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Control of bacterial spot in stone fruit orchards

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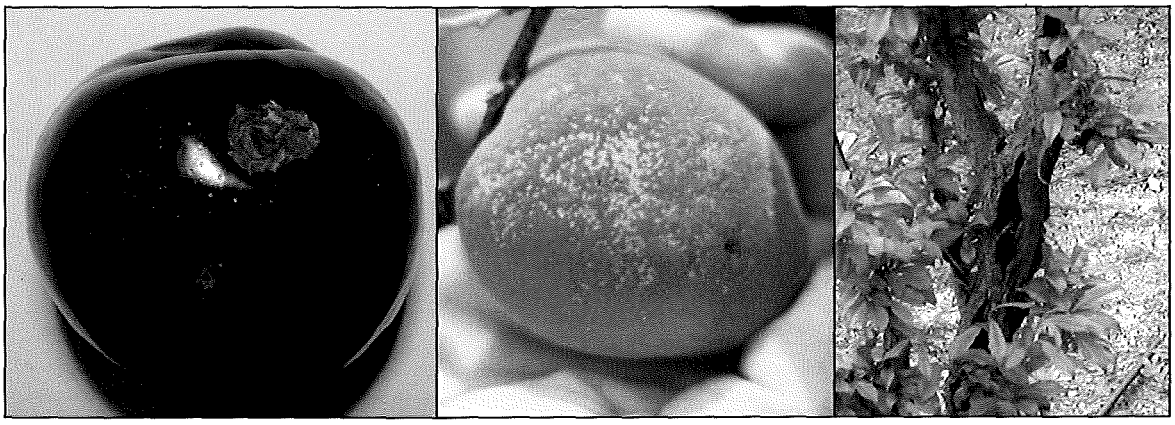

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This document is the final report for the Summerfruit Australia Limited funded project "Control of bacterial spot in stonefruit orchards", and as such contains the details of all scientific work carried out in this project.

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Media Summary

This project has successfully identified new ways of managing one of the most devastating diseases of stonefruit crops, bacterial spot.

Bacterial spot (caused by *Xanthomonas arboricola* pv. *pruni,* or *Xap*) is the most devastating bacterial disease currently affecting Australian stonefruit crops. It has been estimated that bacterial spot affects more than one third of all stonefruit growers in Australia in wet spring/summer seasons, with fruit losses of up to 70% in highly susceptible varieties. This disease significantly reduces the numbers of saleable fruit, and if left untreated can cause long term effects such as reduced tree vigour (leading to poor fruit set and quality), branch loss and tree death in plums. The extent of the problem is so severe that some varieties are unviable for commercial production without a method of bacterial spot control.

As a result of this project, the stonefruit industry is better equipped to fight the disease. Our achievements include:

1. developing a more specific detection method
2. increasing our understanding of the pathogen's lifecycle
3. improving field management methods by developing a targeted copper spray program.

By modifying a technique known as polymerase chain reaction (PCR) to suit the *Xap* bacterium, PhD candidate Emma Ballard has provided an identification test for bacterial spot that can be done quickly, allowing many samples to be processed simultaneously.

The project has also shed new light on the pathogen's behaviour. It has revealed that:

- the most important factors influencing the level of infection and disease spread in Queensland is wet, humid weather and susceptible varieties
- symptoms are randomly distributed over the fruit surface, emphasising the need for good fruit coverage during spraying
- using the targeted copper program significantly reduces bacterial spot canker symptoms on annual shoots
- bacterial spot fruit lesions are no more likely to form on hail marks than on the rest of the fruit.

Possibly of most importance in the short term, particularly to growers and HAL, is the targeted copper spray program developed in the project. This program has been thoroughly tested during four seasons of research station trials, and two seasons of grower sprayed field trials on commercial orchards in Queensland, and has proved successful in managing bacterial spot. During the course of this project we have started to extend the program to growers through on farm trials, information evenings and articles in *Summerfruit Quarterly* and *Tree Fruit* magazines. This program is ready for registration and wide scale trialling throughout Australia.

Technical Summary

Bacterial spot (caused by *Xanthomonas arboricola* pv. *pruni; Xap*) is the most devastating bacterial disease currently affecting Australian stonefruit crops. In the most recent disease survey of Australian stonefruit growers, undertaken by Dr Shane Hetherington during the development of the Australian Summerfruit Integrated Pest and Disease Management manual (Hetherington 2007), bacterial spot was the second most significant disease after brown rot (a fungal disease caused by *Monilinia fructicola*).

It has previously been estimated that bacterial spot affects more than one third of all stonefruit growers in Australia in wet spring/summer seasons, with fruit losses of up to 70% in highly susceptible varieties.

Bacterial spot symptoms include fruit spots, leaf lesions, premature leaf fall, stem lesions and branch cankers. This disease significantly reduces the numbers of saleable fruit, and if left untreated can cause long term effects such as reduced tree vigour, leading to poor fruit set and quality. Longer term effects of bacterial spot include branch loss and tree death. The extent of the problem is so severe that some varieties are commercially unviable without a method of bacterial spot control.

This project has addressed the problem of bacterial spot in three ways:

1. Development of a more specific detection method.
2. Increased understanding of the pathogen's lifecycle.
3. Improved field management methods, with the development of a targeted copper spray program.

An *Xap* specific PCR test developed by PhD candidate Emma Ballard (nee George), provides a rapid, high throughput identification test to aid in stone fruit disease diagnostics and bacterial spot disease epidemiology studies.

Epidemiological studies undertaken in this project have revealed that:

1. The most important factors influencing the level of *Xap* infection and bacterial spot disease development in Queensland orchards, are varietal susceptibility and wet, humid weather.
2. Bacterial spot symptoms are randomly distributed over the fruit surface, emphasizing the need for good coverage of fruit during spray applications.
3. The use of the targeted copper program will result in a significant reduction in bacterial spot canker symptoms on annual shoots.
4. Bacterial spot fruit lesions are no more likely to form on hail marks than on the rest of the fruit.

Of most immediate relevance to HAL industry partners and growers is the targeted copper spray program. This program has been thoroughly tested during four seasons of research station trials, and two seasons of grower sprayed field trials on commercial orchards in Queensland and Victoria. During this project we have started to extend the program to growers through on-farm trials, information evenings and articles in 'Summerfruit Quarterly' and 'Tree Fruit' magazines. The targeted copper spray program is ready for registration and wide scale trialling throughout Australia.

1. Bacterial spot of stone fruit

1.1 Review of relevant international literature

The following review of literature describes the disease, bacterial spot of stone fruit (caused by *Xanthomonas arboricola* pv. *pruni*; *Xap*), as it occurs in parts of the world other than Australia. A description of the disease in Australia follows. It is important to remember that bacterial spot can produce a range of symptoms, not all of which occur in each stone fruit variety or each country where the disease is present. The information presented in this section was obtained from research on European and Japanese plums, peaches, apricots and nectarines. The majority of stone fruit affected by bacterial spot in Australia are Japanese plum varieties, with some infection also occurring on nectarines, peaches and apricots.

1.1.1 Distribution and importance

Bacterial spot occurs in most countries that produce stone fruit. This includes Australia, Brazil, Canada, parts of Europe, Japan, New Zealand, Russia, South Africa and the United States of America (Ritchie 1995).

In Europe and the countries of the Mediterranean basin, *Xap* is locally established in Austria, Bulgaria, France, Italy, Lebanon, Netherlands, Romania and the ex-USSR. It is found, but not established, in Cyprus and Switzerland (Zaccardelli, Ceroni *et al.* 1999). Some countries have a long history of bacterial spot infection, with bacterial spot first discovered in Italy in the 1920's (Battilani, Rossi *et al.* 1999). Other countries, such as France, where bacterial spot was first diagnosed in 1995, have only detected the disease relatively recently (Boudon, Nottéghem *et al.* 2005).

The geographic origin of the pathogen is suspected to be North America, where it was first described (Smith 1903). Random Fragment Length Polymorphism studies demonstrated the origin of the French outbreak in 1995 to be the United States of America (USA). Adaptation of a local bacterial species from a wild host, to a new host, may also have occurred in multiple locations throughout the world (Boudon, Nottéghem *et al.* 2005).

Bacterial spot is more common and most severe in areas where stone fruits are grown in light, sandy soils, and the environment is humid or moist and warm during the growing season (du Plessis 1988).

Xap *transmission*

Xap can persist on stems and buds of peach and plum trees throughout the entire year, and contaminated buds used in plant propagation can disperse the pathogen to areas previously free of the disease (Goodman and Hattingh 1988; Shepard and Zehr 1994; Zaccardelli, Malaguti *et al.* 1998). In the European Union, *Xap* is subject to phytosanitary legislation through the EEC Directive no. 92/103 (N°219 2003), with specific standard protocols for identification recently released (OEPP/EPPO 2006).

1.1.2 Symptoms

The most recent comprehensive description of bacterial spot symptoms world-wide was provided by Ritchie (1995). This is the description in this review (see general description below). A separate description of the disease in Italy is also provided, as the

disease there differs significantly from the general description of Ritchie (1995). A more recent description of symptoms commonly seen in Europe is provided in the EEC standard for diagnosis (OEPP/EPPO 2006).

World-wide general description

Bacterial spot symptoms occur on leaves, twigs and fruit. Leaf symptoms are first visible as angular, water-soaked lesions, ca. 1-3 mm in size, often located along the midrib, leaf tip, or leaf margin. Lesions first appear when young leaves are expanding, or along damaged edges of mature leaves. As the lesions age and enlarge to several millimetres in diameter, lesion centres become purple and necrotic. If they abscise, a shot-hole appearance results. Multiple lesions result in leaf chlorosis and premature abscission. Severe defoliation can occur on highly susceptible cultivars, by mid-season. Consecutive years of disease with severe premature defoliation may weaken trees and reduce fruit quality. Initial leaf and fruit symptoms may be confused with pesticide injury (Ritchie 1995).

Several types of lesions occur on twigs and are designated as 'spring' or 'summer' cankers or as 'black tip'. Spring cankers occur on twigs of the previous summer's growth, developing from leaf scar infection in late autumn (Feliciano and Daines 1970). Visible about the time of leaf emergence, spring cankers appear as slightly raised, blister-like areas that can extend several centimetres along the twig. Summer cankers are formed on new green shoots and are visible first in late spring or early summer. 'Black tip' is visible in late winter before leaf emergence, and is limited to the terminal bud region of the previous year's growth. The terminal bud usually fails to open, and a dark cankered area, completely surrounding the twig, can extend several centimetres downward from the tip. Both spring cankers and 'black tip' result from infections the previous autumn. Dieback as a result of twig cankers is more severe on plum and apricot than on peach (Ritchie 1995).

On highly susceptible cultivars, the entire fruit crop can be lost in years when bacterial spot is severe. Fruit symptoms are first visible about three to five weeks after petal fall as small, water-soaked, brownish lesions. Early symptoms on fruit can be mistaken for insect damage. During periods of high humidity, gum may exude from these lesions. As the season progresses, lesions become cracked and sunken and may even appear "cavernous". The frequency and severity of disease on fruit does not always correlate with that on leaves (Ritchie 1995).

Italy

It is important to note, that bacterial spot symptoms in Italy differ from the traditional description, with symptoms only appearing on the leaves and fruit of peaches and nectarines, while plums also develop cankers on the branches and trunk. Flowers are always symptom-free (Battilani, Rossi *et al.* 1999).

According to the traditional description of the *Xap* disease cycle, sources of primary inoculum on peach are 'black tips', affected leaf scars, and cankers on branches and trunks. In Italy, affected leaves on the soil surface, and secondary hosts, such as plum, probably play as important a role as these primary sources of inoculum (Zaccardelli, Malaguti *et al.* 1998). Summer cankers develop only on plum following penetration through leaf scars during the previous autumn (Gasperini, Bazzi *et al.* 1984).

Xap is thought to be able to overwinter in terminal and/or auxiliary buds (Dhanvantari 1971). In Italy, the pathogen can overwinter in peach buds, but this does not play any role in primary infections, because primary symptoms usually appear in late May-early June, one month after bud break (Battilani, Rossi *et al.* 1999).

Xap can also be epiphytic on the twigs and buds of peach and plum, despite the absence of symptoms (Shepard and Zehr 1994), but in Italy some authors have failed to isolate *Xap* from symptomless leaves and fruit (Zaccardelli, Malaguti *et al.* 1998). Otherwise, xanthomonads generally can multiply and survive for a least several weeks on the surfaces of plant tissues without causing disease and can also survive and multiply on non-host plants under favourable conditions (Timmer, Marois *et al.* 1987).

1.1.3 Causal pathogen

Bacterial spot of stone fruit is caused by a bacterium, a short gram-negative rod 0.2-0.4 x 0.8-1.0 μm in size. *Xap* is non spore forming, motile with a single polar flagellum, and occurs singly, in pairs or short chains (Civerolo and Hattingh 1993). It is a strict aerobe with optimum growing conditions of 24-29°C in culture. Growth on yeast-dextrose-calcium carbonate medium results in mucoid, yellow-pigmented colonies (Ritchie 1995).

Xap has the following physiological traits: fails to produce gas on any medium; does not reduce nitrates; is oxidase negative; has an oxidative metabolism; is aerobic; is capable of hydrolysing gelatine; is alkaline by litmus milk test; produces acid from the breakdown of L-arabinose, galactose, sucrose and dextrin; produces ammonia; is catalase positive; and can metabolise quinate (Dunegan 1932; Lee, Hildebrand *et al.* 1992; Smith 1903). The thermal death point of *Xap* is 51°C after 10 min (Dunegan 1932; Larsh and Anderson 1948; Smith 1903). *Xap* produces xanthan, a biopolymer of high economic importance in the food, chemical, pharmaceutical, cosmetic and oil industries (Moreira, Vendruscolo *et al.* 2001; Sutherland 1993). The doubling time of *Xap* is 1.53 hours. *Xap* grows *in vitro* at temperatures ranging from 7°C to 37°C, with an optimal temperature of 31°C. *Xap* is not cold tolerant and when grown outside its temperature range its growth rate drops rapidly (Young, Luketina *et al.* 1977).

Nomenclature and taxonomy

Bacterial spot was originally described by Erwin F. Smith in 1903 on Japanese plum (*Prunus salicina* Lindl.) from Michigan (Smith 1903). Smith named the bacterial spot pathogen *Pseudomonas pruni*. There have subsequently been several revisions of the pathogen's name, including *Bacterium pruni* (E.F. Smith), *Phytomonas pruni* (E.F. Smith), *Xanthomonas pruni* (E.F. Smith) and *Xanthomonas campestris* pv. *pruni* (Smith) Dye. More recent revisions of *Xanthomonas* (Rademaker, Louws *et al.* 2005; Vauterin, Hoste *et al.* 1995) have proposed/supported the new species name *Xanthomonas arboricola*, of which *Xanthomonas arboricola* pv. *pruni* (*Xap*) is a member; along with, *Xanthomonas arboricola* pv. *celebensis* (banana), *Xanthomonas arboricola* pv. *corylina* (hazelnut), *Xanthomonas arboricola* pv. *juglandis* (walnut), *Xanthomonas arboricola* pv. *poinsettiicola* (poinsettia), *Xanthomonas arboricola* pv. *populi* (poplar) and *Xanthomonas arboricola* pv. *fragariae* (strawberry).

There are many common names for bacterial spot disease, including bacteriosis, bacterial leaf spot, bacterial shot hole, bacterial crack and black spot (Ritchie 1995). In

this report the term bacterial spot will be used to refer to this disease, and the term *Xap* will be used to refer to the causal bacterium.

Strains of Xap

Strains of *Xap* are generally separated from one another on the basis of varying levels of pathogenicity. There have been very few researchers able to demonstrate a difference between strains of *Xap* based on other physical or genetic characters (Scortichini and Rossi 2003; Zaccardelli, Ceroni *et al.* 1999); indicating a high level of homogeneity between *Xap* populations. However, du Plessis did find minor differences in antigenic and phage sensitivity between strains (du Plessis 1988; du Plessis, Loos *et al.* 1981; du Plessis, Matthee *et al.* 1979).

Different levels of virulence have been recorded for different strains of the bacterium when infecting different host varieties; i.e. peach, plum and apricot cultivars (Ritchie 1995). Pathogenicity tests have shown that *Xap* strains are able to cross-infect species other than that from which they were originally isolated (Scortichini, Janse *et al.* 1996). Strains of *Xap* may vary in their degree of virulence, with some strains highly pathogenic while others are only mildly so. There can also be great variability in the susceptibility of the host (Scortichini, Janse *et al.* 1996).

Genetic analysis

Genetic diversity studies have shown *Xap* cultures from around the world to consistently cluster together when compared to other *Xanthomonas arboricola* species. Genetic analysis of *Xap* has been undertaken in several countries: the USA using plasmid analysis (Randhawa and Civerolo 1987) and random amplified polymorphic DNA (RAPD) analysis (Pagani *et al.* 1995; Pagani 2004; Pagani and Ritchie 2002); Italy using amplified fragment length polymorphism (AFLP), repetitive polymerase chain reaction (rep-PCR) and gene cassette array analysis (Barionovi and Scortichini 2006; Scortichini and Rossi 2003; Zaccardelli, Ceroni et al. 1999); France using fluorescent AFLP (Boudon, Nottéghem *et al.* 2005) and Australia using Rep-PCR (Zuli 2003).

Rep-PCR is based on the occurrence of conserved repetitive DNA sequences, comprised of the repetitive extragenic palindromic (Rep) sequences. Primer sets are commonly designed to either the enterobacterial repetitive intergenic consensus (ERIC) and/or the BOX (BOX element of *Streptococcus pneumonias* genome) regions. Primer sets for each region generate 10-30 or more PCR fragments per genome, ranging from less than 200 bp to more than 6 kb (Louws, Rademaker *et al.* 1999). When analysed, these fragments form reproducible patterns that can be used to distinguish DNA sequences (i.e. genomes) from one another.

USA

All strains of *Xap* tested by Randhawa and Civerolo (1987) had plasmids, with 10 size classes identified. The number of plasmids in a strain ranged from one to four. However, there was no relationship between the presence of plasmids, and pathogenicity on detached peach leaves (Randhawa and Civerolo 1987).

Pagani *et al.* (1995) used RAPD analysis to differentiate strains of *Xap* from 51 other bacterial isolates representing strains of different genera, *Xanthomonas* species, and *Xanthomonas campestris* pathovars. Among 61 primers screened, one primer yielded a

distinctive fragment for strains of *Xap*. The DNA amplification pattern generated with this primer revealed two major products of 1.5 kbp and 0.9 kbp among 53 *Xap* strains tested. The 1.5 kbp fragment was present in all *Xanthomonas* and *Pseudomonas* strains examined by not in strains of *Erwinia*. The 0.9 kbp fragment associated with *Xap* strains was used a hybridisation probe to confirm specificity of the fragment. Hybridisation occurred to all *Xap* strains tested but not to any of the other non-*Xap* bacteria. This fragment may provide a sensitive and specific tool for detection and identification of *Xap* strains.

