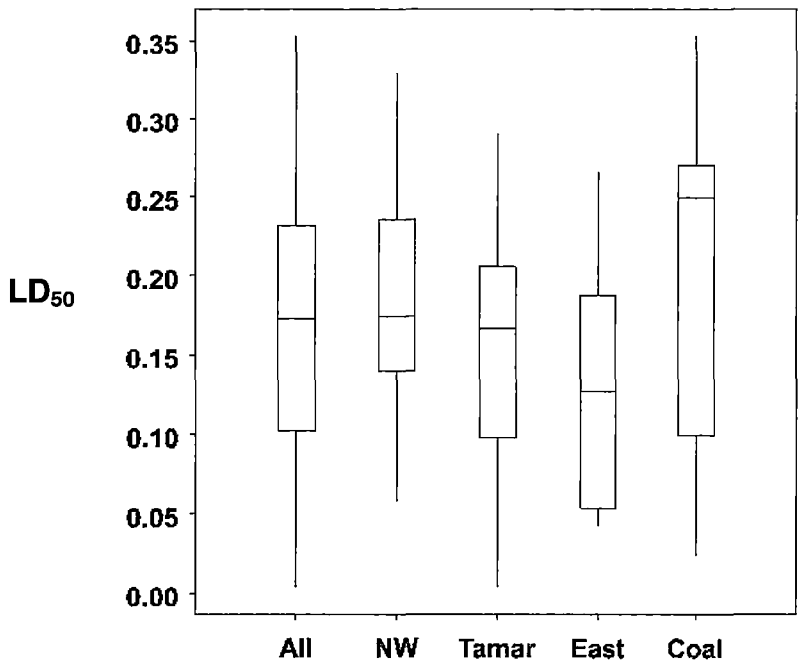


Figure 5.4. Box plot of estimates of LD_{50} for 34 isolates of *Xanthomonas arboricola* collected from commercial walnut orchards, by growing region in Tasmania. The y axis is the LD_{50} or the lethal dose of copper sulphate (mM) for 50% mortality of bacteria.

The spread of values for LD_{50} in the first assay for *Xa* isolates from commercial orchards is presented in Table 5.3 and Fig. 5.4. Most isolates had a LD_{50} in the range 0.1 to 0.3 mM, with two of the 34 isolates (US4105, US6505), each from a different region, having a LD_{50} greater than 0.3 (Table 5.3). Among isolates from commercial walnut orchards, the highest estimated LD_{50} was 0.353 mM copper sulfate for isolate US 65 05 from the Coal River Valley and the lowest estimated LD_{50} value was 0.024 mM copper sulfate for isolate US 71 05, also from the Coal River Valley.

The percentage survival of strains as a function of the concentration of copper sulfate appeared to be lower for isolates from the Tamar Valley and the East Coast when compared to the Coal River Valley and the North West of Tasmania (Fig. 5.5). However, there were no significant differences among regions in the numbers of isolates in each LD_{50} category (Table 5.3), according to Pearson's chi-squared test with simulated P-value ($P = 0.5902$).

Considering all isolates of *Xanthomonas arboricola* from Tasmania, isolates from

Serr and Franquette appeared to be more sensitive to copper sulfate than isolates form Chandler and Vina (Fig. 5.6). However, the sample size for each walnut variety was small and variable.

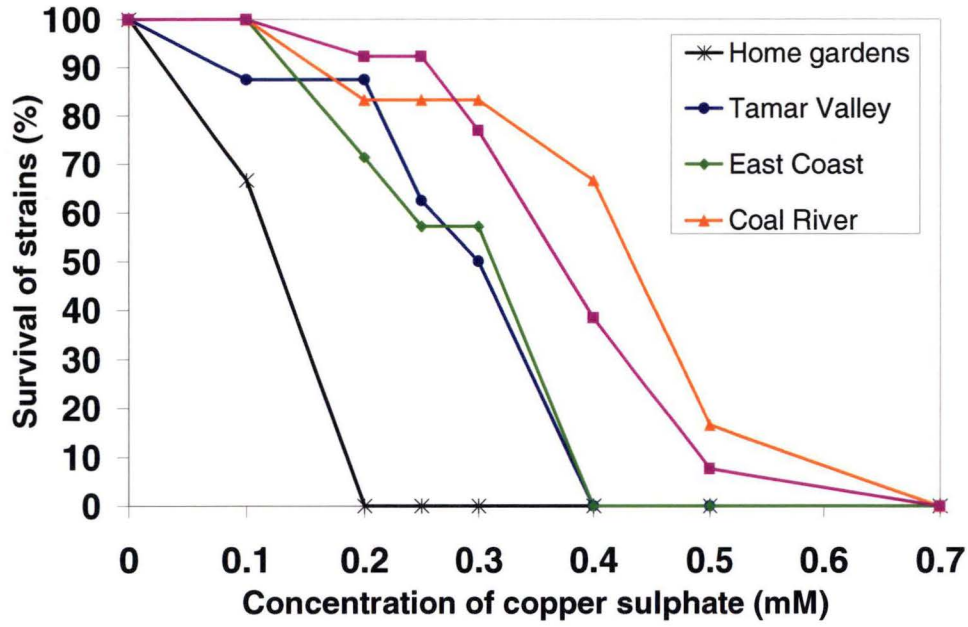


Figure 5.5 Survival of *Xanthomonas* spp. strains from home gardens (three isolates) or specific walnut growing regions (34 isolates) in Tasmania as a percentage of the total number of strains tested, as a function of copper sulfate concentration.

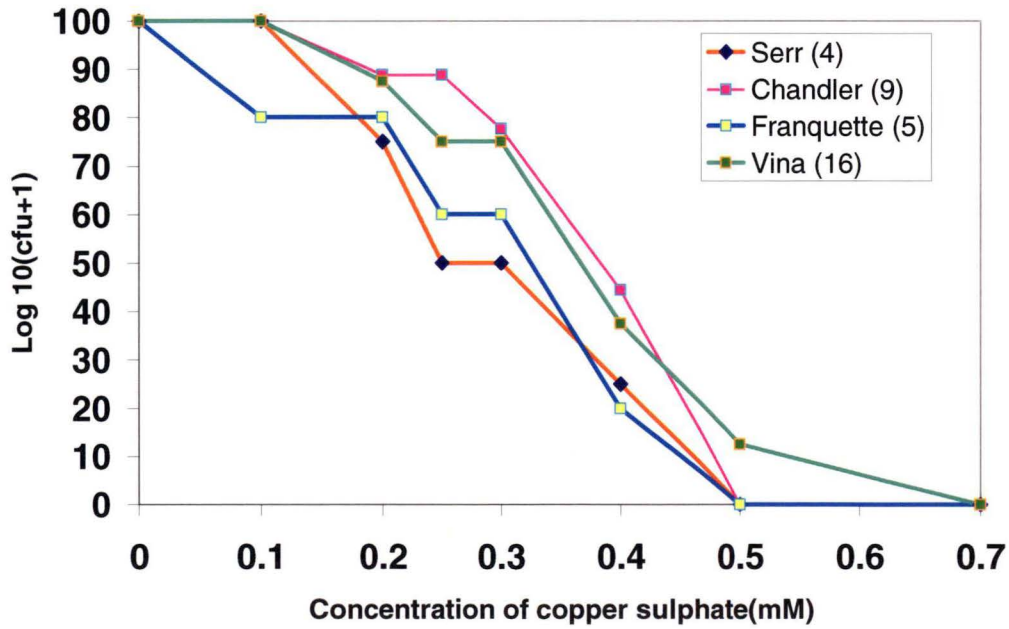


Figure 5.6 Survival of *Xanthomonas* spp. strains isolated from different commercially grown walnut varieties as a percentage of the total number of strains tested, as a function of copper sulfate concentration. The number of isolates tested for each variety is listed in parentheses in the figure legend.

When the assay for copper sensitivity was repeated with nine isolates of *Xa* the cfu in the absence of copper sulfate was higher than the previous assay, ranging from 1.4 x 10^7 (US5305) to 1.6 x 10^9 cfu (US4405), with a median of 1.4 x 10^8 cfu (Table 5.2, Appendix 2). When values of MLC were compared per isolate for the first and second assay, the values of MLC were higher in the second assay if the number of cfu in the absence of copper sulfate was also higher (Table 5.2). Given this observation, differences in copper sensitivity among isolates were compared within assays only. The repeat assay for isolate US 53 05 produced the same result with colonies having 100% mortality at 0.3 mM copper sulfate, whereas growth of colonies was observed at 0.5 mM copper sulfate. Unlike the first assay, isolates US0105, US2305 and US4405 in the second assay grew at concentrations of copper sulphate above 0.5 mM, with isolate US2305, from the northwest of Tasmania, having the highest MLC of 0.71 mM copper sulphate.

5.4 Discussion

This study presents the current known range of copper sensitivity among isolates of *Xanthomonas arboricola* from walnut fruit sampled broadly across Tasmania. The fact that all bacterial isolates from home gardens had a MLC of ≤ 0.2 mM suggested that exposure to copper through commercial orchard practices has selected for individuals that can tolerate higher doses of copper than those that have never been exposed.

Regarding data analyses, the second order polynomial model appeared to be sub-optimal for explaining the relationship between LOG_{10}(cfu) and the concentration of copper sulphate for a subset of isolates. Another model or several models will be tested in the future for improving the accuracy of estimates for LD_{50}, MIC and MLC presented below. Despite the scope for improving estimates (by assay design and data analyses), the results can be interpreted in relation to other studies.

The highest MIC observed in this study, 0.4 mM copper sulfate, was lower than the highest value reported for a *Xanthomonas* species, which appears to be 2.4 mM copper sulfate for strains of *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) grown on amended nutrient agar (Bender *et al.*, 1990). The assay for copper sensitivity reported here utilised PYEG broth, a medium in which copper tends to remain in ionic form. It is possible that bacterial strains from Tasmania could have tolerated a higher copper concentration than reported, if this research had been conducted using PYEG agar (solid) media.

Several authors have applied a threshold MIC for identifying bacterial strains that

are considered resistant to copper after assay on solid media. For example, strains of *Xaj* from France were considered copper resistant if their MIC was ≥ 32 μg Cu^{2+}/ml (Garden *et al.*, 1993). Similarly, Scortichini *et al.* (2001) considered that *Xaj* strains that grew at 20-60 μg Cu^{2+}/ml were copper resistant. Strains of *Xaj* that grew above 30 μg Cu^{2+}/ml were considered copper resistant by Lee *et al.* (1993, 1994). Martin *et al.* (2004) considered that strains of *Xcv* that survived at 1.0 mM copper sulfate (64 μg Cu^{2+}/ml) in liquid culture were copper tolerant, if not copper resistant. None of these authors described their criteria for determining their threshold MIC for copper resistance. According to J. Vanneste (HortResearch, New Zealand, personal communication), a bacterial strain is truly copper resistant if genes for copper resistance in the bacterium are characterised and the strain grows on solid media amended with 250 μg Cu^{2+}/ml.

For other bacterial plant pathogens, Scheck *et al.* (1996) analysed copper and streptomycin resistance of *P. syringea* from nurseries in the Pacific Northwest in the USA. They considered strains that grew at 0.32 mM $CuSO_4.5H_2O$ or higher were copper resistant. Similarly, Anderson *et al.* (1990) considered that strains of *P. syringea* that grew at 0.36 mM $CuSO_4.5H_2O$ were copper resistant. Considering the current interpretation of what constitutes copper resistance or tolerance for various phytopathogenic bacteria in the literature, then it might be assumed that strains of *Xa* from walnut that were able to grow at 0.5 mM copper sulfate were tolerant of copper in a technical sense. Based on this interpretation, 2/34 strains of *Xa* from commercial walnut orchards in Tasmania would be considered copper tolerant. This finding does not mean copper sprays will fail to control these strains if present in a walnut orchard. The dose of copper in a commercial spray mixture is at least 30 fold higher than the highest dose of copper tested in this study. However, uneven application of spray droplets and the decay of copper on the leaf or fruit surface means that the dose of copper varies in time and space. The usefulness of the *in vitro* study of copper sensitivity is for application to bacterial populations over time in order to detect changes or shifts in copper sensitivity. Any disease control failures experienced following application of copper in walnut orchards could be explored in terms of copper tolerance, if other factors such as sub-optimal spray timing or coverage are ruled out.

In concurrent studies of walnut blight epidemiology, alternatives to copper sprays and the minimisation of copper inputs are the key objectives toward effective and sustainable control of this disease in Tasmania (Lang and Evans, 2005). Research on reduction in the use of copper is for environmentally friendly purposes: for reducing

accumulation of copper in soil and for protecting soil microflora, while decreasing the potential of bacterial populations to develop tolerance to copper (Radix *et al.*, 1994; Garcin *et al.*, 2001; Ninot *et al.* 2002).