Subsequently a specific PCR primer set and digoxigenin-labeled DNA probe were developed, based on the 0.9 kbp fragment thought to be unique to *Xap* isolates (Pagani and Ritchie 2002; Pagani 2004). This primer set has since been shown to be unable to specifically detect *Xap* isolates from Australia and some *Xanthomonas arboricola* pv. *juglandis* isolates (personal communication, Prof David Ritchie). Pagani (2004) was also responsible for the first report of a putative gene from the genome of *Xap*.

Italy

AFLP analysis performed in Italy was not able to distinguish between clones or strains of *Xap* from different host plants or geographic origin (Zaccardelli, Ceroni *et al.* 1999). This study was able to break up a group of 109 strains of *Xap* into three groups of similar strains, with one outlying strain. However the groups did not correspond to differences in geographic location or host plant (Zaccardelli, Ceroni *et al.* 1999).

The genetic relationship between 26 *Xanthomonas arboricola* pv. *fragariae* strains, 20 strains of *Xanthomonas arboricola* pv. *corylina*, 22 strains of *Xanthomonas arboricola* pv. *juglandis* and 16 strains of *Xap* was assessed by rep-PCR using ERIC and BOX primer sets. This analysis demonstrated significant genetic diversity between the pathovars. There were four clearly different genomic patterns produced; but no significant diversity within pathovars (Scortichini and Rossi 2003).

Variability in the gene cassette array pattern of *Xap* was observed between the *Xap* strains analysed. However, no correlation between the two cassette array patterns shown by the strains of *Xap,* and host plant or strain origin was observed (Barionovi and Scortichini 2006).

Australia

Twenty-one strains of *Xap* isolated from Australia and seven strains isolated from other geographic locations were analysed by Rep-PCR using both BOX and ERIC primer sets. BOX-PCR was unable to distinguish between the *Xap* strains, with some diversity present between *Xanthomonas arboricola* pathovars. However, no *Xap* unique fragments were identified. The ERIC primers on the other hand showed a small amount of variation between *Xap* strains and some differences between *Xanthomonas arboricola* pathovars. A primer set was developed to some of the DNA fragments thought to be specific to *Xap*, but this primer set was later found to be unable to distinguish between *Xap* and *Xanthomonas arboricola* pathovars *corylina* and *juglandis* (Zuli 2003).

France

Xap isolates from France were compared with *Xap* populations from the USA and Italy (Boudon, Nottéghem *et al.* 2005). Four genes and the intergenic transcribed spacer

region were sequenced from a total of 3.9 kb of sequences, and fluorescent AFLP analysis was performed. A collection of 64 *Xap* strains, including 23 strains from France, was analysed. The *Xap* population had a low diversity with no sequence polymorphisms observed. Population diversity revealed by fluorescent AFLP was lower for the West European population than for the American population. The same bacterial genotype was detected from five countries on three continents. This geographic distribution can be explained by human-aided migration of bacteria, and the data supports the hypothesis that the pathogen originated in the United States and has been subsequently disseminated to other stone fruit growing regions of the world (Boudon, Nottéghem *et al.* 2005).

1.1.4 Host plants

The most common commercially cultivated hosts of bacterial spot are peach (*Prunus persica* (L.) Batsch.), nectarine (*Prunus persica* var. *nectarina* [Ait.] Maxim.), Japanese plum (*Prunus salicina* L.), apricot (*Prunus armeniaca* L.) and almond (*Prunus amygdalus* L.). Other hosts include sweet (*Prunus avium* L.) and sour (*Prunus cerasus* L.) cherries, Japanese apricot (*Prunus mume* Sieb. & Succ.), Chinese wild peach (*Prunus dividiana* (Carriere) French.), flowering plum (*Prunus blireiana* L.), European plum (*Prunus domestica* L.), *Prunus buergeriana*, *Prunus crassipes* and *Prunus donarium* (Ritchie 1995; Scortichini, Janse *et al.* 1996). American and European plum are less susceptible than Japanese plum. Cultivars within all of these *Prunus* species vary widely in their susceptibility (Ritchie 1995).

In commercial orchards, the most severe levels of disease can vary between types of stone fruit in each country. For example in Italy, it is mostly peach and Japanese plums that are affected; in France mainly peach and apricot (personal communication, Marco Scortichini); in the south-eastern USA peaches are the most affected (personal communication, David Ritchie); while in Australia, Japanese plums show the most severe symptoms.

1.1.5 Disease cycle and Epidemiology

In autumn, *Xap* invades peach twigs via inoculum from fresh leaf scars. These infections are then expressed as 'spring cankers' or 'black tip' in the following winter to early spring. In plum, the major route of twig infections appears to be by systemic movement of bacteria through leaf petioles from infected leaves. In peach, most primary inoculum is believed to be overwintered bacteria associated with spring cankers and 'black tip'. However, bacteria may overwinter in terminal buds of peach, and have also have been detected in epiphytic association on twigs and buds. If epiphytic survival is proved to be prevalent, cankers may not be essential as overwintering sites. The pathogen has been readily isolated from lateral buds in summer and autumn. On plum and apricot, bacteria can overwinter in summer cankers, and the cankers continue to develop the following spring. The occurrence and development of primary and subsequent secondary infections depends entirely on environmental conditions. Frequent periods of moisture during late bloom to a few weeks after petal fall are very conducive to primary fruit and leaf infections of peach and nectarine. Wind-driven rain may increase disease severity. Similar environmental conditions throughout the growing season allow for the continuation of secondary infections. Few infections occur during hot, dry conditions (Ritchie 1995).

Infection process

The most common entry point for *Xap* into stone fruit leaves is through the stomata (Smith 1903). A continuous film of water from the leaf surface, through the stomata, and into water congested leaf tissues is required for successful infection of peach leaves (Daines 1961). Peach leaves in a water-saturated condition may undergo changes in cell membrane permeability leading to leakage of nutrients into intercellular spaces (Young, Luketina *et al.* 1977). Presumably, *Xap* may use these nutrients as a substrate for growth (Zehr, Shepard *et al.* 1996).

Once *Xap* has made entered the plant through leaf stomata, it passes into plum twigs through the veins of infected leaves (du Plessis 1984), then migrates systemically through twigs to other leaves, producing symptoms on main and secondary leaf veins along the way. Petioles and veins appear to favour the proliferation of *Xap* (du Plessis 1986).

Stomata on leaves are also important outlets for *Xap* cells, which can then act as inoculum for further leaf and fruit infections (Miles, Daines *et al.* 1977; Smith 1903). The leaves act as key sites for proliferation of *Xap* before systemic migration occurs into twigs (du Plessis 1986).

Xap migrates the xylem vessels in the vascular bundles of twigs (du Plessis 1986), and can successfully invade intercellular spaces and parenchymatous cells. However, entrance of *Xap* into xylem vessels appears to be of no pathological importance since the bacteria, once inside, cannot move out of the xylem to infect neighbouring cells (Feliciano and Daines 1970).

Xap also invades peach twigs in the autumn through fresh leaf scars. For successful infection to occur, *Xap* must invade damaged tissues quickly (within 1-3 days), as the suberised protective layers of the tree are formed prior to normal leaf drop, and provide excellent protection against bacterial infection (Feliciano and Daines 1970).

Higher *Xap* populations introduced in plum petioles rather than the main veins of young shoots (4-5 weeks old), favoured effective entry and subsequent summer canker development in shoots. Even low pathogen populations applied to plum petioles, caused reasonably high percentages of cankers at nodes. In contrast, older shoots (7-8 weeks) were immune to summer canker development. Progressive resistance of nodes away from distal ends of shoots demonstrated the restraining effect of older tissue on summer canker development (du Plessis 1987). Resistance of rice and citrus leaf tissue to *Xanthomonas* also increases as leaves mature (Qi and Mew 1985; Stall, Marco *et al.* 1982).

The majority of fruit symptoms are the result of infections that occur during flowering, with most severe fruit infections initiated in the three week period immediately following shuck split (Pagani, Leoni *et al.* 2001).

Systemic migration of *Xap* into fruit through fruit stalks has been observed in South Africa, and produces unusual lesions extending from the exocarp to the endocarp. *Xap* was isolated from the vascular channels of the stalk, seed coat, stony endocarp and mesocarp of infected fruit; but were absent from the starchy endosperm or surface of the diseased exocarp (du Plessis 1990).

Finally, as with all bacterial diseases, open wounds of any kind are prime targets for infection, especially if wounding occurs during wet weather e.g. a rain or hail storm. Fresh leaf scars associated with storm damage to trees in the autumn provide excellent sites for infection that can lead to spring canker development the following season (Feliciano and Daines 1970; Gasperini, Bazzi *et al.* 1984).

Environmental conditions

Water congestion, defined as accumulation of excessive water in intercellular spaces as a result of internal water pressures (Johnson 1947), and leaf wetness, are requisites for the development of bacterial spot (Matthee and Daines 1968). These conditions commonly occur during rains, dews, or periods of high relative humidity coupled with abundant water absorption by trees. A continuous film of water often forms and extends from the leaf surface through stomata into the substomatal chamber of peach foliage, providing highly favourable conditions for *Xap* infection and multiplication (Matthee and Daines 1968; Matthee and Daines 1969).

Temperature also has a significant effect on the initial infection of stone fruit trees by *Xap* and the subsequent development of bacterial spot symptoms. Symptom development is minimal, and appearance delayed (after 12 days), when trees are inoculated at 20°C. Peach trees inoculated and held at ambient temperatures of 22-26°C or at 30°C showed typical symptoms in 5 to 12 days (Zehr, Shepard *et al.* 1996).

Influence of soil type, stomata size and water absorption on susceptibility

There are many references in the literature to the connection between sandy or light soils, increased levels of water congestion and increased incidence of bacterial spot disease (Ritchie 1995; Zehr, Shepard *et al.* 1996), but very few explanations of why this is so.

Stone fruit trees planted in light/sandy/poor water holding soils have a larger number of stomata (per cm^2 of leaf surface area) of a larger size, than trees planted in heavy/clay/water holding soils (Kramer 1945). This increase in stomata size and number provides the trees with the capacity to take up water more quickly, as the rate of absorption of water in moist soil is determined primarily by rate of transpiration (Kramer 1945).

Soil factors which result in the highest susceptibility to bacterial spot symptoms on leaves are abundant aeration, which results in maximum solution absorption. Maximum solution absorption results in maximum stomatal opening, which is followed, under conditions of high humidity, by maximum water congestion. When all of the above conditions combine bacteria are provided with optimal conditions to enter plant tissues through continuous waterways leading from the exterior to the interior of the leaf (Matthee and Daines 1968).

Peach trees grown in well-aerated sand or sandy loam soils exhibited greater susceptibility to bacterial leaf spot caused by *Xap*, than peach trees grown in heavier soils with a lower oxygen-supplying power. Under optimum soil water conditions, trees grown in sands also developed larger stomatal apertures, had more water congestion of the foliage, and had a lower water diffusion pressure deficit than plants growing in heavier, less-aerated loams. The relationship between susceptibility of trees to bacterial

leaf spot and stomatal aperture size, water congestion, and diffusion pressure deficit were correlated with the concentration of dissolved oxygen in the nutrient solutions (Matthee and Daines 1968).

A key feature of stone fruit varieties that are less susceptible to bacterial spot infection is that their leaves have stomata with smaller aperture sizes than more susceptible varieties (Matthee and Daines 1969).

Inoculum sources

The most important sources of *Xap* inoculum vary between different stone fruit types, within and between, different geographical locations.

In peaches, the main source of primary *Xap* inoculum comes from spring cankers occurring in the terminal portions of twigs produced during the previous growing season (Feliciano and Daines 1970). Autumn infection of peach twigs results in the development of cankers the following spring (Goldsworthy and Wilson 1952). Spring canker development is less the further the distance from the terminal shoot (Adam, Powell *et al.* 1955).

The biological cycle of *Xap* on peach in the Po Valley, Italy differs substantially from that of plum. Cankers act as important sources of inoculum for early plum infections, whereas bacterial spot outbreaks are common in peach despite a lack of cankers (Bazzi and Mazzucchi 1980; Zaccardelli, Ceroni *et al.* 1999; Zaccardelli, Malaguti *et al.* 1998).

In South Africa, summer and spring cankers on plum are due to systemic movement of *Xap* from infected leaf and shoot tissues (du Plessis 1987). The importance of leaf scar infection of plum (Gasperini, Bazzi *et al.* 1984) in South Africa remains in doubt; as wind-driven rains that dislodge leaves are rare in South African fruit-growing areas (du Plessis 1987).

As a general rule, spring and summer cankers, leaf scars and stomata are the main sources of Xap inoculum for the majority of stone fruit worldwide.

Interestingly, *Xap* cells were found to be more prevalent on the lower than on the upper surface of peach leaves (Miles, Daines *et al.* 1977). Bacteria, either as individual cells or in a mass, exuded from stomata as much as six days before infections were visible to the unaided eye, suggesting that they may be available for new infections during much of the presymptomatic period (Miles, Daines *et al.* 1977). Similarly, Shepard and Zehr (1994) found the epiphytic persistence of *Xap* on leaves (especially in the summer after rainy periods) and asymptomatic peach and plum flowers in the USA to be significantly longer than expected.

Disease prediction models

Several researchers have correlated weather conditions with the development of bacterial spot symptoms, either by field observation (Battilani, Rossi *et al.* 1999; Pagani, Leoni *et al.* 2001) or in laboratory experiments to identify the conditions required for *Xap* infection (Zehr, Shepard *et al.* 1996). This information has been used to develop infection prediction systems of varying complexity. Battilani, Rossi *et al.* (1999) developed a mathematical formula using a logistic regression model to predict infection of leaves and fruit; while Zehr, Shepard *et al.* (1996) simply determined the

temperature, relative humidity and time needed for successful leaf infections, and Pagani, Leoni *et al.* (2001) determined the stages of fruit development when inoculation of *Xap* produced the most severe symptoms.

Italy (Battilani, Rossi et al. *1999)*

A study conducted in peach orchards south-east of Lake Garda in the Veneto region of northern Italy between 1993 and 1995 demonstrated a strong correlation between infection events and weather conditions. Leaf infections typically occurred after at least three consecutive days of rainy weather with temperatures between 14 and 19°C. Fruit was less severely affected than leaves. Disease progress was closely correlated with the number of rainy days after disease onset.

USA (Zehr, Shepard et al. *1996; Pagani, Leoni* et al. *2001)*

The sporadic occurrence of bacterial spot in South Carolina peach orchards is often attributed to the variable frequency of rainy, warm weather. Zehr, Shepard *et al.* (1996) clearly demonstrated the relationship between rates of disease development, leaf wetting periods and temperature. Bacterial spot symptoms developed on peach leaves after only three days at 30°C. At 24°C symptoms took 10-14 days to develop. Regardless of leaf wetness period, disease severity and incidence were much greater at 30°C than at 24°C. Exposure to 100% relative humidity for 24-48 hours after inoculation resulted in much greater disease severity than was observed with shorter wetness.

Several equations were produced by Zehr, Shepard *et al.* (1996) to describe the percentage of leaf surface necrosis produced by incubation of inoculated leaves at 24°C and 30°C. Leaf lesions developed more slowly at 24°C than 30°C and did not enlarge after appearance. At 30°C, leaf lesions did not enlarge after appearance if no wetting period followed inoculation. If six hours or more of wetting followed inoculation, necrotic areas near the leaf margins spread inward, enlarging the area of necrotic tissue.

Given the warm temperatures that prevail in South Carolina during late spring and summer, some degree of bacterial spot can reasonably be expected when peach leaves are wet for 36 h or more. Even shorter wetting periods are perhaps sufficient for bacterial spot infection, if such periods occur frequently.

As *Xap* may be found on symptomless leaves (Shepard and Zehr 1994) the above criteria should be considered a guide only as the authors did not test isolates of *Xap* from several sources or locations. It should also be noted that the effects of interrupted periods of wetting when the bacteria are present were not studied. The severity of infection will depend on temperature, length of the wetting periods, soil type, and susceptibility of the cultivar. More precise definitions of these criteria are needed to develop an accurate forecasting model for bacterial spot (Zehr, Shepard *et al.* 1996).

In subsequent work, a simple inoculation study showed that the most severe symptoms of bacterial spot infection occurred with inoculation during the period just prior, to three weeks after shuck split (Pagani, Leoni *et al.* 2001). Pagani, Leoni *et al.* (2001) suggest that this period would be the most effective time for bacterial spot disease control measures to be applied.

The influence of tree stress on disease

The induction of plant defence responses or pathogenesis-related proteins occurs within 12 hours of exposure of leaves and stems of peach shoot cultures grown *in vitro,* to *Xap* culture filtrates (Thimmapuram, Ko *et al.* 2001).

Nematode infestation

Research in South Carolina has shown that peach trees planted in to soil infested with the plant parasitic nematode *Criconemella xenoplax* (*Cx*) developed more severe bacterial spot disease symptoms than those planted into uninfested soil. Nematode infestation correlated with a larger percentage of leaf area affected by disease symptoms, and larger and more numerous lesions after inoculation with *Xap.* Premature defoliation following leaf infection occurred earlier and more often in trees grown in *Cx* infested soil (Shepard, Zehr *et al.* 1999).

Mojtahedi, Lownsbery *et al.* (1975) found that water stress was higher in leaves of *Cx* infested trees, than for trees grown in non-infested soil. Some reports (Lownsbery, English *et al.* 1973; Mojtahedi, Lownsbery *et al.* 1975) suggest that *Cx* affects susceptibility to bacterial spot directly through feeding damage to roots, which subsequently results in water stress throughout the tree. However, Shepard, Zehr *et al.* (1999) demonstrated an additional host response to feeding by *Cx* that permits extensive development of bacterial spot symptoms, with no visible evidence of water stress in trees observed in their study.

Shepard, Zehr *et al.* (1999) also point out the potential for peach varieties that were previously considered to be less susceptible to bacterial spot to become more susceptible, or experience more severe symptoms, if they become infested with *Cx.*

Tree nutrition

Adequate fertility should be maintained to avoid excessive foliar growth or the weakening of trees through poor nutrition, as both of these factors enhance bacterial spot development (Ritchie 1995).

High nitrogen and low potassium levels increase the susceptibility of many plants to certain diseases and increased the susceptibility of two peach varieties to both water congestion and bacterial spot (Matthee and Daines 1969). High nitrogen increased disease incidence in peach fruit, while high potassium reduced bacterial spot incidence on peach fruit (Bachelder, Daines *et al.* 1956).