General discussion

Xanthomonas arboricola pv *juglandis* (*Xaj*) is considered to be the causal agent of walnut blight in the major walnut production regions of the world (CABI, 1987; Lindow 2002). According to GC-FAME analyses, 32 of 45 isolates of bacteria from symptomatic walnut fruit in Tasmania were *Xanthomonas arboricola.* The frequent isolation of *Xanthomonas arboricola* from blight lesions on walnut fruit using the non-selective NA medium suggested that this organism was associated with the disease. However, non-Xanthomonad bacteria were associated with walnut blight lesions and their presence might be explained by ineffective surface sterilisation of saprophytic bacteria, combined with semi-selective media that allowed the growth of genera such as *Erwinia, Pantoea, Pseudomonas* and *Serratia.*

Strains of *Pseudomonas* producing yellow cellular pigment and fluorescent pigment have been isolated from cankers on walnut trees (Hildebrand *et al.*, 1994). The strains did not produce any blight symptoms when inoculated on walnut leaves and shoot tips, but were easily recovered from injured areas one month after inoculation, indicating that the strains were residents but not pathogenic. When these same Pseudomonad strains were co-inoculated with *Xaj*, typical blight lesions were produced. In this study, the question remains as to whether bacteria such as *Erwinia, Pantoea, Pseudomonas* and *Serratia* were saprophytes that were co-isolated with Xanthomonads or whether they contributed to walnut blight symptoms. Du Plessis and van der Westhuizen (1995) isolated a strain of *Xanthomonas arboricola* pv *pruni* from walnut shoots but demonstrated that this isolate was pathogenic on plum leaves but not on walnut leaves. Therefore, some bacterial taxa may have a resident phase on walnut without causing disease symptoms. *Xa* pv. *pruni* was named frequently during GC-FAME analyses, although GC-FAME does not distinguish pathovars of *Xanthomonas* well (Yang *et al.* 1993). Further research is required to ascertain whether or not *Xa* pv. *pruni* is resident and/or pathogenic on walnut in Tasmania. In concurrent studies, M. Lang (MSc student, University of Tasmania) is screening suitable methods to determine the pathogenicity of both Xanthomonad and non-Xanthomonad bacteria isolated in Tasmania to walnut fruit and for satisfying Koch's postulates.

For isolation of *Xanthomonas* at a high frequency from blight lesions in Tasmania, it is recommended that GYCA medium be used to screen bacterial isolates for mucoid

growth. Scortichini *et al.* (2001) and Scortichini and Rossi (2002) also used this medium for isolating *Xanthomonas*. SQ media can also be used concurrently for confirming the almost universal character of *Xaj* for metabolising quinate. In contrast, Murlean and Schroth (1981) used BS media to recover isolates of *Xa* and Scortichini *et al.* (2001) found it useful for isolating *Xaj* from leaves and twigs. The use of BS media for selection of *Xanthomonas* was not useful in this study, given the weak to moderate growth of bacterial isolates observed. Du Plessis and van der Westhuizen (1995) also reported a low recovery rate for *Xaj* from symptomatic walnuts from South Africa when using BS media, with some isolates lacking hydrolytic activity, as observed in this study.

Results of this study were consistent with the range of phytopathogenic and/or saprophytic bacteria reported to metabolise quinate on SQ media. For example, species of *Erwinia* and *Pseudomonas* that metabolised quinate were isolated from walnut blight lesions, consistent with the findings of Lee and Chou (2003) and Lee *et al.*, (1992). All vigorously growing isolates of putative *Xanthomonas arboricola* (*Xa*) were able to metabolise quinate on SQ media, except for isolate US3505. The reason for this finding remains obscure because quinate metabolism appears to be a stable character for *Xaj* (Lee *et al.*, 1992; Scortichini *et al.* 2001). Isolate US3505, unlike *Xa* isolate US7105 which metabolised quinate, was not well separated genetically from other isolates of *Xa* when analysed by rep-PCR. The genetic and phenotypic characters of isolates US3505 and US7105 should be investigated further.

It was clear from this research that phenotypic characters alone cannot differentiate pathovars of *Xa* or differentiate *Xa* from non Xanthomonads. G-C FAME allowed isolates to be determined to the species level, but it was considered unreliable at discriminating *X. arboricola* at the pathovar level (Yang *et al.* 1993). The results emphasised the importance of adopting more than one diagnostic technique, including molecular-genetic techniques for resolving issues of taxonomy and for discriminating closely-related bacteria. Methods for rep-PCR were adopted and modified to produce DNA fingerprints that were easy to score. The preliminary study of genetic variation in *Xa* demonstrated the ability of rep-PCR to differentiate Xanthomonads and non-Xanthomonads. Isolates of *Xa* from Tasmania that have different rep-PCR profiles can now be selected for studies of pathogenicity in walnut that include other species of Xanthomonas and non-Xanthomonad for determining which isolates cause walnut blight symptoms.

The next step in the development of rep-PCR is to obtain reproducible DNA fingerprints for all 37 isolates of *Xa* with each of the three sets of primers (BOX, ERIC and

REP). This information would allow a more precise study of genetic variation within *Xa* from Tasmania, especially if the study included isolates of *Xaj* from other regions, other pathovars of *Xa,* other *Xanthomonas* species and several non-Xanthomonads as out-groups. Given current evidence that rep-PCR technique can resolve individuals at the sub-specific level, then genetic variation in *Xa* should be studied at the appropriate level of plant organisation, as discussed in Chapter 4. Knowledge of the genetic structure of the walnut blight pathogen could be related to information on pathogenicity, which might inform strategies for disease management.

Having characterised bacterial isolates associated with blight lesions on walnut fruit in Tasmania, it was possible to select the 37 isolates of *Xa* for addressing the major objective of this research, which was to estimate the range of copper sensitivity among isolates of *Xa* collected on a broad geographical scale. A major finding of this study was that two of 34 isolates of *Xa* from commercial walnut orchards in Tasmania were classified as copper tolerant following *in vitro* assay. This is the first report of copper tolerant strains of the walnut blight bacterium from Australia and the southern hemisphere. This finding adds to the increasing number of regions where copper tolerant isolates of *Xaj* have been reported, including the most recent report from Portugal (Scortichini *et al.*, 2001).

There is circumstantial evidence from this study to suggest that copper is exerting an environmental selection pressure towards reduced copper sensitivity among some populations of *Xa* in Tasmania. The first piece of evidence is the detection of two copper tolerant strains in the relatively small sample of 34 isolates, plus the fact that these two isolates originated from orchards where copper had been applied to walnut trees for more than five growing seasons. The second piece of evidence is the high sensitivity to copper of all three isolates from home gardens where copper had never been applied to the walnut canopy. Two non-Xanthomonad bacteria from home gardens were also highly sensitive to copper (data not presented). Shifts in populations of phytopathogenic Xanthomonads to lower copper sensitivity in response to selection by copper in the environment have been well documented (Martin *et al.* 2004) and demonstrate the importance of establishing baseline information on copper sensitivity, as reported in this study.

Determining whether or not copper tolerance is a widespread issue for walnut growers in Tasmania will require adoption of a hierarchical sampling strategy to study the sensitivity of bacterial populations within and among walnut orchards over time. In the study conducted by Martin *et al.* (2004), strains of *Xcv* isolated pre-1987 were more

sensitive to copper than strains isolated post 1987. This result was explained by the greater exposure of post-1987 populations of the bacterium to copper sprays when compared with pre-1987 populations. A similar change in the sensitivity of populations of *P. syringea* over time was reported by Scheck *et al.* (1996). In the future, intensive sampling of walnut orchards within and among growing seasons may also be useful in detecting shifts in the bacterial population in their sensitivity to copper in relation to the intensity of copper applications. The relatively small sample size meant that the apparent differences in copper sensitivity of groupings of isolates according to region sampled or host variety were not statistically significant. Larger sample sizes and a suitable sampling strategy may assist in detecting regional and/or varietal differences in the future.

The results of this study highlight the urgent need to reduce inputs of copper in commercial walnut production by development of more sustainable solutions for managing bacterial diseases. Studies of walnut blight epidemiology, alternatives to copper sprays and the minimisation of copper inputs are the key objectives toward effective and sustainable control of this disease in Tasmania (Lang and Evans, 2005). A reduction in the use of copper, while delaying the development of copper tolerance, may also prevent the build up of this heavy metal in soil, which has been shown to be detrimental to the soil microflora (Radix *et al.*, 1994; Garcin *et al.*, 2001). Effective sampling strategies for studies of copper sensitivity and genetic variation will be necessary in order to fully understand the nature of the bacterial population and its capacity to evolve in response to its environment and disease management.

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Appendix 1

Abbreviations used below:

LD_{50} = lethal dose of copper sulphate at which 50% of bacterial cells are killed

MLC = minimal lethal concentration, lowest concentration that kills the bacterial strain

SQRT = square root

Steps in the calculation of LD_{50} and MLC from the quadratic function f(x) or $y = ax^2+bx+c$

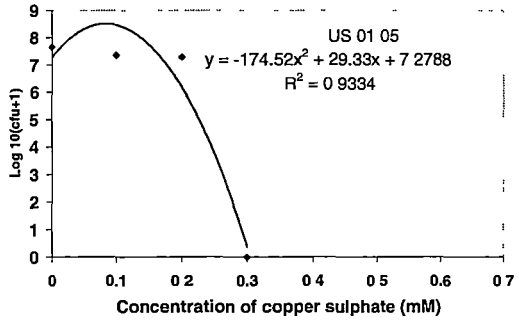
1. Calculate the value of y when x = 0. (If x = 0, then y = c)
2. Maximum (estimated) cfu = $(10\text{^}y_{x=0})-1$.
   The LD_{50} was then defined as the value of x (a positive number) at 50% of the maximum estimated cfu.
3. y at (maximum cfu/2) = $Log_{10}((\text{maximum cfu}/2)+1)$
4. Calculate x for y = $Log_{10}((\text{maximum cfu}/2)+1)$
5. LD_{50} = $(-b-SQRT(b^2-4a(c-(Log_{10}((\text{maximum cfu}/2)+1))))/2a$

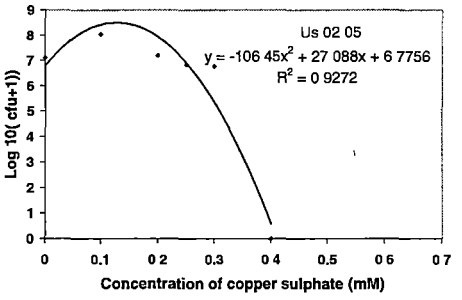
Calculation of MLC (x) at y = 0:

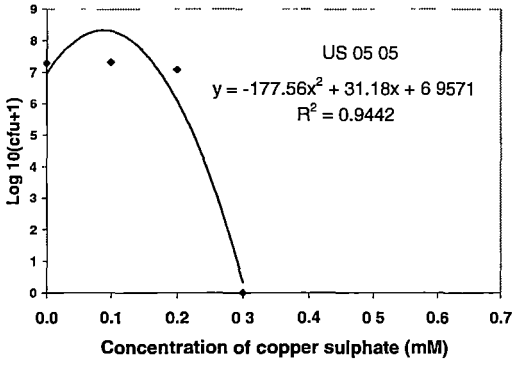
$x = (-b - (SQRT\ (b^2-4ac))\ /2a$

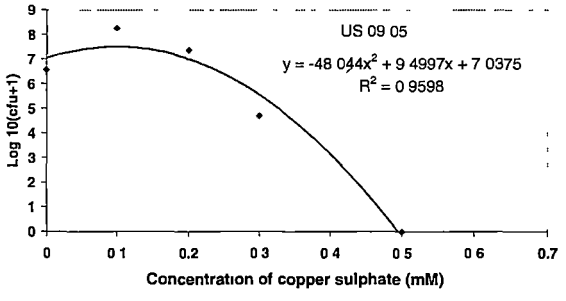
Appendix 2

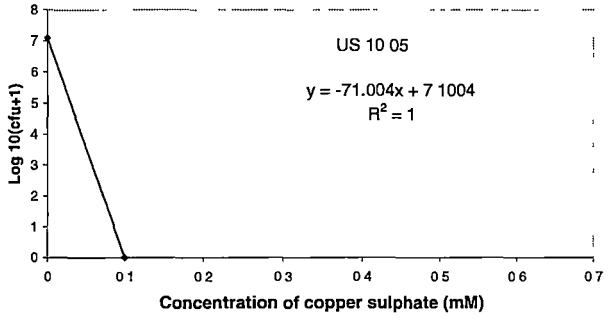
A. Quadratic functions estimated for each of 37 isolates of *Xanthomonas arboricola* assayed for copper sensitivity.