***Epiphytic populations of* Xap**

Epiphytic persistence of *Xap* on peach (*Prunus persica*) and plum (*Prunus domestica*) trees was studied by sampling leaves, twigs, buds, flowers and fruits of a susceptible cultivar of each species in South Carolina, USA (Shepard and Zehr 1994). The bacterium was found on all symptomless organs sampled during a 13 month period in 1984-1985. Bacterial populations observed were variable among replicates and organs on all sampling dates, but populations were consistently found on some organs during winter and summer months alike. No bud colonisation was evident before bud break, even though external colonisation of peach and plum floral and leaf buds was detected in all trees sampled. *Xap* can persist year-round on surfaces of peach and plum trees even in the absence of symptoms of bacterial spot. Bacterial spot may develop rapidly after rainy periods in spring even in orchards that were previously free of disease

symptoms (Shepard and Zehr 1994). Similarly in southern Georgia, USA, *Xap* colonised peach leaf surfaces after artificial inoculation in spring, and persisted until autumn (Gitaitis and Bertrand 1986).

Shepard and Zehr (1994) suspected that epiphytic populations are important in the sudden, widespread appearance of bacterial spot on stone fruits during periods of rainy weather in the south-eastern USA. Large populations of *Xap* were found on peach and plum trees at any time of the year, during dry periods as well as rainy weather. This has important implications for bacterial spot management, in particular the selection of budwood. Symptomless buds used in plant propagation may transmit the disease to young trees. In older trees, bacterial spot outbreaks shortly after flowering result in significant losses, and so the application of bactericidal sprays just prior to flowering is especially helpful in reducing losses.

The removal of diseased twigs during pruning may therefore not be that useful in disease prevention (Shepard and Zehr 1994).

Many bacteria have potential for growth on plant surfaces (Hirano and Upper 1983), and hence long-term persistence on perennial plants. Plant-pathogenic bacteria on plant surfaces can serve as inoculum for disease development when the environment is conducive (Hirano and Upper 1983).

Overwintering sites for Xap

As with the other aspects of this disease, there is great variation between stone fruit types and countries when it comes to overwintering sites of *Xap* inoculum.

In Italy, *Xap* cells have been found in peach bud/leaf scars and infected leaf debris up to seven months after inoculation, and due to the frequent lack of cankers in Italian peach trees are thought to be the primary source of inoculum for outbreaks in some seasons (Zaccardelli, Malaguti *et al.* 1998).

Twig cankers have been cited as the probable overwintering site of the bacterium in the USA (Feliciano and Daines 1970; Foster and Petersen 1954), but their importance as sources of inoculum has not been confirmed in the eastern USA. Moreover, severe outbreaks of bacterial spot may appear in orchards where twig cankers are rarely found (Shepard and Zehr 1994). In South Africa, however, cankers do appear to be important sites for overwintering bacteria in plum (du Plessis 1987).

In Ontario, Canada, terminal and axillary buds have been reported as overwintering sites for *Xap*. Epiphytic populations were also found on leaves in summer, especially after periods of precipitation (Dhanvantari 1973).

1.1.6 Identification Techniques

Rapid and accurate isolation and identification methods for *Xap* infection of stone fruit have been widely researched over a long period of time (104 years). Many different techniques have been used with varying degrees of success. The main difficulties have been due to the close genetic relationships and physical characteristics shared by Xanthomonads, and *Xanthomonas arboricola* pathovars in particular.

Selective media

A variety of selective and differential media have been developed for the isolation and growth of *Xap* from plant material, including XPSM (Civerolo, Sasser *et al.* 1982), medium D5 (Kado and Heskett 1970) and SX (Schaad and White 1974). More recently, several authors have used GYCA media, especially when growing bacteria for use in molecular techniques (Schaad 1988; Scortichini, Janse *et al.* 1996; Scortichini, Rossi *et al.* 2002; Vauterin, Swings *et al.* 1991; Yang, De Vos *et al.* 1993).

Strains of *Xap* only show minor differences in their growth on selective media. A simple selective medium (XPSM) was tested with nine *Xap* strains, 14 strains of eight other *Xanthomonas nomen* species and several bacteria from other genera (Civerolo, Sasser *et al.* 1982). Added *Xap* was recovered from soil containing ca. 102-103 colony forming units/g and from *Prunus* leaf extracts containing <102-103 colony forming units/ml. It was also readily detected in, and isolated from, extracts of lesions in naturally infected apricot leaves. Soil and leaf bacteria were generally suppressed on XPSM and only an occasional fungal colony developed from the soil samples.

This selective medium contains 2 g/L alginic acid, 0.2 g/L 8-azaguanine, 2 mg/L nicotinic acid, 3 mg/L cysteine in basal media comprised of 1.5% Difco Bacto-agar, 0.08% monobasic potassium phosphate (KH_2PO_4), 0.08% dibasic potassium phosphate (K_2HPO_4) and 0.01% $MgSO_4$. After autoclaving, 80 µg/ml of cholothalonil and 16 µg/ml of kasugamycin were added (Civerolo, Sasser *et al.* 1982).

In most cases, a simpler, less selective medium is often useful. When diagnosing the causal agent from unusual symptoms, a medium such as Nutrient Agar (Amyl Media, Dandenong) can be used to detect a range of bacterial pathogens including *Xap*, which forms yellow, mucoid colonies on this medium when grown at 28°C for 24-48 h .

Refractive index

The technique of Gitaitis, Hamm *et al.* (1988) described below has not been used by other *Xap* researchers. This may be because the characteristics that make the technique work are common to other bacteria.

Epiphytic populations of *Xap* were detected on peach leaves by spreading 0.1 ml aliquots from serial dilutions of leaf washes onto the surface of nutrient agar plates. After incubation at 30°C for 48-72 hours, plates were positioned 15 cm above a clear plastic template with horizontal black lines spaced 3 mm apart. The lined template was sub-illuminated by a fluorescent light box. When the template was viewed through the bacterial colonies, various patterns were associated with different colony types. The clarity and refractive quality of colonies of *Xap* created an undistorted image of discrete straight lines. Other yellow colonies made distorted patterns or were opaque (Gitaitis, Hamm *et al.* 1988), but the identity of identity of these colonies was not provided.

Fatty-acids analysis

Comparison of the fatty-acids content of bacteria is a relatively common method used by bacteriologists to determine the identity of unknown specimens. The technique analyses bacterial samples by either high-performance liquid chromatography or gas-liquid chromatography, to develop a lipid profile that is, hopefully, specific to the species or pathovar of bacteria being tested.

Unfortunately, fatty-acids analysis has not proven to be very successful at separating *Xap* isolates from one another, although it is excellent for differentiating *Xap* from other *Xanthomonas arboricola* pathovars (Scortichini, Janse *et al.* 1996; Wells, Civerolo *et al.* 1993; Vauterin, Yang *et al.* 1992).

Fatty-acid analysis of 14 *Xap* isolates from four different countries (over three continents) for four different *Prunus* species, including apricot, European plum, peach and Japanese plum was undertaken by Scortichini, Janse *et al.* (1996). Comparisons of the *Xap* strains with other Xanthomonads, including *Xanthomonas campestris* pv. *campestris, X.c.* pv. *graminis, X.c.* pv. *hyacinthii, X.c.* pv. *pelargonii* and *X.c.* pv. *vasculorum*, showed a remarkable homogeneity in fatty-acids content and whole-cell protein profiles. Principal component and cluster analysis did not reveal any grouping according to original host or geographical origin. However, *Xap* strains could be grouped apart from the other *Xanthomonas* pathovars (Scortichini, Janse *et al.* 1996).

Serological methods

The use of antibodies to detect and identify plant pathogens, is the basis for many commonly used commercially available test kits. These tests rely on the development of antibodies to specific proteins on the surface of bacteria, fungi and viruses. Prior to the development of nucleic acid detection techniques, they were the cheapest and most reliable way to distinguish many plant pathogenic bacteria from other closely related species.

Although very effective at selectively identifying pathogens, these tests rely on the identification of bacterial cell surface proteins that are specific to the species, sub-species or pathovar of interest. In a species such as *Xanthomonas arboricola* which is renowned for the high levels of similarity between pathovars, these methods have become superseded by nucleic acid technologies, which are able to more easily detect minor differences between closely related bacteria.

Strains of *Xap* show only very minor differences in antigenicity, variation in cell surface proteins. Ten isolates from peach, plum and apricot in South Africa, Argentina, New Zealand and the USA were serologically typed by cross-agglutination and Ouchterlong gel diffusion techniques (du Plessis, Matthee *et al.* 1979). Five South African isolates were not host-specific when cross-inoculated on the three hosts, and all isolates showed a close serological relationship, with only minor antigenic differences.

A slide agglutination and a quantitative indirect immunofluorescence staining method (IFAS) were developed in Italy. The IFAS method was difficult to use due to the strong adherence of *Xap* cells to the bud and leaf tissue tested. False negative IFAS reactions, coupled with the variability seen between the numbers of fluorescent cells counted in repetitions, limited the usefulness of the IFAS technique (Zaccardelli, Consiglio *et al.* 1995). This technique has not been cited in other papers.

Bacteriophage sensitivity

Bacteriophage sensitivity was used by authors to specifically identify *Xap* isolates from diseased material (Daines and Feliciano 1971; Feliciano and Daines 1970), but with the advent of quicker techniques has not been used for identification in more recent times.

Strains of *Xap* tested in South Africa showed only minor differences in bacteriophage sensitivity (du Plessis, Loos *et al.* 1981). Three bacteriophages of *Xap* isolated from soil beneath diseased plum trees were used to type six South African and four other isolates which had previously been shown to be closely related serologically (du Plessis, Matthee *et al.* 1979). Three distinct groups were identified. Isolates from South Africa, USA and Argentina were lysed by all three bacteriophages with a high degree of efficiency, whereas the two New Zealand isolates showed no lysis or lysis with low efficiency. The phage typing provided no evidence that peach, apricot and plum trees in South Africa were infected by different strains of *Xap*, but indicated that phage typing may be more useful than serological typing for the recognition of different strains of this pathovar (du Plessis, Loos *et al.* 1981).

DNA detection systems

Two DNA detection and/or identification systems have previously been developed for *Xap*, including PCR primers (Pagani 2004; Pagani and Ritchie 2002; Zuli 2003) and Southern hydridisation dot-blot (Pagani 2004; Pagani and Ritchie 2002).

North Carolina, USA

Two tests for the specific detection of *Xap* were derived from a DNA fragment generated by RAPD analysis (Pagani 2004; Pagani and Ritchie 2002). This unique DNA fragment was conserved among 50 *Xap* strains obtained from various locations and hosts including peaches, plums, nectarines and apricots. The DNA fragment was cloned, and sequenced, and specific PCR primers were designed for the detection of that sequence. Primers Y17CoF and Y17CoR amplified a 943-bp DNA fragment in all strains previously identified as *Xap,* on the basis of biochemical and physiological tests, and failed to amplify DNA from other xanthomonads and non-xanthomonads including saprophytes and epiphytes associated with *Prunus* species. The PCR assay detection limits were as low as 25 and 50 cells of *Xap* per reaction.

A digoxigenin-labeled DNA probe, XPRUNI14, was developed and used to assay a collection of 167 non-*Xap* strains and 138 strains of *Xap* through dot-blot and Southern analysis. Results indicated that *Xap* could be accurately detected and identified by PCR analysis and Southern hybridisations on symptomatic and asymptomatic plant materials avoiding the need for prior isolation of this phytopathogen (Pagani 2004). It was possible to target and amplify a DNA sequence specific to *Xap* even in the presence of plant extracts and without prior isolation of the pathogen from plant tissues. In addition to its sensitivity, this detection system ensures selectivity against non-target microorganisms that are usually associated with *Prunus* plants and plant parts (Pagani 2004).

Xap could be accurately detected and identified by both PCR and probe hybridizations on asymptomatic samples from orchard trees in which bacterial spot had occurred the previous year. Thus, these two assays are considered important tools for detection and identification of *Xap* in overwintering sites on symptomless tissues of peach trees (Pagani and Ritchie 2002). Since then, this test has been found unable to distinguish between Xap and *Xanthomonas arboricola* pv. *juglandis*, causal agent of bacterial spot of walnut (personal communication David Ritchie).

Queensland, Australia

A small study was undertaken at the University of Queensland, and involved the screening of Australian strains of Xap with a range of PCR primers, previously described in the section on genetic analysis. Zuli (2003) selected a section of sequence which appeared to be specific to *Xap*, based on Rep-PCR results (discussed previously) and developed a set of PCR primers to specifically detect this sequence. However, when the primers were tested against a larger range of isolates, the primers were found to also detect the closely related *Xanthomonas arboricola* pv. *corylina,* and one strain of *Xanthomonas arboricola* pv. *juglandis.*

1.1.7 Disease management

Chemical control

Successful chemical control of bacterial spot may depend highly on the timing of applications. Once the disease is observed, it is difficult to control effectively (Ritchie 1995).

Copper

Autumn applications of copper near leaf drop and/or early-season applications of fixed copper can prevent leaf scar infections, and reduce overwintering inoculum on the tree surface, and inhibit bacterial movement from overwintering cankers to newly emerging leaves and fruit. Peaches and some other stone fruits are very sensitive to copper, so the use of copper has previously be limited to dormant and early season sprays. Limited success has been obtained with very low rates of copper used during the growing season (Ritchie 1995).

Copper is currently registered for use against bacterial spot of stone fruit in Queensland, and for the prevention of bacterial spot infection after hail damage in Queensland, New South Wales, South Australia and Tasmania (PER 9503). The use of copper for control of bacterial spot in the other states of Australia is not allowed.

Antibiotics

The use of antibiotics against bacterial spot disease in the USA was very successful in the 1970's, with trunk injection much more effective in providing long term protection than weekly foliar sprays (Keil 1979; Keil and Civerolo 1979; Keil and Weaver 1970). The widespread use of antibiotics can be expensive, and requires careful management to avoid the development of resistance.

Antibiotics are not registered for use in plant-based agriculture in Australia, and are unlikely to be registered in the future.

Sulphur

Sulphur has been effective in reducing bacterial blast (caused by *Pseudomonas syringae* pv. *syringae* or *Pseudomonas syringae.* pv. *persicae*) in New Zealand nectarines (McLaren, Vanneste *et al.* 2005) and apricots (McLaren 2006). The mode of action of sulphur is unclear, but it may have some potential for use as an alternative chemical for bacterial spot disease management in stone fruit.

Fungicides

Interestingly, fungicides have been shown to reduce levels of bacterial spot infection, including Dodine® in combination with Captan®, and zinc-containing chemicals such as

ziram and zinc sulphate (Ritchie 1995). The use of Dodine® and Captan® is no longer recommended in either Australia or the USA, due to the potential for severe russeting if applied while fruit are on the tree (personal communication, David Ritchie).

Cultural methods

Variety selection

Planting of highly susceptible varieties should be avoided in areas where bacterial spot is a problem. However, when environmental conditions are highly conducive to disease development, no cultivar is immune (Boudon, Notteghem *et al.* 2005), and the severity of disease can vary yearly on the same cultivar. Some cultivars have been developed with a degree of tolerance to bacterial spot. If susceptible cultivars are planted they should not be planted in adjacent blocks (Ritchie 1995), but separated by less susceptible *Xap* cultivars.

In South Africa, the virulence of five strains of *Xap* was determined by inoculating the leaves of trees of 10 cultivars of peach, plum and apricot grown in the glasshouse (du Plessis 1988). There was a highly significant interaction between strains and cultivars, suggesting that the level of disease symptoms produced by *Xap* infection is a combination of the virulence of the *Xap* strain and the susceptibility of the host plant.

In Europe, peach and Japanese plum cultivars that were licensed as resistant to *Xap,* became infected by some strains of *Xap* in a pathogenicity study. Symptoms on the resistant varieties were less severe than those produced on *Xap* susceptible cultivars. Strains of *Xap* several locations and original hosts were inoculated onto a range of commercial stone fruit hosts. For this Germplasm, the term tolerant appears more appropriate than 'resistant' (Scortichini, Janse *et al.* 1996).

The capacity of a single strain to cross-infect species other than that from which it was originally isolated is epidemiologically relevant (Scortichini, Janse *et al.* 1996), particularly when blocks of different *Prunus* spp are closely planted in orchards.

These results show that the selection and evaluation of bacterial spot resistance of apricot, peach and plum cultivars might be influenced substantially by the strains of *Xap* used in screening (du Plessis 1988).

Quarantine/prevention

Efforts should be made to avoid the introduction of bacterial spot into new stone-fruit growing areas where environmental conditions are favourable for disease development. The pathogen can survive in lateral buds during the summer and early autumn, so the use of budwood from orchards with diseased trees should be avoided (Ritchie 1995).

Inoculum reduction

The existence of large numbers of epiphytic bacteria on peach branches, leaves, fruit and flowers is thought to significantly reduce the importance of removing infected tissues from orchards for disease control (Shepard and Zehr 1994). As the source of primary inoculum can vary greatly between countries and stone fruit types, it would seem prudent to determine the primary source of inoculum in seasonal outbreaks, before making such a decision.

Biological control

Biological control of bacterial spot of stone fruit has mainly focused on the use of bacteriophages, viruses that infect bacteria. Extensive research was undertaken into the use of bacteriophages to manage bacterial spot in the USA in the 1970's (Civerolo and Keil, 1969; Civerolo 1970, 1973, 1974, 1976; Randhawa and Civerolo 1986), and in Italy in the early 1990's (Zaccardelli, Saccardi *et al.* 1992; Saccardi, Gambin *et al.* 1993), with promising results.

The use of bacteriophages for effective control of bacterial diseases can be complex, and has many constraints that the use of chemical sprays does not. For instance, the need for the application of phage suspension to occur at a specific time in the infection cycle and environmental conditions conducive to bacteriophage survival, and the need for careful monitoring to ensure bacteriophage resistance does not develop in *Xap* populations.

In low disease pressure situations and in combination with other integrated management strategies, there may be a role for the use of bacteriophages in bacterial spot management (Saccardi, Gambin *et al.* 1993).

1.2 Bacterial spot of stonefruit in Australia

This section only contains information generated in Australia, and primarily reviews the experimental and field experience of the authors, their predecessors (Heaton, Dullahide *et al.* 1993) in plant pathology research at DPI&F, Queensland and the recently published IPDM for Australian Summerfruit (Hetherington 2006).

1.2.1 Distribution and importance

Bacterial spot is a major disease of susceptible stone fruit in Queensland, particularly in wet seasons. Japanese plums are most severely affected, with peaches, apricots and nectarines affected to a lesser extent.