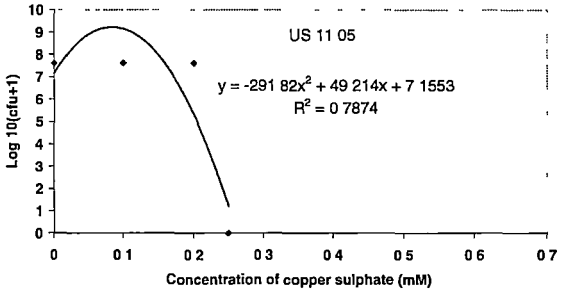


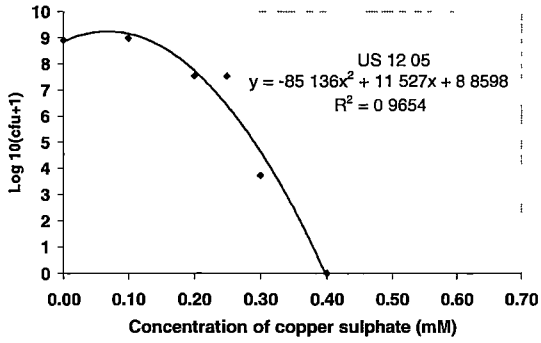


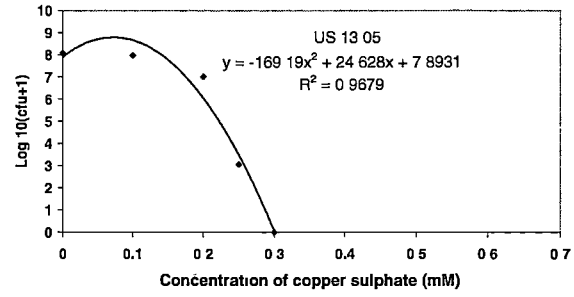


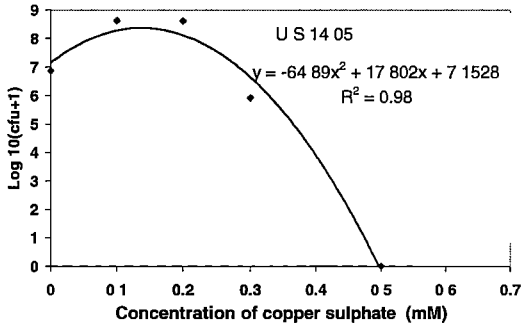


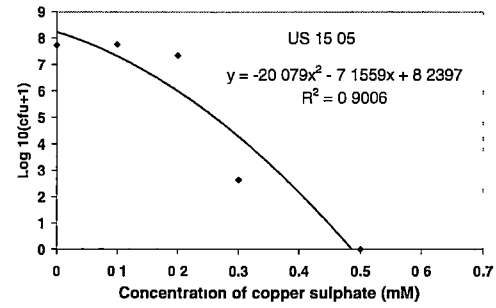


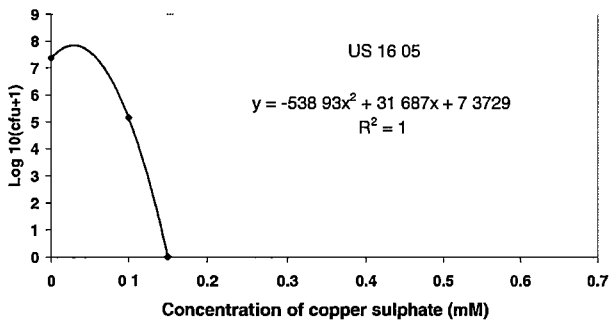


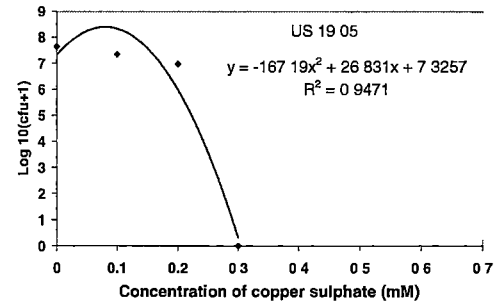

US 12 05
y = -85 136x² + 11 527x + 8 8598
R² = 0 9654
Log 10(cfu+1)
Concentration of copper sulphate (mM)

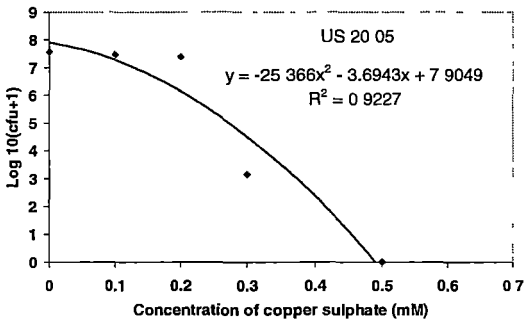

US 13 05
y = -169 19x² + 24 628x + 7 8931
R² = 0 9679
Log 10(cfu+1)
Concentration of copper sulphate (mM)

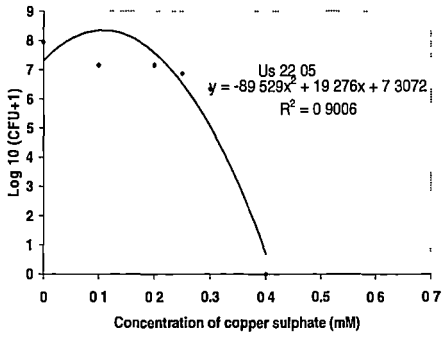

U S 14 05
y = -64 89x² + 17 802x + 7 1528
R² = 0.98
Log 10(cfu+1)
Concentration of copper sulphate (mM)

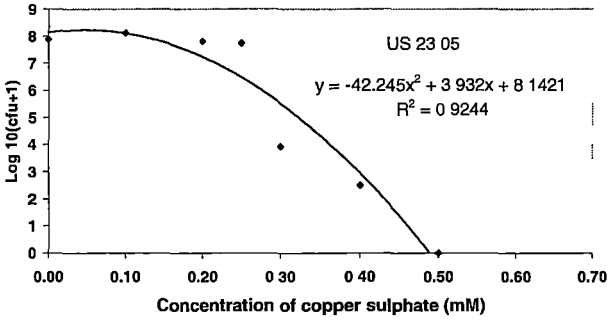

US 15 05
y = -20 079x² - 7 1559x + 8 2397
R² = 0 9006
Log 10(cfu+1)
Concentration of copper sulphate (mM)

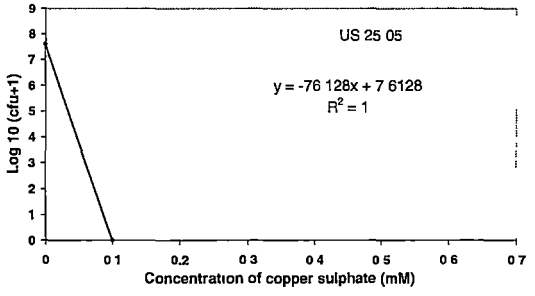

US 16 05
y = -538 93x² + 31 687x + 7 3729
R² = 1
Log 10(cfu+1)
Concentration of copper sulphate (mM)

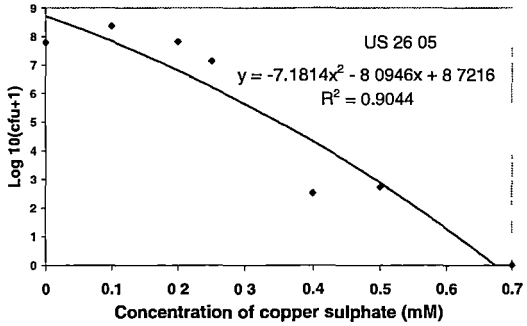

US 19 05
y = -167 19x² + 26 831x + 7 3257
R² = 0 9471
Log 10(cfu+1)
Concentration of copper sulphate (mM)

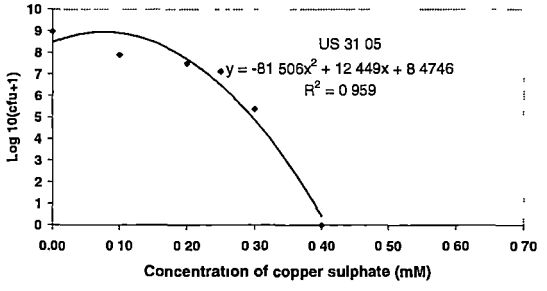

US 20 05
y = -25 366x² - 3.6943x + 7 9049
R² = 0 9227
Log 10(cfu+1)
Concentration of copper sulphate (mM)

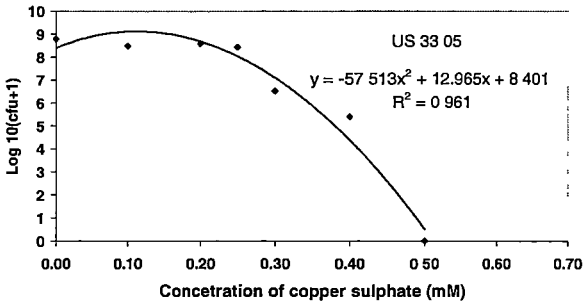

Us 22 05
y = -89 529x² + 19 276x + 7 3072
R² = 0 9006
Log 10 (CFU+1)
Concentration of copper sulphate (mM)

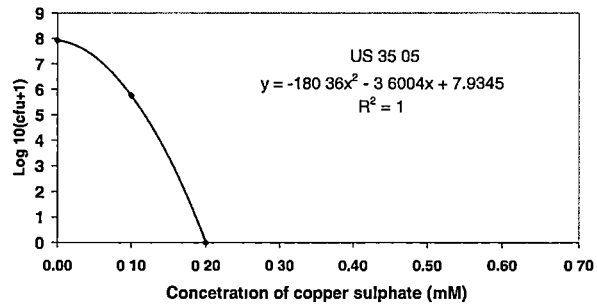

US 23 05
y = -42.245x² + 3 932x + 8 1421
R² = 0 9244
Log 10(cfu+1)
Concentration of copper sulphate (mM)

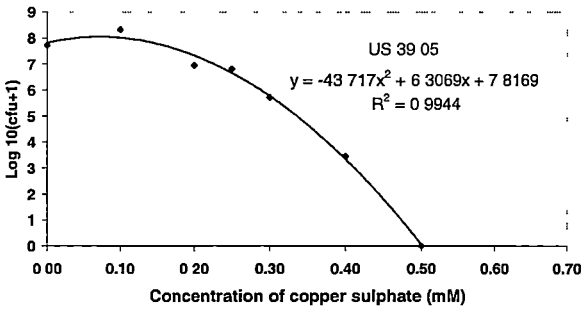

US 25 05
y = -76 128x + 7 6128
R² = 1
Log 10 (cfu+1)
Concentration of copper sulphate (mM)

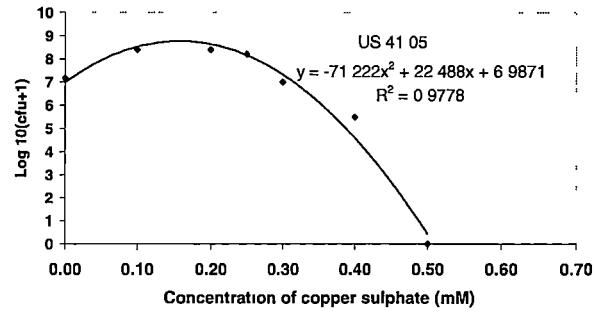

US 26 05
y = -7.1814x² - 8 0946x + 8 7216
R² = 0.9044
Log 10(cfu+1)
Concentration of copper sulphate (mM)

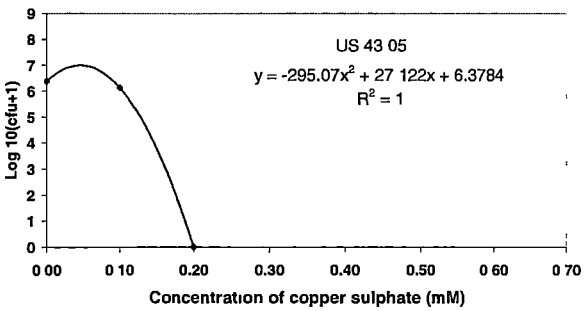

US 31 05
y = -81 506x² + 12 449x + 8 4746
R² = 0 959
Log 10(cfu+1)
Concentration of copper sulphate (mM)

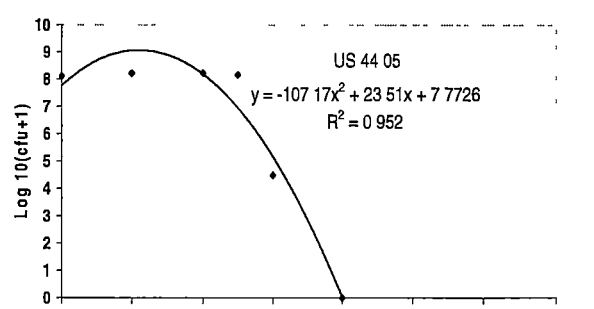

US 33 05
y = -57 513x² + 12.965x + 8 401
R² = 0 961
Log 10(cfu+1)
Concetration of copper sulphate (mM)

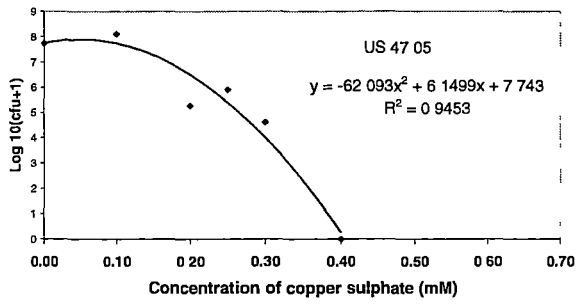

US 35 05
y = -180 36x² - 3 6004x + 7.9345
R² = 1
Log 10(cfu+1)
Concetration of copper sulphate (mM)

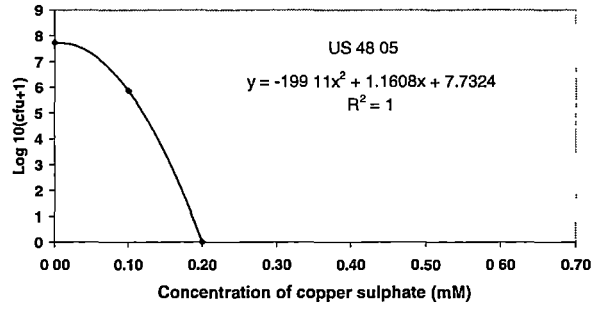

US 39 05
y = -43 717x² + 6 3069x + 7 8169
R² = 0 9944
Log 10(cfu+1)
Concentration of copper sulphate (mM)

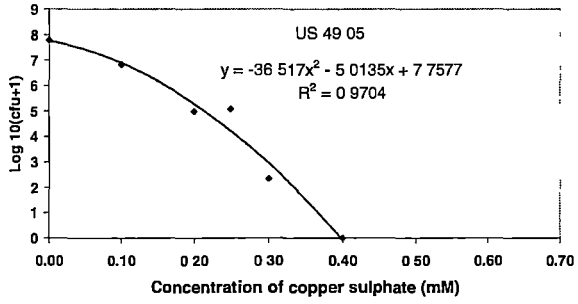

US 41 05
y = -71 222x² + 22 488x + 6 9871
R² = 0 9778
Log 10(cfu+1)
Concentration of copper sulphate (mM)