The Japanese plum cultivars Doris, Santa Rosa, Red Ace, Mariposa, Friar and Laroda as well as those imported from the Californian breeding program are very susceptible. Some Japanese plums e.g. Wilson, Narrabeen and Burbank, appear resist to the disease, and have been used in Queensland breeding programs to produce bacterial spot-resistant, high-quality plum cultivars.

European plums, a different species from Japanese plums, are unaffected by the disease.

Bacterial spot has been a serious regional issue during the last 10 years in the following districts:

- New South Wales: Alstonville, Granite Belt, Southwest Slopes, Sydney Basin
- Queensland: Granite Belt
- South Australia: Adelaide Hills, Riverlands
- Tasmania
- Victoria: Goulbourn Valley, Swan Hill
- Western Australia: Perth Hills, Manjimup, Donnybrook

1.2.2 Disease description

Symptoms

In Australia, bacterial spot symptoms are observed on buds, leaves, stems and fruit of apricots, nectarines, peaches and plums.

Buds

Expanded buds become blighted and may fail to unfurl.

Leaves

Leaf spots appear in spring as greasy or water-soaked angular areas, partly confined by leaf veins (Figure 1.1). Spots dry to a light tan, then darken with age and become dark brown to black. As the leaves expand, diseased tissue separates from the surrounding healthy tissue and may drop out to give a shot-hole symptom. This is easily confused with fungal shot-hole caused by the fungal pathogen *Wilsonomyces carpophilus,* or symptoms of copper phytotoxicity (Figure 1.1). The bacterial disease can usually be recognised by the oily sheen and sharp angles of the young lesions.

The spots often join, and where infection is heavy, affected areas become pale yellow-green or reddish. Extensive spotting results in ripping and tattering of the leaves. Premature defoliation may occur.

1 2 3 4

Figure 1.1. From left to right: Images 1-3 are bacterial spot lesions on leaves. Note the greasy or watersoaked lesion edges in the first two images. Image 4 is shot hole caused by copper phytotoxicity.

Twigs/stems

Twigs develop greasy, dark green, elongated areas which develop into tan, sunken areas called cankers, during spring and summer.

Small greasy lesions appear on the rapidly growing young branches in early spring. They become elongated, depressed and tan. Cracks may form in the lesions and develop into open cankers from which gum exudes (spring canker from Figure 1.2). Cankers may also develop during the summer after leaf symptoms are well developed (summer canker from Figure 1.2). Stem cankers are rarely larger than 1-2 cm, but if numerous they may cause shoot distortion or dieback.

If allowed to progress unchecked, spring or summer cankers when formed on young trees can develop into deep, scaffold branch cankers (Figure 1.2).

Fruit

Typical fruit lesions are shown in Figure 1.3. On peach, nectarine and apricot fruit, tan, pinpoint spots occur which crack to form pits on the surface. Cracks and pits may be quite extensive and are often associated with a gummy material. On ripe fruit, a green halo may surround the pits.

Plums develop fewer, but larger, circular greasy spots which darken and crack in the centre as the fruit grows.

Lesions appear in late spring as circular greasy spots that become sunken and darken as the fruit enlarges. The centre of each spot frequently cracks and may ooze gum. Roughened cork tissue develops on the edges of lesions as the fruit continues to expand.

1 2 3

Figure 1.2. Spring (Image 1) and summer (Image 2) cankers on young shoots, and deep scaffold branch cankers (Image 3).

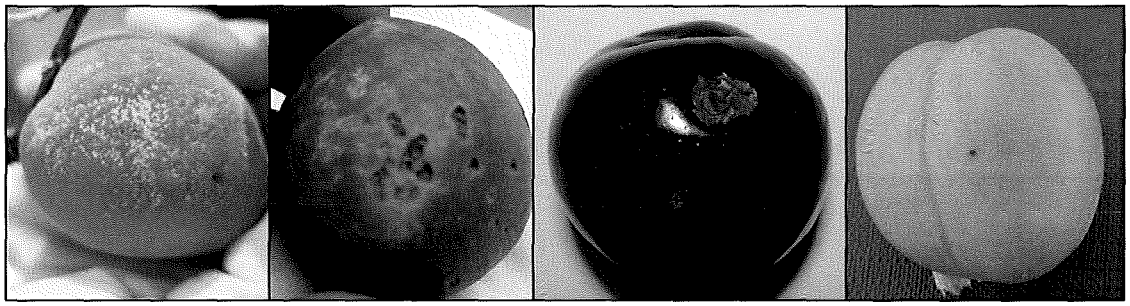


Figure 1.3. (Left to Right) Bacterial spot lesions on mature fruit of nectarine, peach and plum, and an immature plum with the beginning of a bacterial spot lesion (far right). Note the greasy/watersoaked appearance of the young lesion.

Source of infection and spread

Bacterial spot is favoured by high summer rainfall, high humidity and moderate temperatures. The bacteria are spread by rain and wind from diseased trees to adjacent trees through the growing season, especially during hailstorms which damage plants and provide entry points for bacteria.

At leaf fall in autumn, bacteria enter leaf scars and remain dormant until late winter, when buds swell. Bacteria then spread from the scars and the branch cankers formed in the previous season.

During spring rain, bacteria are washed from cracks in branch cankers to the young twigs and foliage, allowing new branch cankers and leaf spots to develop. Bacteria invade and kill young swollen buds, and may blight young branches. Further spread occurs in spring and early summer, affecting leaves, twigs, branches and fruit.

1.2.3 Management

Copper is registered for use, in a standard seasonal pattern, against bacterial spot on stone fruit trees only in Queensland. This use pattern involves the application of copper at early leaf fall in autumn, and again in spring at bud movement and 7-10 days later.

Copper is registered for use against bacterial spot of stone fruit after hail damage in all states of Australia (PER9503).

Other chemical management methods also used in Australia including the use of hydrogen peroxide sprays, very low concentrations of bleach/chlorine and some other 'alternative' products. Growers should keep in mind that these chemicals are not likely to provide much assistance in areas with high levels of disease, and are not recommended for use in high spring/summer rainfall areas.

Prevention

All bacterial diseases are difficult to control once established, and it is therefore important that growers are vigilant in preventing the disease from entering their orchard.

Growers should also consider removing nearby wild or neglected *Prunus* trees, as they can act as reservoirs of the disease. Avoid planting new blocks nears blocks that have the disease.

Choosing species and varieties

Bacterial spot affects all commercial stone fruit grown in Australia. The most serious symptoms occur on plums. Although no varieties of plum are resistant, some are more susceptible than others (Table 1.1). Buy and plant only vigorous, disease-free trees from a reputable nursery.

Orchard design

Orchards in exposed locations are more vulnerable to attack by the disease than those in sheltered situations. Avoid low-lying sites with poor air movement and soil drainage.

Overhead irrigation is a serious obstacle to disease control. Avoid irrigation systems that wet the leaves. Planting windbreaks will reduce the chance of disease spread by windblown rain. However, dense wind breaks can block the spring and summer breezes that dry the trees. Therefore, make sure that the undergrowth at the base of the windbreaks allows some gentle wind movement.

Pruning and shaping trees

Pruning to allow thorough spray penetration and more rapid drying of the canopy helps to reduce the severity of the disease, and increases tree vigour. Do not prune or train trees during wet weather. Pruning of older, inactive cankers, especially those on scaffold branches, is of questionable value. However, pruning of infected fruit, leaves, twigs and mummies from the orchard is an important method of inoculum reduction and should be performed as a priority during thinning, harvest, at the end of the growing season and during annual pruning of trees. Ensure that all of these infected materials are either removed from the orchard or completely broken down before the start of the new season.

Maintaining soil fertility

Trees under nutrient stress or stress caused by the presence of other diseases or insect pests are more susceptible to bacterial spot infection. Therefore it is advisable to maintain high levels of soil fertility and use a regular, high quality pest management program.

Table 1.1. Susceptibility of plum varieties to bacterial spot

Name	Susceptibility*
Autumn Giant	1
Blackamber	1
Durado	1
Friar	1
Roysum	1
Stirling	1
Tegan Blue	1
Doris	2
Queen Rosa	2
Queensland Red Ace	2
Red Beaut	2
Casselman	3
Red Gold	3
Ruby Blood	3
Santa Rosa	3
Satsuma	3
Bellerosa	4
Black Santa Rosa	4
Kelsey	4
Simka	4
Byron Gold	5
Donsworth	5
Earlisweet	5
Explorer	5
Mariposa	5
Narrabeen	5
Radiance	5
Wilson	5

Rated from 1 (very susceptible) to 5 (less susceptible). (Russell, Topp *et al.* 2004)

2. Development of a Molecular Diagnostic Test for the detection of Xap and its comparison with other specific diagnostic tests

Emma Ballard (nee George), (PhD Candidate, University of Queensland).

Introduction

Correct identification of a pathogen is essential to the study of the disease it causes. Although there are many methods available to detect a pathogen PCR is at the forefront of technology and has many advantages over traditional methodologies. PCR basically involves *in vitro* amplification of DNA via an enzymatic reaction, resulting in many copies of the target DNA. The technique is specific to the target DNA, reproducible, simple and rapid.

Development of a set of *Xap* specific primers has proven difficult due to the lack of genetic diversity amongst the *Xanthomonas arboricola* pathovars. Although primer sets have been attempted, thus far they have failed to be specific to *Xap*. Cross-specificity has occurred with closely related *Xanthomonas arboricola* pv. *corylina* and *Xanthomonas arboricola* pv. *juglandis* isolates (Zuli 2003; personal communication David Ritchie). To obtain *Xap* specific fragments, suppression subtractive hybridisation was attempted using *Xanthomonas arboricola* pv. *corylina* as the driver and *Xap* as the tester strain. This technique involved the DNA hybridisation of tester and driver strains, resulting in only tester specific DNA being amplified. The tester specific DNA was cloned, checked by southern blotting to be tester specific, and primer sets were designed. One set of primers proved to be specific to *Xap*.

Aim

The aim of this section was to development a molecular diagnostic test specific to the detection of *Xap*. The section is divided into two parts:

- the development of the test;
- a comparison of the test with the other molecular diagnostic tests available for *Xap* detection.

2.1 Development of a molecular diagnostic test specific to the detection of Xanthomonas arboricola pv. pruni.

Materials and methods

2.1.1 Subtractive Hybridisation

2.1.1.1 Isolation of Bacterial Genomic DNA

The tester strain was Xp22, a typed strain (ICMP 51) isolated from Japanese plum fruit in New Zealand. The driver strain was Xc3, a typed strain (ICMP 5726 aQ) isolated from Filbert, a type of hazelnut in the USA. Both were stored on cyropreservation beads at -80°C. Two beads were placed on to GYCA agar, streaked and incubated for 2 days at 28°C. A loopful of culture was extracted using the ChargeSwitch® gDNA Mini Bacteria Kit (Invitrogen, Mount Waverley, VIC, Australia) as per kit instructions. DNA was quantified using the BioSpec mini (Shimadzu Biotech, Mount Waverley, VIC, Australia).

2.1.1.2 *Suppression Subtractive Hybridisation Procedure*

The BD PCR-Select™ Bacterial Genome Subtraction Kit (Clontech, Mountain View, CA) was used to obtain tester specific DNA according to the manufacturer's protocol. Analysis of the ligation efficiency of the tester strain required two PCR reactions including the standard PCR reaction (described in the manual) to observe successful ligation of both adaptors. The primer set by Zuli (2003) was used to check the quality of the ligated DNA. The Zuli (2003) primers (Forward primer XP92F = 5'-ATG CGC GGC GTT GAT G-3', Reverse primer XP92R = 5'-CTG TTG CGG ACG AGG TCA CGA-3') (GeneWorks, Adelaide, Australia) were used at a final concentration of 0.24 pmol/µl with an annealing temperature of 59°C, all other factors were as per the manual. All amplifications were performed in a PTC-100 Programmable Thermal Controller (MJ Research, Regents Park, NSW, Australia). The secondary PCR products from the tester specific subtracted DNA were purified using the QIAquick® PCR Purification Kit (Qiagen, Doncaster, VIC, Australia) for subsequent cloning.

2.1.2 *Cloning*

2.1.2.1 *Ligation of tester specific DNA into vector*

Three microlitres of the purified secondary PCR products from the tester (Xp22) was ligated into pGEM®–T EasyVector, using the pGEM®–T EasyVector System 1 (Promega, Madison, WI) as per manufacture's instructions.

2.1.2.2 *Transformation of ligated tester specific DNA into competent cells*

The ligated tester specific PCR products were transformed into XL10-Gold Ultracompetent Cells (Stratagene, La Jolla, CA) following manufacture's instructions. The reactions were downsized to use a 25 µl aliquot of cells.

2.1.2.3 *Insert Checks*

One hundred and eighty-seven white colonies were selected to check for the presence of an insert. Each 50 µl PCR reaction contained a final concentration of 0.025 U/µl of AmpliTaq Gold® (Applied Biosystems, Foster City, CA), 1 µM each of the M13 forward (5'-GTA AAA CGA CGG CCA G-3') and reverse primers (5'-CAG GAA ACA GCT ATG AC-3') (GeneWorks, Adelaide, Australia), 1.5 mM magnesium chloride, 0.2 mM dNTP's (Invitrogen, Mount Waverley, VIC, Australia), 1 × GeneAmp® 10X GeneAmp® PCR Buffer II (Applied Biosystems, Foster City, CA). Template was added by touching the tip of a sterile pipette to the colony and adding it directly to the PCR mix. Amplifications were performed in a PTC-100 Programmable Thermal Controller (MJ Research, Regents Park, NSW, Australia) with the following cycling conditions: Initial denaturation at 95°C for 5 min, thirty-five cycles of denaturation at 95°C for 30 s, annealing at 48°C for 1 min, extension 72°C for 1 min followed by a final extension at 72°C for 10 min and cooling to room temperature, 28°C for 1 min. PCR products (5 µl) were run on a 2% agarose gel in 1 x TAE at 5V/cm for 30 min and visualised with staining in 0.2 µg/ml ethidium bromide (EtBr) under UV light (Hoeffer UV Transilluminator, Hoefer, San Francisco, CA).

2.1.3 *Differential screening to confirm tester specific sequences*

2.1.3.1 *Rsa I digestion and labelling of tester and driver genomic DNA*

Six micrograms of the tester and driver genomic DNA was digested with *Rsa* I (supplied in the BD PCR-Select™ Bacterial Genome Subtraction Kit) and purified according to the manufacture's instruction. Successful *Rsa* I digestion was determined by running the digested samples on a 2% agarose gel containing 1 x TAE, running at 5V/cm for 30 min and visualised with staining in 0.2 μg/ml EtBr under UV light (Hoeffer UV Transilluminator, Hoefer, San Francisco, CA).

Three micrograms of digested DNA was Digoxigenin-11-dUTP labelled using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics, Castle Hill, NSW, Australia), following kit instructions. Labelling reactions were incubated at 37°C for 17 h. The efficiency of the labelled products was determined following the manufactures protocol on a positively charged nylon membranes (Roche Diagnostics, Castle Hill, NSW, Australia).

2.1.3.2 PCR amplification of selected clones, denaturation and membrane transfer

For tester screening a total of 41 clones was selected (based on estimated insert sizes ranging from 800 to 1300 bp) and amplified as described in Section 2.1.2.3. A 16s PCR reaction was applied to *Agrobacterium tumefaciens* (ACM 297), Xp22 and Xc3 to for the purpose of controls. The 50 μl reactions contained a final concentration of: 0.025 U/μl of AmpliTaq Gold® (Applied Biosystems, Foster City, CA), 1 pmol/μl each of the 16s forward (27F = 5'-AGA GTT TGA TCM TGG CTC AG -3') and reverse primer (1492R = 5'-TAC GGY TAC CTT GTT ACG ACT T-3') (GeneWorks, Adelaide, Australia), 1.5 mM magnesium chloride, 0.2 mM dNTP's (Invitrogen, Mount Waverley, VIC, Australia), 1x GeneAmp® 10X PCR Buffer II (Applied Biosystems, Foster City, CA). *Agrobacterium tumefaciens* template was added by touching the tip of a sterile pipette to a colony and added directly to the PCR mix. For Xp22 and Xc3 a final concentration of 1 ng/μl of DNA was added. Amplifications were performed in a PTC-100 Programmable Thermal Controller (MJ Research, Regents Park, NSW, Australia) with the following cycling conditions: Initial denaturation at 95°C for 5 min, thirty five cycles of denaturation at 95°C for 30 s, annealing at 48°C for 1 min, extension at 72°C for 2 min followed by a final extension at 72°C for 10 min and cooling to room temperature, 28°C for 1 min. PCR products (5 μl) were run on a 2% agarose gel in 1 × TAE at 5V/cm for 30 min and visualised with staining in 0.2 μg/ml EtBr under UV light (Hoeffer UV Transilluminator, Hoefer, San Francisco, CA).

PCR amplicons were roughly quantified using Hyperladder I (Bioline, London, UK) via visual estimation through gel electrophoresis. Approximately 150 ng of each PCR product and 150 ng of lambda DNA, as a negative control, were subsequently used for the southern blot. The DNA was added to 60 μl of denaturation solution (400 mM NaOH, 10 mM EDTA), incubated for 15 min and transferred to a positively charged nylon membrane (Roche Diagnostics, Castle Hill, NSW, Australia) under vacuum using the Hoefer PR 648 slot blot manifold (Hoefer, San Francisco, CA). Two negative controls containing only denaturation solution were also applied to the membrane. The samples were fixed by UV cross-linking onto the membrane for 3 min on a Hoeffer UV Transilluminato (Hoefer, San Francisco, CA). The process was repeated for testing of the driver DIG-labelled DNA probes.

2.1.3.3 Hybridisation and stringency washes

All hybridisation steps were performed in a Hybaid Hybridisation oven (Hybaid, UK). Membranes were prehybridised in 'DIG Easy Hyb' (Roche Diagnostics, Castle Hill, NSW, Australia) hybridisation buffer, at 65°C for 30 min. Membranes were hybridised overnight at 65°C with 3.5 ml of DIG Easy Hyb buffer containing 25 ng/ml of either tester or driver DIG labelled *Rsa* I digested DNA probe.

Following overnight hybridisation, the membrane was washed using high stringency conditions to remove any unbound probe. The membrane was washed twice in 2 × SSC, 0.1% SDS for 5 min at room temperature and then twice in pre-warmed 0.1 × SSC, 0.1% SDS at 68°C for 15 min.

2.1.3.4 Detection

Immunological detection of hybridised probes resulted from anti-digoxigenin-AP Fab fragments which were visualised with the colorimetric substrates NBT/BCIP as per the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics, Castle Hill, NSW, Australia), instructions.