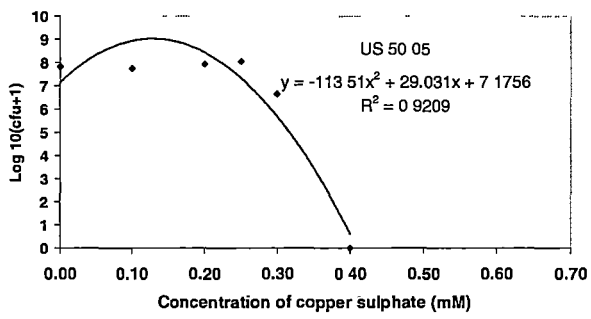

US 43 05
y = -295.07x² + 27 122x + 6.3784
R² = 1
Log 10(cfu+1)
Concentration of copper sulphate (mM)

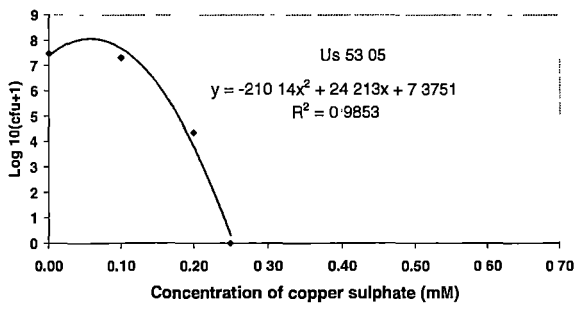

US 44 05
y = -107 17x² + 23 51x + 7 7726
R² = 0 952
Log 10(cfu+1)
Concentration of copper sulphate (mM)

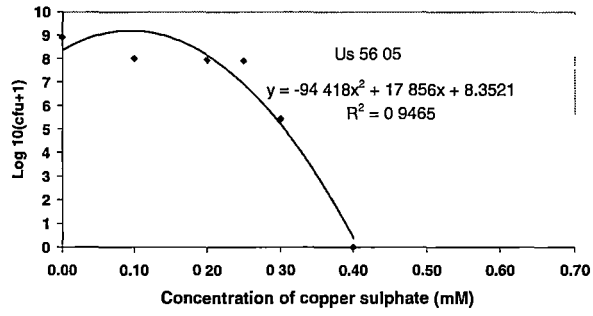

US 47 05
y = -62 093x² + 6 1499x + 7 743
R² = 0 9453
Log 10(cfu+1)
Concentration of copper sulphate (mM)

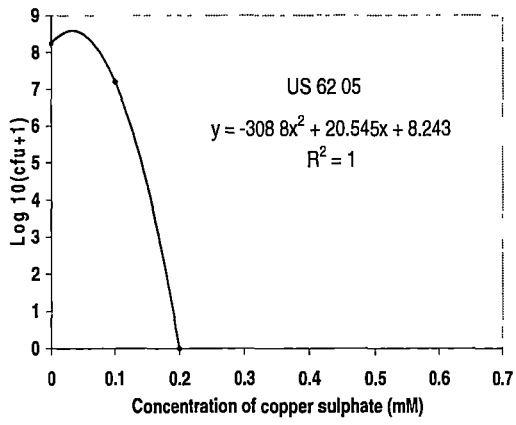

US 48 05
y = -199 11x² + 1.1608x + 7.7324
R² = 1
Log 10(cfu+1)
Concentration of copper sulphate (mM)

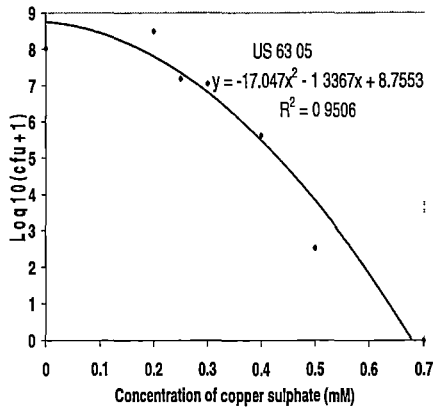

US 49 05
y = -36 517x² - 5 0135x + 7 7577
R² = 0 9704
Log 10(cfu+1)
Concentration of copper sulphate (mM)

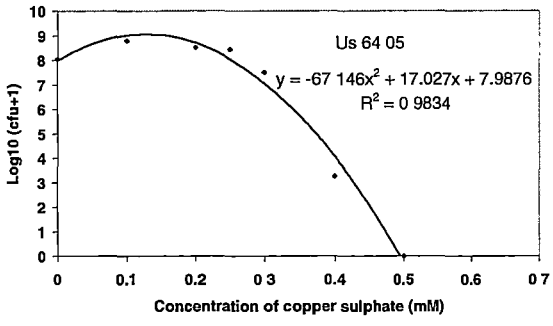

US 50 05
y = -113 51x² + 29.031x + 7 1756
R² = 0 9209
Log 10(cfu+1)
Concentration of copper sulphate (mM)

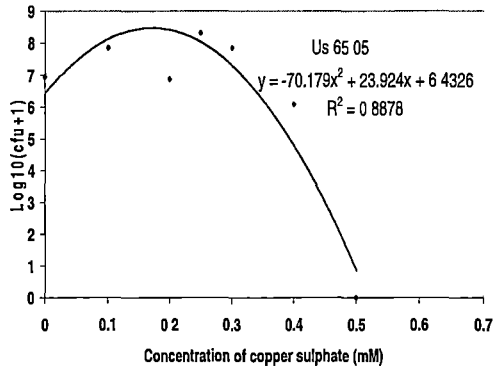

Us 53 05
y = -210 14x² + 24 213x + 7 3751
R² = 0 9853
Log 10(cfu+1)
Concentration of copper sulphate (mM)

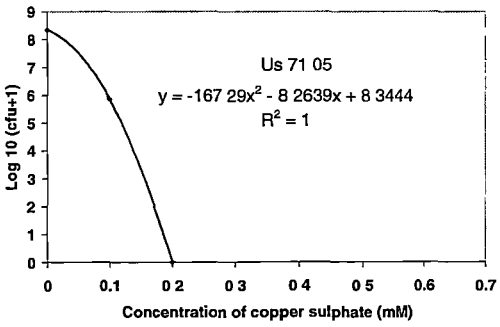

Us 56 05
y = -94 418x² + 17 856x + 8.3521
R² = 0 9465
Log 10(cfu+1)
Concentration of copper sulphate (mM)

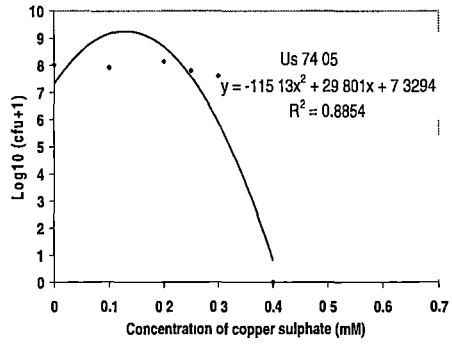

US 62 05
y = -308 8x² + 20.545x + 8.243
R² = 1
Log 10(cfu+1)
Concentration of copper sulphate (mM)