2.1.3.5 Sequencing of selected clones

The colonies chosen for sequencing were selected according to the southern blot results. Twenty-six colonies showing a differential result and five showing a negative result were selected for sequencing. Template for the sequencing reactions, prepared in Section 2.1.2.3, was purified using the QIAquick® PCR Purification Kit (Qiagen, Doncaster, VIC, Australia) as per manufacture's instructions. PCR amplicons were roughly quantified using Hyperladder I (Bioline, London, UK) via visual estimation through gel electrophoresis. Ten microliter reactions containing a final concentration of 12.5 ng of purified template and 6.4 pmol/µl of M13 forward primer (5'-GTA AAA CGA CGG CCA G-3') were submitted to the Australian Genome Research Facility for DNA labelling, cleanup and subsequent sequencing on the Applied Biosystems automatic DNA sequencer 3730xl (Applied Biosystems, Foster City, CA). The sequences were viewed using the ContigExpress component of the Vector NTI Advance 10.1.1 (Invitrogen, Mount Waverley, VIC, Australia).

2.1.3.6 Bioinformatic analysis of Xap *specific DNA fragments*

The ContigExpress component of the Vector NTI Advance 10.1.1 (Invitrogen, Mount Waverley, VIC, Australia) was used to search for and remove the pGEM®-T Easy Vector (Promega, Madison, WI) from the DNA fragments. Putative tester-specific sequences were then saved in FASTA format and submitted to NCBI BLAST (National Centre for Biotechnology Information, Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST) for BLASTn and BLASTx searches to identify closely related nucleotide and protein sequences respectively.

2.1.3.7 Primer Development

Four primer sets were designed (Table 2.1) to have an annealing temperature of between 55-57°C, with product sizes between 300-400 bp.

Table 2.1: Summary of primer sets.

Primer Name	Sequence	Annealing Temperature (°C)	Expected Product Size (bp)
150 F 150 R	5'-CCG AAG ATT TCC GCA ATT AC-3' 5'-GCT GGT GGC AAC ATC AGA CGC-3'	57	387
29 F 29 R	5'-GTA CCG CAT TTC AGG CCG TCA-3' 5'-AAG TAG CCA ACG CGG AAT TT-3'	56	322
106 F 106 R	5'-GGA CAA TGC TAT TCG CTA CGG-3' 5'-TGC GGC ACG GTC AGA T-3'	56	461
99 F 99 R	5'-AAT CGC TTC TTC GCA TCA ATG-3' 5'-CCC GTT ATG CAG CTA TGG AA-3'	55	322

2.1.4 Primer Specificity

2.1.4.1 Gel Based Assay

Initially to test primer specificity, a panel containing only *Xap* isolates was used for testing all of the primer sets. If the primer sets could detect all *Xap* isolates they were tested on closely related *X. arboricola* pathovars. To test for primer specificity 1 µl of a dense cell suspension containing approximately 10^8 cfu/ml was PCR tested. Each 25 µl PCR reaction contained a final concentration of 0.025 U/µl of AmpliTaq Gold® (Applied Biosystems, Foster City, CA), 1 µM each of the forward and reverse primer, 1.5 mM magnesium chloride, 0.2 mM dNTP's (Invitrogen, Mount Waverley, VIC, Australia), 1× GeneAmp® 10X GeneAmp® PCR Buffer II (Applied Biosystems, Foster City, CA). Amplifications were performed in the PalmCycler (Corbett Research, Mortlake, NSW, Australia) with the following cycling conditions: initial denaturation at 95°C for 10 min, thirty cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension 72°C for 30 s followed by a final extension at 72°C for 10 min and cooling to room temperature, 28°C for 1 min. PCR products (5 µl) were run on a 2% agarose gel in 1× TAE at 5V/cm for 30 min and visualised with staining in 0.2 µg/ml ethidium bromide under ultraviolet light (Hoeffer UV Transilluminator, Hoefer, San Francisco, CA).

2.1.4.2 Real Time Assay

Primer set 29 was tested on the panel of isolates described in Table A.1, Appendix A. A one in ten dilution was made containing approximately 10^7 cfu/ml of each isolate. 1 µl of each cell suspension was added to each PCR reaction. Each 10 µl PCR reaction contained a final concentration of 1× SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK), 0.2 µM each of 29F and 29R (GeneWorks, Adelaide, Australia), 1 mg/ml of Bovine Serum Albumen (Sigma-Aldrich, Castle Hill, NSW, Australia). Amplifications were performed on the ABI 7900 with the following cycling conditions: initial denaturation at 95°C for 10 min, forty-five cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension 72°C for 30 s followed by cycling to determine dissociation curve for products of 95°C for 2 min, 60°C for 15 s, and 95°C for 15 s.

2.1.5 Primer Sensitivity

2.1.5.1 Cell suspensions

A dense cell suspension of Xp22 was made from two day old cultures in sterile deionised water and 12, ten times serial dilutions were made. The exact number of cells was determined by plating 100 µl of each the dilutions onto GYCA media in triplicate and incubating the plates at 28°C for four days.

2.1.5.2 DNA

One loopful of two-day-old culture from Xp22 was placed directly into extraction buffer and extracted as per the method described by Llop (Llop, Caruso *et al.* 1999). Ten times serial dilutions were made and the resultant DNA was checked visually for quality and quality through gel electrophoresis. The DNA was roughly quantified using Hyperladder I (Bioline, London, UK).

2.1.5.3 Gel based assay

One microlitre of each dilution was PCR tested. Each 25 µl PCR reaction contained a final concentration of 0.025 U/µl of AmpliTaq Gold® (Applied Biosystems, Foster City, CA), 1 µM each of 29F and 29R, 1.5 mM magnesium chloride, 0.2 mM dNTP's (Invitrogen, Mount Waverley, VIC, Australia), 1× GeneAmp® 10X GeneAmp® PCR Buffer II (Applied Biosystems, Foster City, CA). Amplifications were performed on the PalmCycler (Corbett Research, Mortlake, NSW, Australia) with the following cycling conditions: Initial denaturation at 95°C for 10 min, thirty cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension 72°C for 30 s followed by a final extension at 72°C for 10 min and cooling to room temperature, 28°C for 1 min. PCR products (5 µl) were run on a 2% agarose gel in 1× TAE at 5V/cm for 30 min and visualised with staining in 0.2 µg/ml ethidium bromide under UV light (Hoeffer UV Transilluminator, Hoefer, San Francisco, CA).

2.1.5.4 Real time assay

One microliter of each dilution was tested. Each 10 µl PCR reaction contained a final concentration of 1X SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK), 0.2 µM each of 29F and 29R (GeneWorks, Adelaide, Australia) and 1 mg/ml of bovine serum albumin (Sigma-Aldrich, Castle Hill, NSW, Australia). Amplifications were performed on the ABI 7900 (Applied Biosystems, Foster City, CA) with the following cycling conditions: initial denaturation at 95°C for 10 min, forty-five cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension 72°C for 30 s followed by cycling to determine dissociation curve for products of 95°C for 2 min, 60°C for 15 s, and 95°C for 15 s.

Results

2.1.6 Analysis of RsaI digested tester, driver and control E.coli genomic DNAs

Genomic DNA was successfully extracted from the tester *Xap* strain Xp22 and the driver *Xanthomonas arboricola* pv. *corylina* strain Xc3. Each undigested genomic DNA was visible as an expected high molecular weight fragment, with a molecular weight greater than 12 kb, observed for the tester Xp22 and the driver Xc3.

The digested DNA was visible as a smear from 0.2kb to 5kb for both the tester Xp22 and driver Xc3. The digested *E. coli* control DNA was visible as a smear from 0.2 kb to

greater than 12 kb with no distinct fragments present in the smear. Adaptors 1 and 2R were ligated to the *Rsa*I digested tester and *E.coli* control.

2.1.7 Analysis of PCR products after secondary PCR

In the secondary PCR, nested PCR was used to further reduce background and enrich for tester-specific sequences. The secondary PCR required 14 cycles for banding to become visible in both the tester (Xp22) and *E.coli* control. Both the unsubtracted tester (Xp22) and the unsubtracted *E. coli* control DNA appeared as a smear from 0.2-2 kb. The subtracted Xp22, the subtracted *E. coli* control DNA and the PCR control subtracted DNA provided in the kit showed distinct bands against a slight background smear. The subtracted *E. coli* control DNA and the PCR control subtracted DNA clearly demonstrated similar banding patterns as expected.

2.1.8 Cloning of subtracted Xp22 cDNA clone library

A 1 in 100 dilution of the transformed tester specific cells onto Luria broth agar containing ampicillin, IPTG and X-Gal resulted in 212 white and 104 blue colonies being present. The plates growing undiluted transformed cells contained too many colonies to be counted.

2.1.9 PCR screening for presence of inserts

One hundred and eighty-seven clones from the tester (Xp22) subtracted DNA library were selected for PCR screening. Of these, 167 clones contained a potential tester-specific insert and three contained more than one fragment. The size of each insert ranged from 400 to 1400 bp.

2.1.10 Southern Blot Analysis

2.1.10.1 Sequence analysis of subtracted Xp22 cDNA clone library

Twenty-six colonies showing a differential result and five showing a negative result were selected for sequencing. The M13 primer sequences were identified in the raw sequence data from selected clones, to identify only the putative tester-specific sequences for BLAST analysis. Trimmed sequences were submitted as a query to conduct searches using the NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Best alignment matches from the NCBI database BLASTn analysis and NCBI Blastx analysis are summarised in Tables A.2 and A.3 in Appendix A, respectively.

2.1.11 Specificity testing of primer set 29

The gel based test (Figure 2.1) detects all of the Australian and international *Xap* isolates (Lanes 1-48, full details in Table A.1, Appendix A), but does not detect any of the closely related *Xanthomonas arboricola* pathovars (lanes 49-62). The water sample did not show the presence of a product, indicating cross contamination of template did not occur.

2.1.11.2 Real time PCR test

Amplification

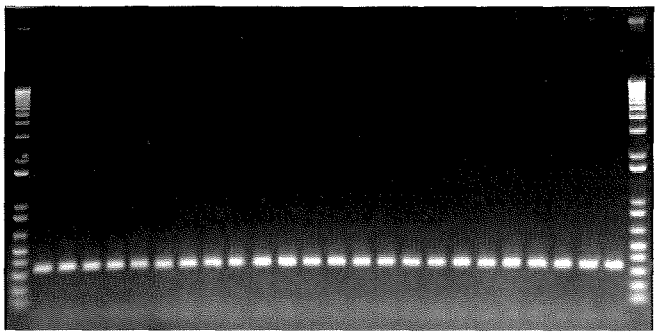
All of the *Xap* isolates were detected, while none of the isolates of the other closely related *Xanthomonas arboricola* pathovars were detected (Figure 2.2). At 43 cycles there could possibly be some sort of late amplification set in the Xj6 sample, a *Xanthomonas arboricola* pv. *juglandis* isolate. Such a late amplification indicates

inhibition. At high template levels the sample produced a very inhibited amplification-like reaction occurring amongst the background, and at lower template concentrations the sample provided a negative result. At no time is a peak detected on the dissociation curve for this isolate. Since the possible amplification does not under any circumstances come close to crossing the threshold nor is a peak present on the dissociation curve, this sample cannot be considered as a positive result.

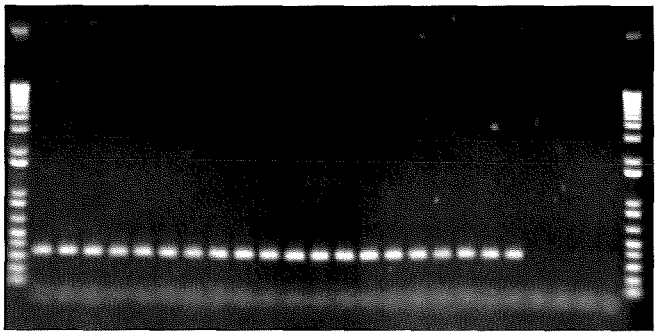
Dissociation Curve

Figure 2.3 shows that the melting temperature for the product produced by primer set 29 is around 82°C.

2.1.11.1 Gel Based Test



(a)



(b)

(c)

Figure 2.1. PCR amplification using primer set 29 on *Xap* and closely related *X. arboricola* pathovars. Gel pictures contain Australian *Xap* isolates Gel (a) lanes 1-20 and Gel (b) lanes 29-42; International *Xap* isolates Gel (a) 20-24 and Gel (b) lanes 25-28 and lanes 43-48. Other pathovars of *Xanthomonas arboricola* are located in Gel (c) lanes 49-62, lane 63 contains water only. See Table A.1, Appendix A for full sample details.

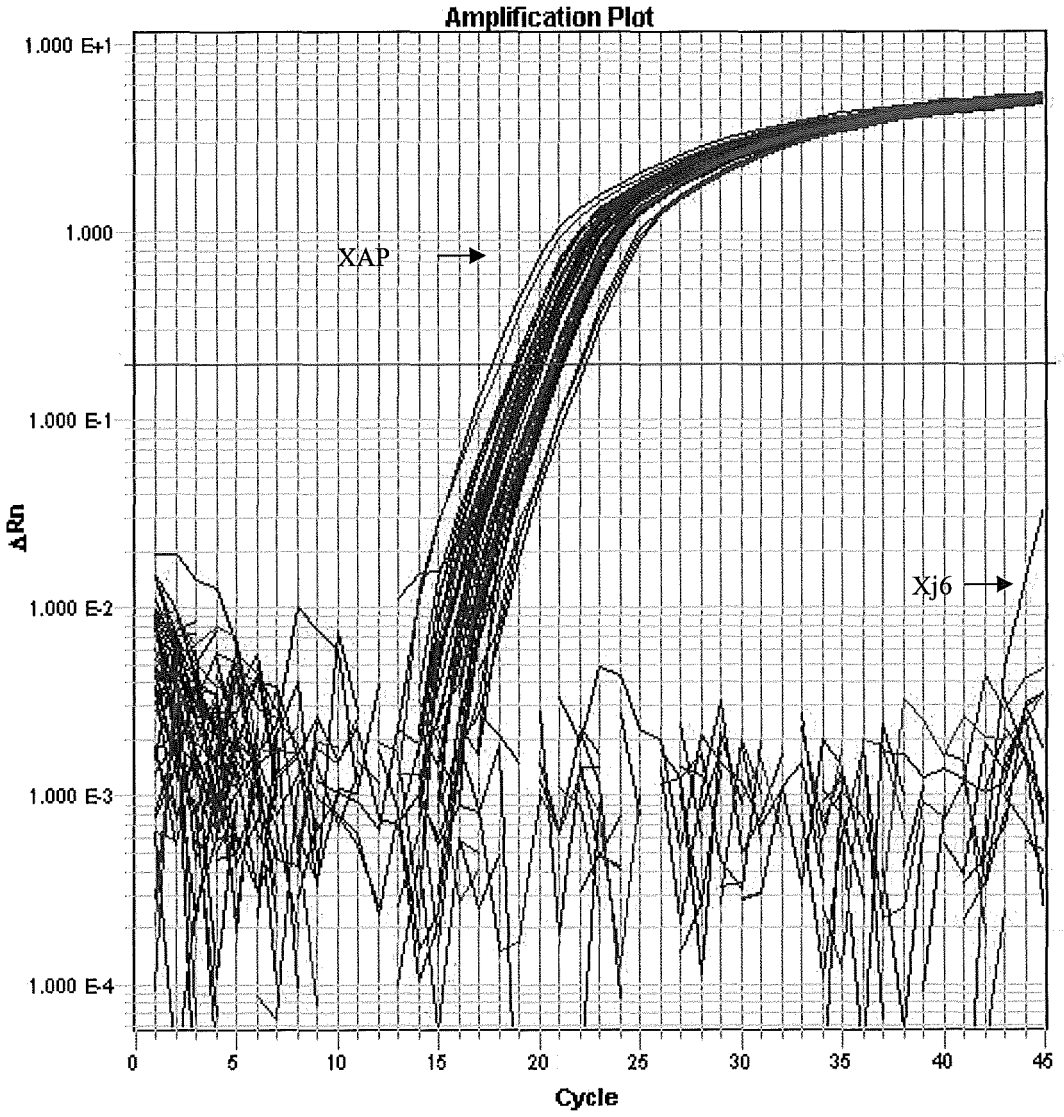


Figure 2.2. Amplification curve for SYBR real time testing of cell suspensions containing approximately 10^7 CFU/mL. Figure shows results for sample numbers 1 to 62. Sample 63 contains water only. See Table A.1, Appendix A for full sample details.

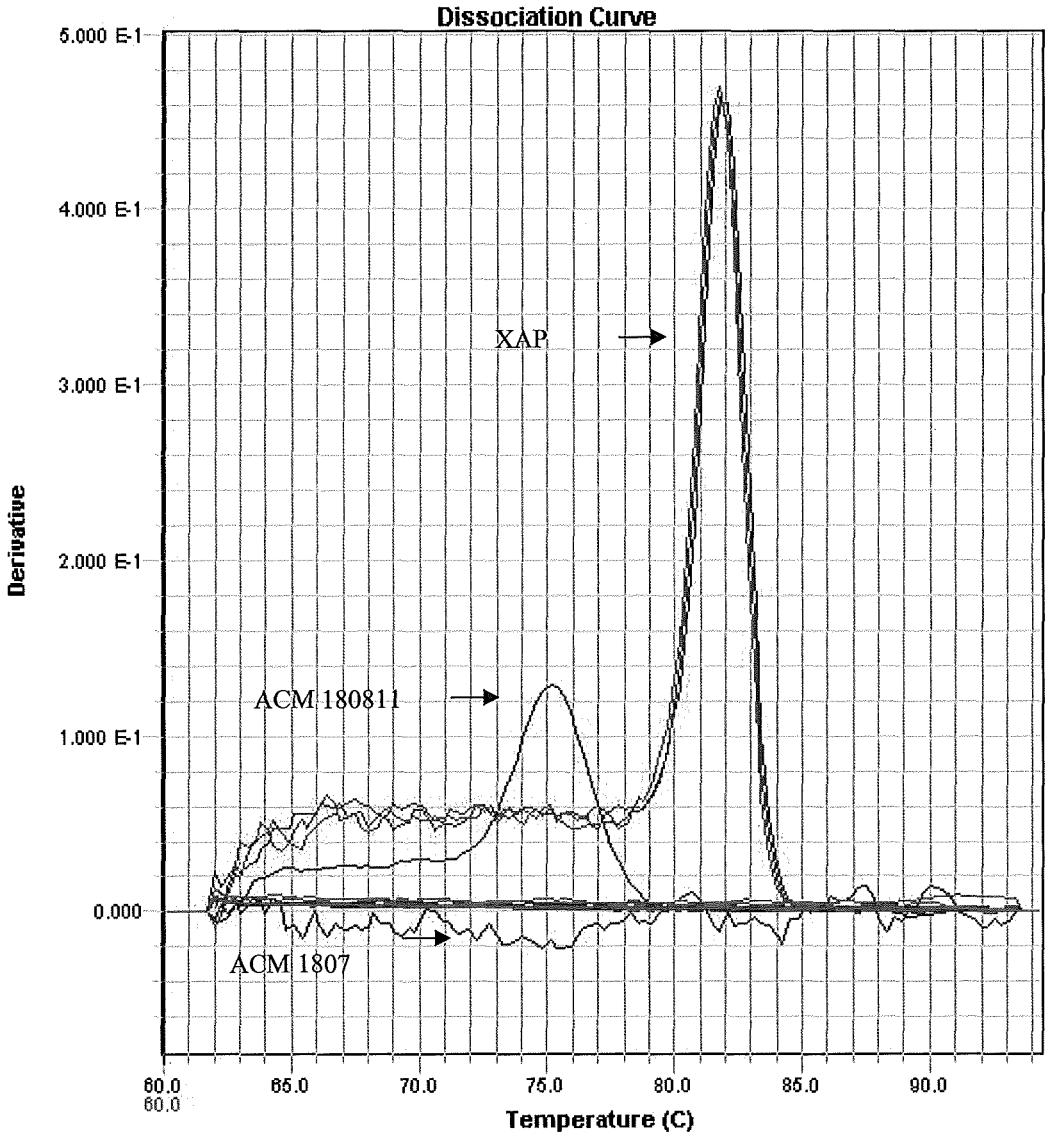


Figure 2.3. Dissociation curve for SYBR real time testing of cell suspensions containing approximately 10^7 CFU/ml. Figure shows results for sample numbers 1 to 62. Sample 63 contains water only. See Table A.1, Appendix A for full sample details.

2.1.12 Sensitivity testing

2.1.12.1 Gel based

Cell suspension dilution series

The number of cfu/ml in the original cell suspension was determined to be approximately 3 x 10^8 cfu/ml. The PCR test possibly detected down to 30 cfu/ml. No product was found to be present in the water sample.

DNA dilution series

The concentration of DNA in the original sample was 200 ng/µl. The PCR test can detect down to 0.02 ng/µl. The water control was negative.

2.1.12.2 *Real time assay*

Cell suspension dilution series

The number of cfu/ml in the original cell suspension was determined to be approximately 4×10^7 cfu/mL. The real time test could detect something in the serial dilution below the 4 cfu/ml dilution. Since it was possible for some cells to be present in this sample this result is valid. No amplification occurred in the next sample in the series. Between 4×10^2 cfu/ml to 0.4 cfu/ml all of the samples clustered around 33-36 cycles. This is obviously the limit for the SYBR detection for pure cell suspensions. Nothing was detected in water. The 10^8 cfu/µl was not tested in this example but always demonstrated an inhibited reaction.

DNA dilution series

The amount of DNA in the first sample was 10 µg/µl. The amount of DNA was clearly too much for the reaction resulting in an inhibited amplification. The real time test could detect down to 0.1 pg/µl. It was unable to detect 0.01 pg/ul. Nothing was detected in water.

2.2 Comparison of the author's Xanthomonas arboricola pv. pruni specific PCR test with the other readily available PCR diagnostic tests.

Materials and methods

2.2.1 Isolation of Bacterial Genomic DNA

The panel of bacterial and fungal isolates described in table A.1 (Appendix A) was used in this section. All *Xap*, *Xanthomonas aboricola* pathovars and *Pseudomonas syringae* isolates (ACM 1807 and ACM 180811) were stored on cyropreservation beads at -80°C. Two beads were placed on to GYCA agar, streaked and incubated for two days at 28°C. *Xanthomonas arboricola* pv. *fragariae* (*Xaf*) and *Pseudomonas syringae* (044) were freeze dried specimens rehydrated in a volume of 200 µl of sterile water, streaked onto GYCA agar and incubated for two days at 28°C. *Phytophthora cinnamomi* isolates (UQ 3206, UQ 3198, 1690) were from agar squares stored under water and pieces of agar were placed onto V8 agar and incubated for five days at 28°C. All field isolates (P11 C1, P4 C2, E6 F2, L14 F2, F4 F1, L3 F1, E8 L3 and F4 L2) were cultured from dense cell suspension in sterile water. A loopful of each was streaked onto GYCA agar and incubated for two days at 28°C. All isolates were extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA). For bacterial isolates the 'Isolation of genomic DNA from gram positive and gram negative bacteria' procedure was used and for fungal isolates the 'Isolation of genomic DNA from plant tissue' procedure was used. To grind the fungal material 0.2 g of 0.2 mm glass beads were added to the extraction buffer and shaken in the Fast Prep FP120 (BIO 101, CA) at a speed of 6 for 30 s. DNA was roughly quantified using Hyperladder I (Bioline, London, UK) via visual estimation through gel electrophoresis.

2.2.2 Zuli Protocol (Zuli 2003)

One microliter of DNA at 25 ng/µl was PCR tested. Each 25 µl PCR reaction contained a final concentration of 0.025 U/µl of AmpliTaq Gold® (Applied Biosystems, Foster City, CA), 0.24 pmol/µl each of the forward primer XP92F = 5'-ATG CGC GGC GTT GAT G-3'and the reverse primer XP92R = 5'-CTG TTG CGG ACG AGG TCA CGA-

3' (GeneWorks, Adelaide, Australia), 1.5 mM magnesium chloride, 0.2 mM dNTP's (Invitrogen, Mount Waverley, VIC, Australia), 1× the 10X GeneAmp® PCR Buffer II (Applied Biosystems, Foster City, CA). Amplifications were performed in the PalmCycler (Corbett Research, Mortlake, NSW, Australia) with the following cycling conditions: Initial denaturation at 96°C for 5 min, thirty cycles of denaturation at 94°C for 15 s, annealing at 59°C for 30 s, extension 72°C for 30 s followed by a final extension at 72°C for 10 min and cooling to room temperature, 28°C for 1 min. PCR products (5 µl) were run on a 2% agarose gel in 1× TAE at 5V/cm for 30 min and visualised with staining in 0.2 µg/ml EtBr under UV light (Hoefer UV Transilluminator, Hoefer, San Francisco, CA).

2.2.3 Pagani Protocol (Pagani 2004)

Two microliters of DNA at 25 ng/µl was PCR tested. Each 25 µL PCR reaction contained a final concentration of 0.025 U/µl of AmpliTaq Gold® (Applied Biosystems, Foster City, CA), 1 µM each of the forward primer Y17CoF = 5'-GAC GTG GTG ATC AGC GAG TCA TTC-3'and the reverse primer Y17CoR= 5'-GAC GTG GTG ATG ATG ATC TGC-3' (GeneWorks, Adelaide, Australia), 2 mM magnesium chloride, 0.2 mM dNTP's (Invitrogen, Mount Waverley, VIC, Australia), 1× the 10X GeneAmp® PCR Buffer II (Applied Biosystems, Foster City, CA). Amplifications were performed in the PalmCycler (Corbett Research, Mortlake, NSW, Australia) with the following cycling conditions: Initial denaturation at 95°C for 5 min, thirty cycles of denaturation at 92°C for 1 min, annealing at 53°C for 1 min, extension 72°C for 2 min followed by a final extension at 72°C for 5 min and cooling to room temperature, 28°C for 1 min. PCR products (5 µl) were run on a 2% agarose gel in 1× TAE at 5V/cm for 30 min and visualised with staining in 0.2 µg/ml EtBr under UV light (Hoeffer UV Transilluminator, Hoefer, San Francisco, CA).

2.2.4 Author's Gel Based Assay Protocol

One microliter of DNA at 25 ng/µl was PCR tested. Each 25 µl PCR reaction contained a final concentration of 0.025 U/µl of AmpliTaq Gold® (Applied Biosystems, Foster City, CA), 1 µM each of the forward primer 29F = 5' GTA CCG CAT TTC AGG CCG TCA -3'and the reverse primer 29R = 5'- AAG TAG CCA ACG CGG AAT TT -3' (GeneWorks, Adelaide, Australia), 1.5 mM magnesium chloride, 0.2 mM dNTP's (Invitrogen, Mount Waverley, VIC, Australia), 1× the 10X GeneAmp® PCR Buffer II (Applied Biosystems, Foster City, CA). Amplifications were performed in the PalmCycler (Corbett Research, Mortlake, NSW, Australia) with the following cycling conditions: Initial denaturation at 95°C for 10 min, thirty cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension 72°C for 30 s followed by a final extension at 72°C for 10 min and cooling to room temperature, 28°C for 1 min. PCR products (5 µl) were run on a 2% agarose gel in 1× TAE at 5V/cm for 30 min and visualised with staining in 0.2 µg/ml EtBr under UV light (Hoeffer UV Transilluminator, Hoefer, San Francisco, CA).

2.2.5 Author's Real Time Assay Protocol

One microliter of DNA at 25 ng/µl was tested. Each 10 µ PCR reaction contained a final concentration of 1X SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK), 0.2 µM each of 29F and 29R (GeneWorks, Adelaide, Australia), 1 mg/ml of BSA (Sigma-Aldrich, Castle Hill, NSW, Australia). Amplifications were performed on the ABI 7900 (Applied Biosystems, Foster City, CA) with the following cycling conditions: Initial denaturation at 95°C for 10 min, forty-five cycles of

denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension 72°C for 30 s followed by cycling to determine dissociation curve for products of 95°C for 2 min, 60°C for 15 s, and 95°C for 15 s.

Results

2.2.6 PCR test comparison

The only protocol to detect all of the *Xanthomonas arboricola* pv. *pruni* and exlude all of the non-*Xanthomonas* isolates was the Authors PCR primer set using both the gel based and real time assay. None of the tests used detected non-*Xanthomonas* isolates. The Zuli (2003) protocol detected all of the *Xap* isolates, all of the *Xanthomonas arboricola* pv. *corylina* isolates and one *Xanthomonas arboricola* pv. *juglandis* (Xj5) isolate. The Pagani, Ritchie *et al.* 1995 protocol did not detect all of the *Xap* isolates, and produced a product of the same size for both of the *Xanthomonas arboricol*a pv. *poinsettiicola,* one *Xanthomonas arboricola* pv. *corylina* (Xc5) isolate and one *Xanthomonas arboricola* pv. *juglandis* (Xj5). Both Xc5 and Xj5 were detected by the alternative tests. Five of the 38 Australian isolates were not detected along with an isolate each from the USA, Canada and Brazil.

Table 2.2. PCR detection results for *Xap* panel isolates using PCR detection systems developed by the author (Ballard), Zuli (2003) and Pagani (2004).

#	Sample*	Ballard Gel based PCR	Ballard Real Time PCR	Zuli (2003)	Pagani (2004)
1	Xc1	Negative	Negative	Positive	Negative
2	Xc2	Negative	Negative	Positive	Negative
3	Xc3	Negative	Negative	Positive	Negative
4	Xc4	Negative	Negative	Positive	Negative
5	Xc5	Negative	Negative	Positive	Positive
6	Xpop1	Negative	Negative	Negative	Negative
7	Xpop2	Negative	Negative	Negative	Negative
8	Xpop3	Negative	Negative	Negative	Negative
9	Xpop4	Negative	Negative	Negative	Negative
10	Xpoi1	Negative	Negative	Negative	Positive
11	Xpoi2	Negative	Negative	Negative	Positive
12	Xcel 1	Negative	Negative	Negative	Negative
13	Xj2	Negative	Negative	Negative	Negative
14	Xj3	Negative	Negative	Negative	Negative
15	Xj4	Negative	Negative	Negative	Negative
16	Xj5	Negative	Negative	Positive	Positive

#	Sample*	Ballard Gel based PCR	Ballard Real Time PCR	Zuli (2003)	Pagani (2004)
17	Xj6	Negative	Negative	Negative	Negative
18	Xp1	Positive	Positive	Positive	Positive
19	Xp2	Positive	Positive	Positive	Positive
20	Xp3	Positive	Positive	Positive	Negative
21	Xp4	Positive	Positive	Positive	Negative
22	Xp5	Positive	Positive	Positive	Positive
23	Xp6	Positive	Positive	Positive	Positive
24	Xp7	Positive	Positive	Positive	Positive
25	Xp8	Positive	Positive	Positive	Positive
26	Xp9	Positive	Positive	Positive	Positive
27	Xp10	Positive	Positive	Positive	Positive
28	Xp11	Positive	Positive	Positive	Negative
29	Xp12	Positive	Positive	Positive	Positive
30	Xp13	Positive	Positive	Positive	Positive
31	Xp16	Positive	Positive	Positive	Positive
32	Xp17	Positive	Positive	Positive	Positive
33	Xp18	Positive	Positive	Positive	Positive
34	Xp19	Positive	Positive	Positive	Positive
35	Xp20	Positive	Positive	Positive	Positive
36	Xp22	Positive	Positive	Positive	Positive
37	Xp23	Positive	Positive	Positive	Positive
38	Xp24	Positive	Positive	Positive	Positive
39	Xp25	Positive	Positive	Positive	Negative
40	Xp26	Positive	Positive	Positive	Negative
41	Xp27	Positive	Positive	Positive	Positive
42	Xp28	Positive	Positive	Positive	Negative
43	Xp29	Positive	Positive	Positive	Negative
44	Xp30	Positive	Positive	Positive	Negative
45	Xp32	Positive	Positive	Positive	Positive
46	Xp33	Positive	Positive	Positive	Positive
47	Xp34	Positive	Positive	Positive	Positive

#	Sample*	Ballard Gel based PCR	Ballard Real Time PCR	Zuli (2003)	Pagani (2004)
48	Xp35	Positive	Positive	Positive	Positive
49	Xp37	Positive	Positive	Positive	Positive
50	Xp38	Positive	Positive	Positive	Positive
51	Xp39	Positive	Positive	Positive	Positive
52	Xp40	Positive	Positive	Positive	Positive
53	Xp41	Positive	Positive	Positive	Positive
54	Xp42	Positive	Positive	Positive	Positive
55	Xp48	Positive	Positive	Positive	Positive
56	Water	Negative	Negative	Negative	Negative

*For full details of isolates refer to Table A.1, Appendix A.

The Zuli (2003) protocol produced a 179 bp product (Figure 2.4). The amplification products for all PCR reactions were of this size.

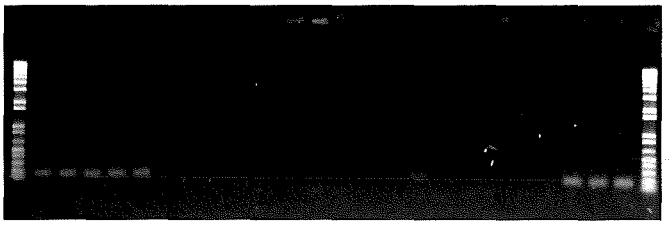


Figure 2.4. PCR amplification using Zuli (2003) protocol from panel isolates described in Table 2.2. Gel picture contains samples 1-24 (Table 2.2). See Table A.1, Appendix A for full sample details.

The Pagani (2004) protocol produced a 943bp product (Figure 2.5). The amplifications produced for all PCR reactions were of this size.

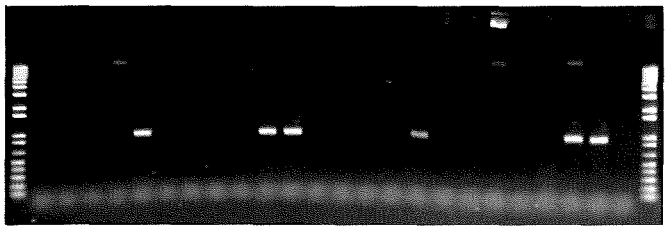


Figure 2.5. PCR amplification using Pagani, Ritchie *et al.* (1995) protocol from panel described in Table 2.2. Gel picture contains samples 1-24 (Table 2.2). See Table A.1, Appendix A for full sample details.

The authors' primer set produced a 344 bp product (Figure 2.6). The amplifications produced for all PCR reactions were of this size.

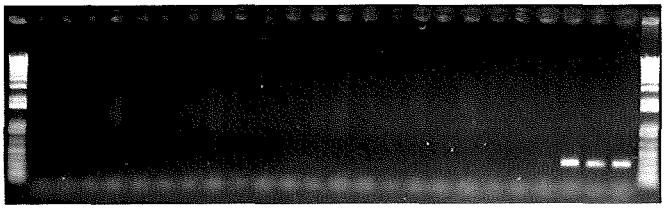


Figure 2.6. PCR amplification using the newly developed primer set protocol from panel described in Table 2.2. Gel picture contains samples 1-24 (Table 2.2). See Table A.1, Appendix A for full sample details.

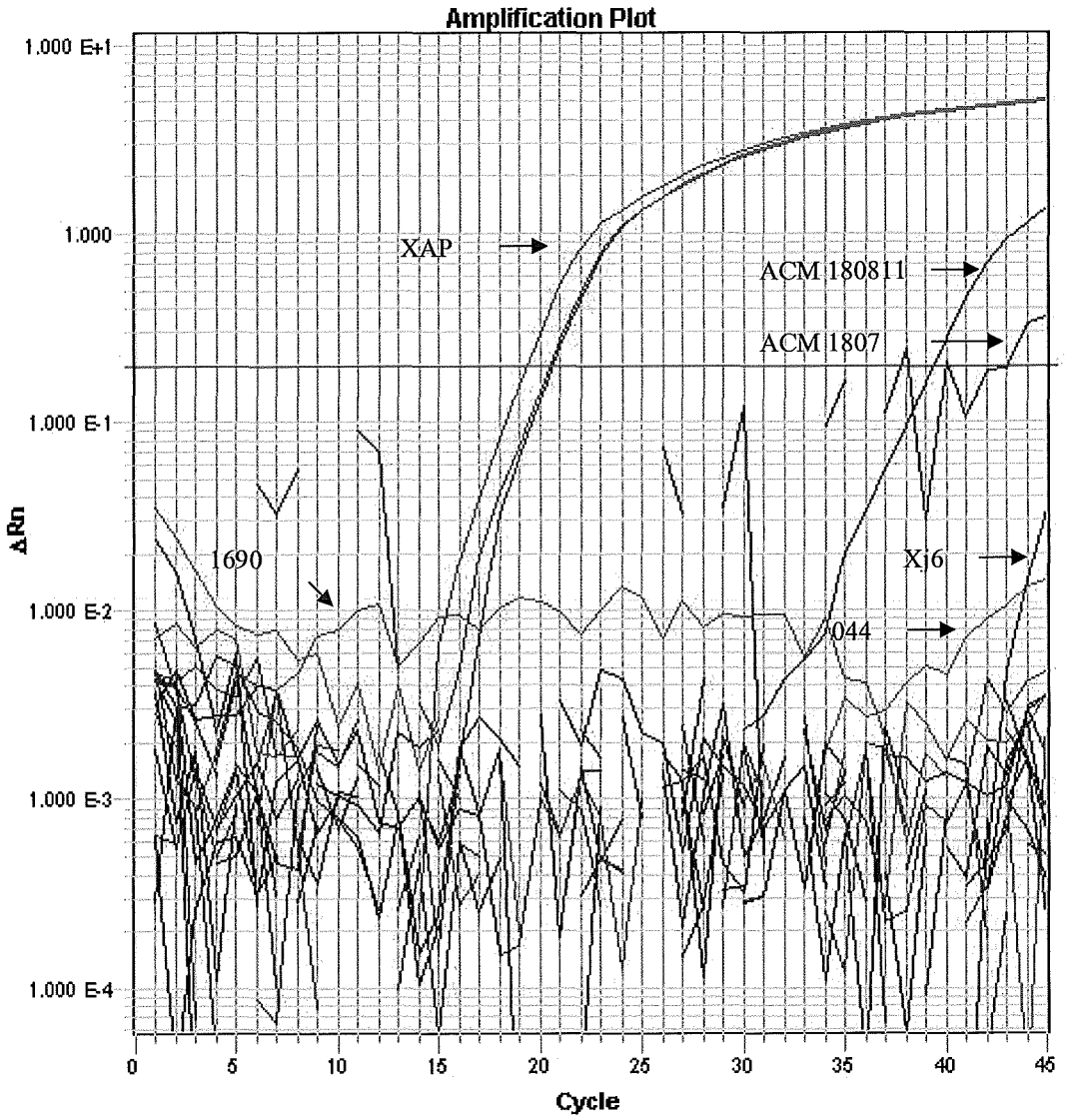


Figure 2.7. Amplification curve for SYBR real time testing from panel described in Table A.1, Appendix A. Picture shows samples 1-24. See Table A.1, Appendix A for full sample details.