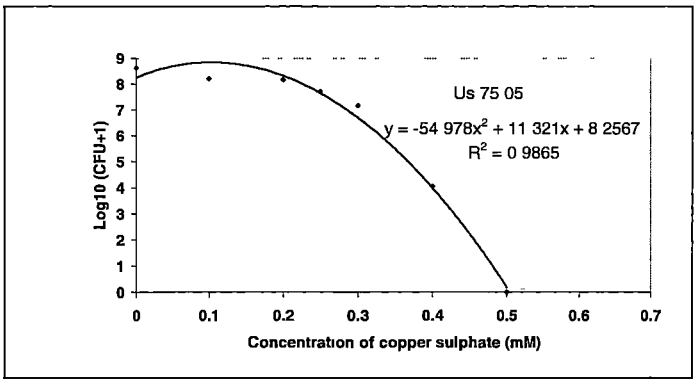

US 63 05
y = -17.047x² - 1 3367x + 8.7553
R² = 0 9506
Log 10(cfu+1)
Concentration of copper sulphate (mM)


Us 64 05
y = -67 146x² + 17.027x + 7.9876
R² = 0 9834
Log10 (cfu+1)
Concentration of copper sulphate (mM)

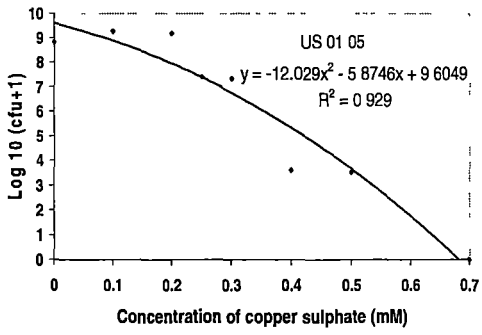

Us 65 05
y = -70.179x² + 23.924x + 6 4326
R² = 0 8878
Log10(cfu+1)
Concentration of copper sulphate (mM)

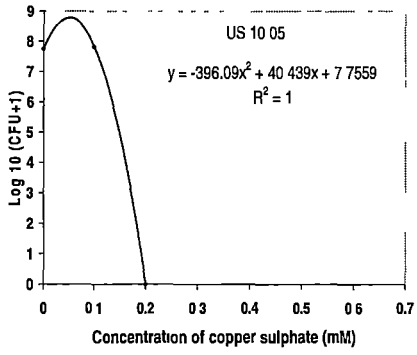

Us 71 05
y = -167 29x² - 8 2639x + 8 3444
R² = 1
Log 10 (cfu+1)
Concentration of copper sulphate (mM)

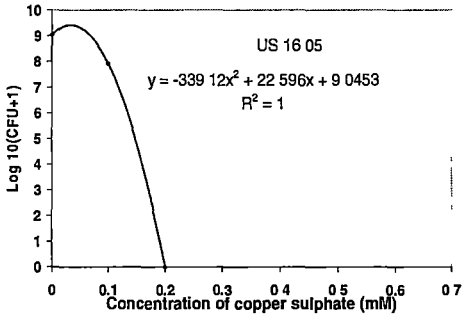

Us 74 05
y = -115 13x² + 29 801x + 7 3294
R² = 0.8854
Log10 (cfu+1)
Concentration of copper sulphate (mM)

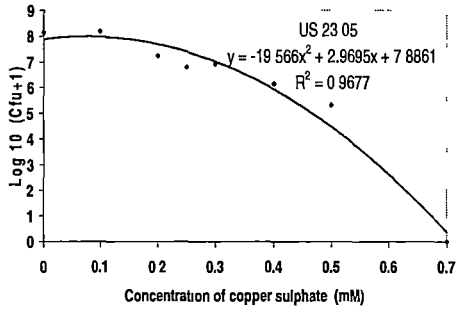

Us 75 05
y = -54 978x² + 11 321x + 8 2567
R² = 0 9865
Log10 (CFU+1)
Concentration of copper sulphate (mM)
0
1
2
3
4
5
6
7
8
9
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7

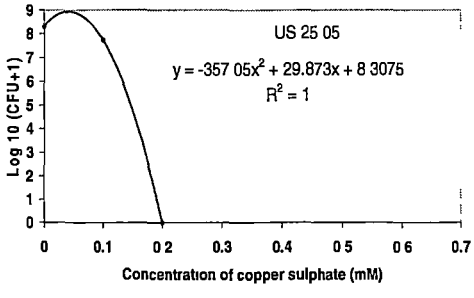
B. Quadratic functions of copper sensitivity of nine isolates of *Xanthomonas arboricola* in the repeat assays.

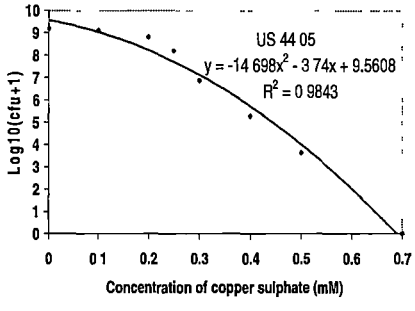


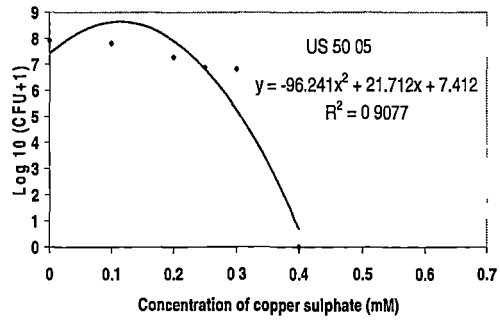


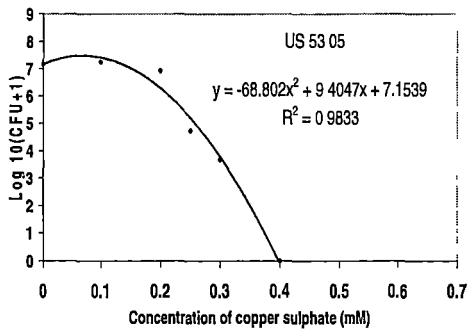


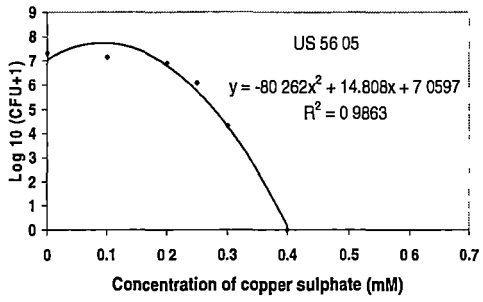







US 50 05
y = -96.241x² + 21.712x + 7.412
R² = 0 9077
Log 10 (CFU+1)
Concentration of copper sulphate (mM)


US 53 05
y = -68.802x² + 9 4047x + 7.1539
R² = 0 9833
Log 10(CFU+1)
Concentration of copper sulphate (mM)


US 56 05
y = -80 262x² + 14.808x + 7 0597
R² = 0 9863
Log 10 (CFU+1)
Concentration of copper sulphate (mM)