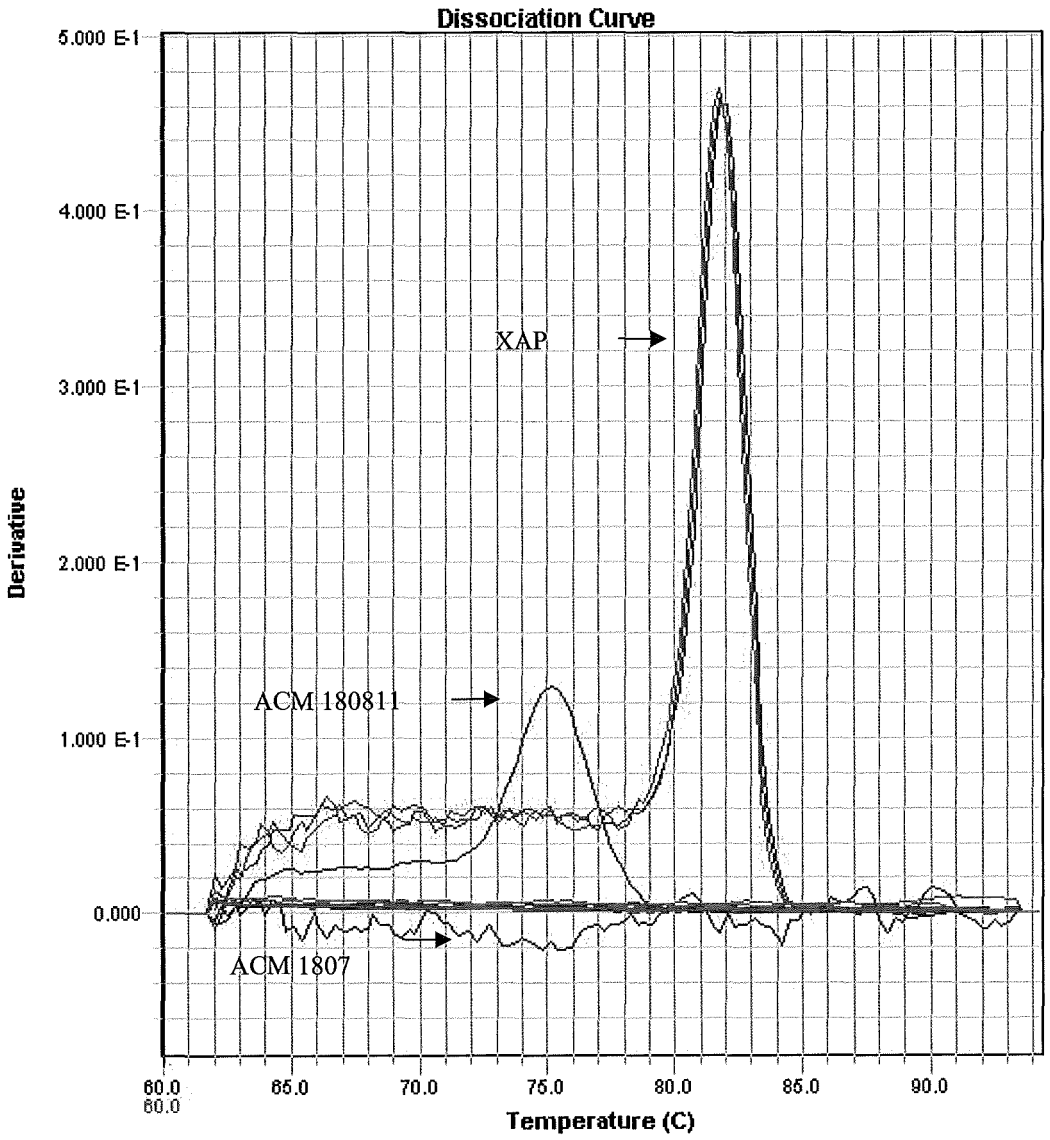


Figure 2.8. Dissociation curve for SYBR real time testing from panel described in Table A.1, Appendix A. Picture shows samples 1-24. See Table A.1, Appendix A for full sample details.

The real time SYBR protocol for the Author's primer set detected a few potential issues. The real time SYBR amplification showed a strong amplification for ACM 180811 and an inhibited reaction for ACM 1807 (Figure 2.7). When the dissociation curve was examined the melting temperature for the PCR product produced from ACM 1807 had a melting temperature of around 75°C compared with the *Xap* isolate which had a melting temperature of around 82°C. Due to the inhibited reaction produced by ACM 18011 no specific melting temperature could be determined. Since the melting temperature for ACM 1807 was significantly different than that of the *Xap* isolates it can be easily distinguish as being a false reaction when the dissociation curve is observed. At 43 cycles there could possibly be some sort of late amplification was seen in the Xj6 sample a *Xanthomonas arboricola* pv. *juglandis* isolate. Samples 044 (*Pseudomonas syringae*) and 1690 (*Erwinia herbicola*) also had amplification-like reactions occurring amongst the background. None of these three produced a peak of any kind on the

dissociation curve. These amplification reactions indicate inhibition and at high template levels these samples produced a very inhibited amplification-like reaction occurring amongst the background, with no peak on the dissociation curve and at lower template concentrations these samples gave a negative result. Since the possible amplification of these samples does not under any circumstances come close to crossing the threshold and no peaks are observed on the dissociation curve, these samples can not be considered as positive results.

2.2.7 Sensitivity of Protocols

Pagani, Ritchie *et al.* (1995) published that the tests limit of detection was 5000 cfu/ml. Zuli (2003) published that the tests limit of detection was 10000 cfu/ml. The limit of detection for the Authors primer set is 30 cfu/ml for the gel based test and the real time assay detects down to approximately 1 cfu/ml. The newly developed primer set has a significantly higher level of sensitivity than the other protocols.

Discussion

A primer set specific to the detection of *Xap* was developed using a technique that involves suppression subtractive hybridisation to identify target DNA specific fragments (*Xap* specific sequences). This technique involves the DNA hybridisation of tester and driver strains resulting in only tester specific DNA being amplified. The author (Ballard) used *X. arboricola* pv. *corylina* as the driver and *Xap* as the tester strain.

This primer set has been developed for use as either a gel based assay or a SYBR real time assay. Protocols have been developed for testing cell suspensions made from bacterial colonies growing on plates (BIO-PCR), DNA and directly from crushed symptomatic tissue.

The gel based assay does not demonstrate any cross-specificity problems, with other closely related *Xanthomonas arboricola* pathovars. This is a problem commonly associated with other readily available *Xap* PCR diagnostic assays (Zuli 2003; Pagani 2004). The real time assay shows late amplification reactions (after 30 cycles) on *Pseudomonas syringae*, Xj6 and unknown cultures isolated (not shown here) from the field. However, these amplifications are clearly distinguishable from *Xap* by looking at the dissociation curve, which shows either no peak or a significantly different melting temperature for these cultures. *Xap* cultures can be clearly distinguished from non-*Xap* cultures with this assay. These isolates could not be induced to produce a product through the gel based protocol, even by ramping up the cycle number. As the gel based test only completes 30 cycles, it will not create a problem.

The gel based test reliably detects *Xap* in suspensions with thirty colony forming units/ml or 0.02 ng/µl of *Xap* DNA. The real time test detects *Xap* in suspensions with only one colony forming unit/ml or 0.1 pg/µl of *Xap* DNA. The sensitivity of these tests compares very favourably against other tests, with Zuli's (2003) protocol only able to detect concentration of 10 000 colony forming units/ml and Pagani's (2004) protocol 5000 colony forming units/ml.

2.3 *Use of the author's Xanthomonas arboricola pv. pruni specific PCR test on field samples*

This section details the use of the authors PCR diagnostic test to diagnose field samples of plum tissues for the epidemiological studies described in Section 3.8.

The method used was based on a combination of techniques previously published (Barnes 1965; Pruvost, Boher *et al.* 2002; Zaccardelli, Consiglio *et al.* 1995).

2.3.1 Preparation of symptomatic leaf, fruit, stem and branch tissues

Infected leaves and fruit from each sampled tree was weighed and surface sterilised in bulk, by swirling in 75% ethanol for 2 min. Infected tissues were then rinsed with sterile distilled water and sterilised with 1% sodium hypochlorite bleach for a further 2min. After the second surface sterilisation, tissues were rinsed three times in sterile distilled water, and dried in a laminar flow cabinet. Once dry, pieces of leaf or fruit spots were ground in a mortar and pestle with 500 µl of sterile distilled water. Stem or branch canker material was placed between two pieces of sterile filter paper and crushed with a hammer. After crushing, 10 ml of sterile distilled water was added. Crushed or ground material was then shaken for 1 h at 200 rpm in an orbital shaker at 28°C. After shaking, the suspensions were allowed to stand for 10 min at 28°C to allow the crushed plant material to settle. The liquid at the top of the sample (wash solution) was then pipetted off, and either plated on to bacterial growth media (Section 2.3.2.1) or used directly in the template extraction step (Section 2.3.2.2).

2.3.2 Preparation of non symptomatic tissues

Leaves, flowers, fruit, twigs, leaf scars (>one-year-old and one-year-old), cankers (>one-year-old and one-year-old), soil and debris were examined. The tissues were weighed and washed in a known volume of water for 3 min at 28°C and samples. The samples were allowed to stand for 10 min at room temperature and the liquid at the top of the sample (wash solution) was then pipetted off, and either plated on to bacterial growth media (Section 2.3.2.1) or used directly in the template extraction step (Section 2.3.2.2). Leaf scars and cankers were surface sterilised in bulk, by swirling in 75% ethanol for 2 min, rinsed with sterile distilled water and sterilised with 1% sodium hypochlorite bleach for a further 2 min. After the second surface sterilisation, tissues were rinsed three times in sterile distilled water, and dried in a laminar flow cabinet. Once dry, cankers and leaf scars were removed with sterile scalpel and the tissue was crushed between two pieces of sterile filter paper with a hammer. The tissue was resuspended in a known volume of water and shaken for 1 h at 200 rpm on an orbital shaker at 28°C. The sample was allowed to stand for 10 min to allow the plant material to settle. The liquid at the top of the sample (wash solution) was then pipetted off, and either plated on to bacterial growth media (Section 2.3.2.1) or used directly in the template extraction step (Section 2.3.2.2).

2.3.3 Template/DNA extraction

2.3.2.1 Template extraction from bacterial colonies

An aliquot (100 µl) of wash solution serially dilution 1:10 (to a dilution determined on the day) onto GYCA or XPSM and incubated at 28°C for 4 and 6 days respectively. Single colonies were picked off and placed into a small volume of water to make dense cell suspensions containing approximately 10^8 colony forming units/ml.

The suspensions were then boiled (95°C) for 3 min, cooled to room temperature and centrifuged in a bench top centrifuge (ca. 12 500 rpm) for 2 min. The supernatant was then used as template in the gel based PCR protocol, or diluted 1:10 for use in the real time PCR protocol.

2.3.2.2 Template extraction from infected tissue washings

The remainder of the wash solution was boiled (95°C) for 3min, cooled, and centrifuged in a bench top centrifuge (ca. 12 500 rpm) for 2 min. The supernatant was used as template in the gel based PCR protocol, or diluted 1:10 for use in the real time PCR protocol, or stored at -20°C.

2.3.3 PCR assay protocols

Template preparations were then used in the previously described methods for gel based PCR (Section 2.2.4) or real time PCR (Section 2.2.5).

3. Epidemiological studies

In order to develop more effective management strategies it is important to have a strong understanding of the mechanism the pathogen uses to initiate infection. This includes understanding the environmental conditions needed for fruit infection, the age/stage of development during which the host is most susceptible, the sources of inoculum within the orchard and the influence of other factors on symptom development.

3.1 Conditions for symptom development and infection

Christine Horlock and Duncan Cameron (DPI&F, Queensland).

Two of the most important factors in successful infection of stone fruit trees by *Xap* are the susceptibility of the host (du Plessis 1988) combined with conducive environmental conditions (Mathee and Daines 1968 and 1969). In this section, we confirm the preliminary tests of Stephens (2002), indicating the most susceptible developmental stages for bacterial spot infection of fruit, as well as examine the differences in weather conditions between a rain event and average spring weather in the Granite Belt of Queensland.

Aim

To determine the susceptibility of stone fruit trees to infection by *Xap* at differing developmental stages of the fruit and leaves.

To examine the effect on relative humidity and leaf wetness caused by early spring rainfall in the Granite Belt, Queensland.

Materials and methods

3.1.1 Effect of developmental stage on fruit susceptibility

Experimental trees

Varieties used include the highly susceptible plums: Pizzazz, Durado and highly susceptible peach Faye Elberta.

Fully dormant trees of all varieties were planted into 30 L plastic planter bags, and placed into pre-dug holes in the Applethorpe Research Station experimental stone fruit orchard in winter 2003. The 'potted' trees were then surrounded by sawdust, to allow easier removal later in the season, and the soil surface in the bags covered with pine bark to reduce evaporation. All trees were watered with individual drippers from an irrigation system.

Inoculum

Inoculum suspensions were produced by mixing bacterial cells from six strains of *Xap*, originally collected from bacterial spot infected plum fruit in the Granite Belt, and stored by cryopreservation between seasons. Each strain was grown individually in nutrient broth solutions. Broths were inoculated with a single loopful of cells, and incubated at 28°C in a shaking incubator, until reaching the early stationary phase of population growth (ca. 18 h). Cultures were centrifuged; cells from each strain were resuspended 1:10 in sterile distilled water. Cells from all six strains were then

combined to make a final suspension of cell 1×10^6 – 1×10^8 cells/ml in sterile distilled water.

Inoculation process

Trees were removed from the orchard in their bags, and transferred to a humidity tent within a glasshouse. The relative humidity was maintained above 95%, using water misters, and the temperature at 25±5°C. Tree roots were watered, and the trees kept saturated at field capacity for the duration of the inoculation process.

Excess fruit was thinned from trees, and the location on the tree and diameter of the remaining fruit recorded. Fruit were exposed by trimming the leaves immediately around the fruit to allow even application of the bacterial suspension.

Trees were inoculated by spraying a fine mist of freshly prepared bacterial suspension over the fruit, growing tips and leaves. The trees were kept in the humidity tent for 48 h after application of inoculum. After 48 h, humidity was slowly reduced by turning off water misters, and leaving the trees in the glasshouse humidity tent with flaps open for 24 h. Trees were finally returned to the orchard 72 h after inoculation.

Inoculated fruit and leaves were monitored weekly for the development of bacterial spot symptoms. Ratings included: number of infection sites (bacterial spot lesions); percentage of total fruit area covered by lesions, and the diameter of the lesions.

3.1.2 Effect of environmental conditions on field infection of plum trees

No specific experiments were performed to determine the environmental conditions required for field infection of plum trees in Queensland. Our method involved measuring environmental conditions (rainfall, maximum and minimum temperatures, leaf wetness, relative humidity and wind speed) within the Applethorpe Research Station experimental stone fruit orchard, and comparing fruit and leaf bacterial spot symptom development data to identify likely infection events.

This work was undertaken over all four seasons of experimental trials (2002/03 – 2005/06) at Applethorpe. During this time there were three relatively dry/low rainfall seasons (2002/03, 2004/05 and 2005/06), and one moderately wet season (2003/04). The occurrence of relatively dry seasons made the determination of infection events much easier, with 2004/05 and 2005/06 only producing one or two possible infection events each season.

Weather data presented in this report from early spring 2005, is typical of the infection events observed over previous seasons.

Results

3.1.3 Effect of developmental stage on fruit susceptibility

Sequential inoculations of Dorado and Pizzazz fruit from the same varieties confirmed that the most severe bacterial spot symptoms occurred on the smallest fruit inoculated (Table 3.1). No significant levels of infection were recorded on the Faye Elberta peaches, regardless of fruit size. This lack of infection may have been due to the very dry conditions experienced in the orchard after inoculations.

Table 3.1. The number of infections initiated by *Xap* inoculation at different developmental stages.

Variety	Fruit diameter at inoculation when the majority of infection occurred (mm)	Fruit numbers of this size infected (%)	Time of successful inoculations (Days after shuck off)
Durado (plum)	12.5	77	16 (Mid-October)
Pizzazz (plum)	6.6	89	8 (Early October)

3.1.4 Effect of environmental conditions on field infection of plum trees

Figure 3.1 shows the relative humidity, leaf wetness and rainfall totals at 15 minute intervals for a 24 h period covering a rainfall event on the 16-17 September 2005. Figure 3.2 shows the relative humidity and leaf wetness recorded for the three following rain free days, 18-21 September 2005.

The patterns of relative humidity and leaf wetness, during and after rainfall (Figure 3.1) show a total of 11 h of 100% relative humidity and just over 6 h of >95% leaf wetness. While the relative humidity recorded for the following three days does reach similar levels for substantial periods (6-8 h) overnight, the amount of time with leaf wetness >95% are much lower (0-45 min per night).

These results indicate that leaf wetness was a more important factor in successful infection than relative humidity in this particular instance. Another interpretation of these results is to reinforce the concept that rainfall, and the raised levels of leaf wetness for extended periods of time that follow, really is an essential part of the *Xap* infection process. And that even though relative humidity and leaf wetness levels are surprisingly high on most nights during early spring in the Granite Belt, these conditions, on their own, are not sufficient to initiate *Xap* infection and consequent bacterial spot disease development.

Discussion

The fruit inoculation results support the findings of Pagani, Leoni *et al.* (2001) and Stephens (2002) that the younger fruit, the more susceptible they are to *Xap* infection. The most severe bacterial spot symptoms were formed on fruit infected at very early developmental stages; with the number and severity of *Xap* infection reducing as the fruit matured. This work also explains the effectiveness of the targeted copper spray program (Section 4.1); which protects fruit from *Xap* infection for four to six weeks after shuck off.

The study of weather conditions surrounding successful infection periods goes someway towards identifying the specific effects induced by rainfall that lead to successful *Xap* infection. However, the interaction between environmental factors is likely to be much more complex, and warrants further investigation.

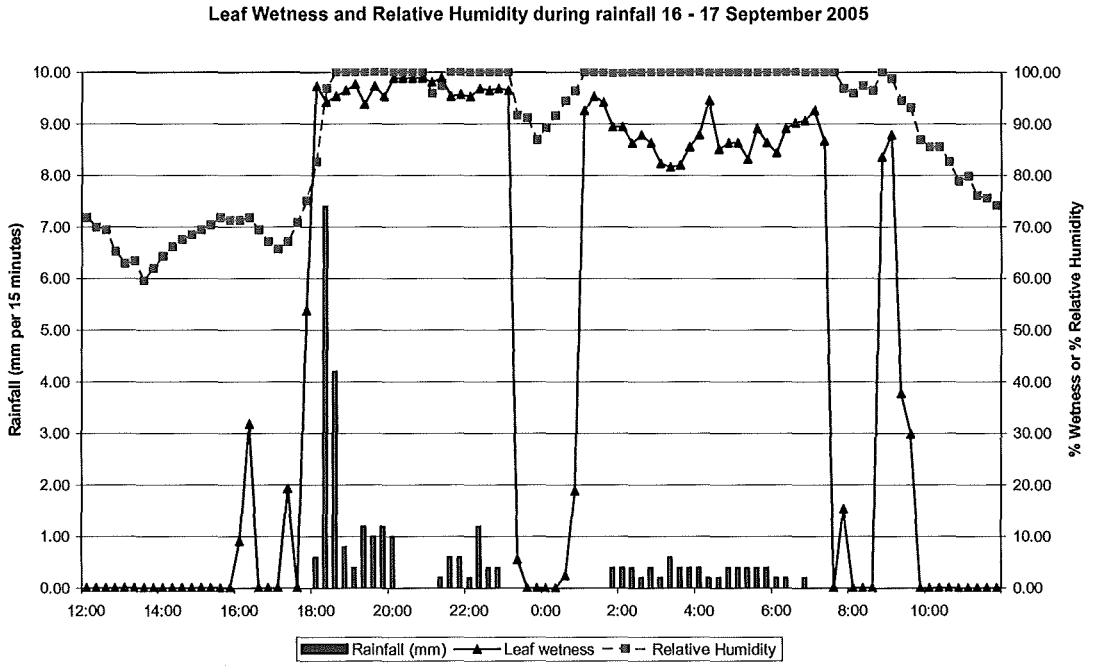


Figure 3.1. Weather conditions for a typical early spring rain event, including rainfall, relative humidity and leaf wetness for the 24 h period from noon 16 September 2005 to noon 17 September 2005.

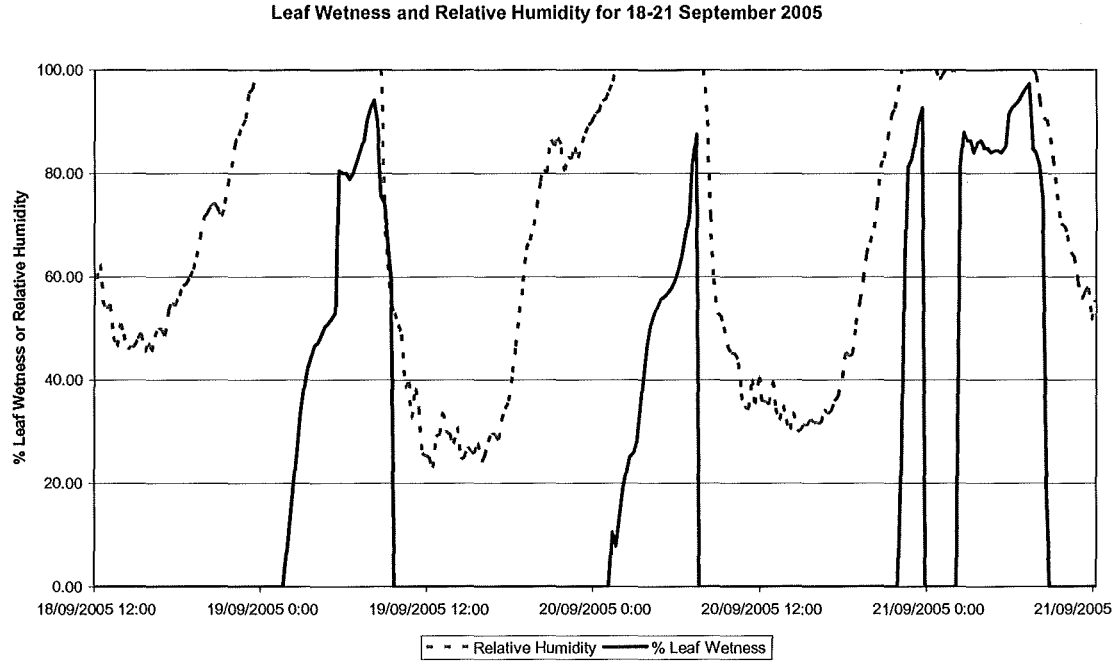


Figure 3.2. Weather conditions for typical early spring days without rain, including relative humidity and leaf wetness for the 72 period from noon 18 September 2005 to noon 21 September 2005.

3.2 Location of overwintering sites for Xap *inoculum*

Christine Horlock and Duncan Cameron (DPI&F, Queensland).

Identifying the location of inoculum sources in an orchard is a vital part of understanding the cycle of any disease. Once the source of infection is known, and the mechanism of survival understood, it is often possible to substantially reduce the level of inoculum surviving from one season to another, and thereby reduce overall infection.

Aim

To determine the length of time *Xap* can survive in infected plum fruit and leaves.

Method

Bacterial spot infected leaves were collected at the end of the 2002/03 experimental season and tested for the presence of *Xap* by grinding of tissues in sterile distilled water, and streaking on nutrient agar plates (Section 4.1.2.5). The leaves were then divided into twelve sample lots, and six lots were mulched into pieces ca. 5 mm^2. A 5% urea solution was applied to three of the mulched, and three of the unmulched, sample lots, with three mulched and three unmulched sample lots left untreated.

Two lots of bacterial spot infected plum fruit was collected for this experiment, the first lot in the spring of 2003, during fruit thinning, and the second after harvest in summer 2004. Representative sample fruit were tested for the presence of *Xap* by grinding of tissues in sterile distilled water, and streaking on nutrient agar (Section 4.1.2.5).

Fruit and leaves were placed in shallow wooden boxes, covered with wire mesh to prevent material blowing away, and placed on the ground in the Applethorpe Research Station orchard between collection and subsequent testing.

Leaves were tested for the presence of *Xap* after winter (77 days). This time was selected as it was the time when most plum varieties in the orchards had reached shuck fall.

Infected fruit were periodically removed from the boxes, over a period of 36 months, and tested for viable *Xap* cells as described above.

Results

Mulched plum leaf pieces collected after 77 days on the orchard floor were found to still contain considerable numbers of viable *Xap* cells, regardless of urea treatment or mulching. There was no significant difference between the numbers of viable cells recovered from mulched or unmulched and urea treated or non-urea treated leaves.

3.2. Number of viable *Xap* cells recovered from bacterial spot infected leaves (a) treated with urea or (b) mulched into 5 mm^2 pieces, after 77 days on a Queensland plum orchard floor.

Treatments	Mulched*	Unmulched*
Urea treated	5.8×10^5 a	2.9×10^4 a
Not Urea treated	1.1×10^6 a	5.4×10^6 a

*Statistical analyses as described in Section 4.1.2.6. Values with the same letter are not significantly different ($p<0.05$). Statistical analyses are valid within and between treatments.

Viable *Xap* cells were isolated from infected fruit mummies 24 months after they were placed on the orchard floor, with all mummies tested recording moderate bacterial populations. No *Xap* cells were isolated from a similar sample of mummies after 36 months on the orchard floor.

These results indicate that infected leaves and fruit mummies are capable of harbouring *Xap* over winter in Queensland plum orchards; and providing substantial amounts of inoculum for at least the following season. Fruit mummies may provide inoculum for two seasons.

Discussion

The recovery of viable *Xap* cells from fruit mummies after 24 months and leaves after 77 days demonstrates the considerable potential for overwintering of bacterial spot in Queensland plum orchards. If left in the orchard at the end of the season, such infected materials provide a considerable source of inoculum for fresh infections in spring. Infected leaves and fruit may also be a good source of inoculum for further infection within the tree during the growing season.

However, survival of these cells alone is not sufficient to result in infection; these viable cells must come into contact with susceptible plant tissues, under conducive environmental conditions for successful infection to occur. The scenario of an infected fruit mummy from the previous season remaining on, or under, trees is not uncommon in many Granite Belt orchards. These results support the need for good cultural practices in orchards affected by bacterial spot. All infected leaves and fruit should be removed from orchards, prior to the start of the next season. Growers should also ensure that activities such as pruning and thinning are undertaken in a hygienic manner, including regular disinfection of hands and equipment.

3.3 Location of infection points on fruit

Christine Horlock and Duncan Cameron (DPI&F, Queensland).

Aim

To determine the location of *Xap* infection points on the fruit of plum trees in an orchard.

To determine the effects of targeted and traditional copper spray programs on the number and distribution of *Xap* infection points on orchard grown plum fruit.

Materials and methods

An experimental orchard at Applethorpe Research Station (the same orchard and trees used in Section 4.1) with a history of bacterial spot infection, and containing five varieties of plums, was monitored for visual signs of the disease on fruit over four seasons (2002/03 to 2005/06). Fruit were monitored from the development of the first bacterial spot symptoms, usually late October, until harvest (late December to February). Final ratings and definitive identification of symptoms (as described in Section 4.1) were undertaken after harvest.

All plums were monitored weekly for signs of bacterial spot infection, and fruit with possible bacterial spot infection were individually identified and tagged. Records were kept of fruit symptom identification, the date symptoms were first observed, height of fruit above the ground (Figure 4.1) and the location of the fruit on the tree (Figure 3.2). The location of all infection sites on the fruit (Figure 3.4) and their size when first found were also recorded. Fruit with atypical bacterial spot symptoms were also marked, and identified after harvest as described above.

It should also be noted that during these ratings there was no consideration given to the orientation of the fruit on the tree.

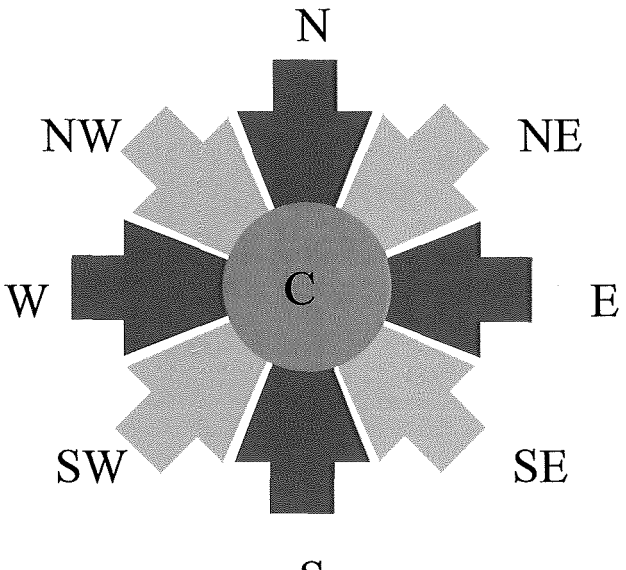


Figure 3.3. The nine aspects of the tree used to record where each infected piece of fruit was found.

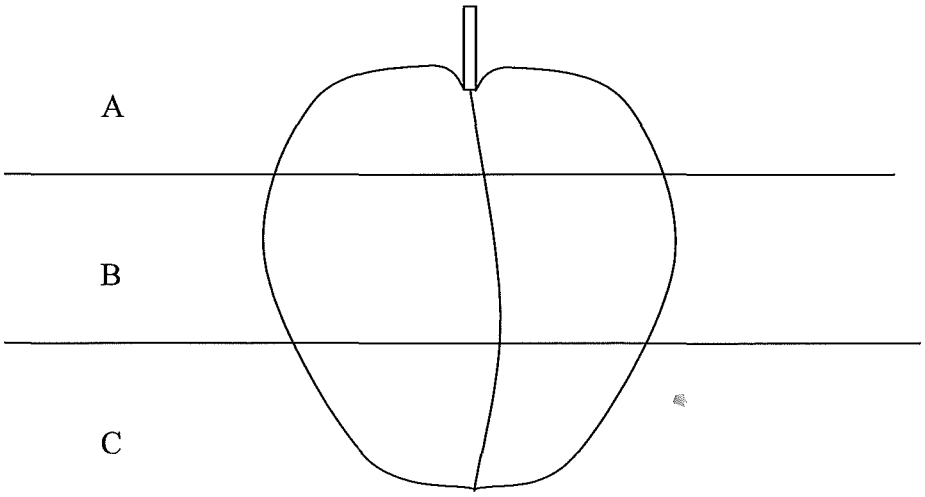


Figure 3.4. The location of each infection site was recorded as top (A), middle (B) or base (C) of the fruit, and the diameter of the spot was recorded.

A weather station in the orchard measured temperature, relative humidity, rainfall, leaf wetness and wind strength at 15 minute intervals. Wind direction was not recorded.

Results

Data is presented from 2003/04 only, as this was the season with the highest levels of bacterial spot infection in the orchard. Similar trends in data were observed for the other seasons, 2002/03, 2004/05 and 2005/06, but not all differences were significant in those seasons.

Environmental conditions in this orchard for all four seasons are presented in Appendix B (Figures B.1-5), full details for the 2003/04 season are provided in Appendix B, Table B.1.

For each of the susceptible plum varieties there were significantly more spots on section B than sections A and C (Table 3.3). Friar and Laroda had significantly more spots on section C than A.

Table 3.3. Location of bacterial spots on individual fruit from susceptible plum varieties in the 2003/04 season.

Variety	Location of spot on fruit (as per Figure 1.2)		
	A*	B*	C*
Eldorado	26.9 a	93.2 d	31.5 ab
Friar	42.1 ab	181.4 e	90.0 d
Laroda	66.3 c	301.9 f	187.1 e

*Statistical analyses as described in Section 4.1.2.6. Values with the same letter are not significantly different ($p<0.05$). Statistical analyses are valid within and between varieties.

Regardless of the copper program used, there were significantly more spots on section B of fruit than section C, which in turn had significantly more spots than section A (Table 3.4).

Table 3.4. Location of bacterial spots on individual fruit from susceptible plum varieties treated with traditional and targeted copper programs in the 2003/04 season.

Treatment	Location of spot on fruit (as per Figure 1.2)		
	A*	B*	C*
Traditional	81.9 c	352.3 e	222.0 d
Targeted	21.7 a	84.2 c	29.5 b

*Statistical analyses as described in Section 4.1.2.6. Values with the same letter are not significantly different ($p<0.05$). Statistical analyses are valid within and between treatments.

There was a trend for more spots on a fruit on the south and west sides of the trees than the North (Table 3.5).

Table 3.5. The effect of fruit location in the tree on number of individual plum fruit with spots. Data is bulked for all three susceptible varieties.

Aspect	Number of plum fruit with spots for each treatment*
N	54.5 ab
NE	36.7 bc
E	63.0 a
SE	46.5 abc
S	61.0 a
SW	49.3 ab
W	65.5 a
NW	27.5 c
LSD	19.14

*Statistical analyses as described in Section 4.1.2.6. Values with the same letter are not significantly different ($p<0.05$). Statistical analyses are valid within and between treatments.

Discussion

It is surprising to see that the mid-section, section B, of the fruit was the area with the largest number of fruit infections. It would seem more likely for either the stem ends (section A) or beaks (section B) of the fruit to have the highest level of infection as these are the areas were moisture tends to collect. However, as section B is the largest section in terms of area, this result may simply indicate a random distribution of infection over the whole fruit surface.

As expected the southern and westerly aspects of trees tended to have higher levels of fruit infection. In this orchard, these two sides of the trees have the highest levels of humidity due to poor exposure to sunlight. The southern sides of southern hemisphere trees always receive the least sun, and the western side of this orchard was shaded by nearby native bushland.

These results confirm the need for good spray application over the whole fruit surface. As well as the need to focus humidity reduction techniques on the southern, or any shaded, aspect of orchard trees; i.e. pruning to increase air flow, ensuring very good coverage of leaves and fruit, and applying sprays during quick drying conditions.

3.4 Location of infection points on orchard trees

Aim

To determine the location of *Xap* infection points on the leaves, twigs and branches of orchard grown plum trees.

To determine the effects of targeted and traditional copper spray programs on the number and distribution of *Xap* infection points on orchard grown plum trees.

Materials and methods

The mature plum trees at Applethorpe Research Station (described in Section 4.1) were surveyed for general tree health on the 26 of April, 2006. Each tree was given an overall rating, with regard to bacterial spot infection. The rating scale was 1 = no visible symptoms, 2 = leaf infection but no dead shoots, 3 = death of minor branches and leaves, 4 = major branch death and 5 = tree death.

Five one-year-old shoots (canes) from around the each of the trees were selected at random. These shoots were measured for length of growth for the season, and the number of cankers per shoot recorded.

Parameters analysed were general tree health, average number of cankers per cane and number of cankers per metre of cane. An analysis of variance (ANOVA) was carried out on the results using the logarithm transformation $y' = \log(y+1)$.

Results

The rating of general tree health rating showed no significant differences between trees of the same variety treated with either the traditional or targeted copper programs (data not shown). No visible symptoms of bacterial spot infection were observed on the trees of the less susceptible varieties, Queen Rosa and Black Amber. The more susceptible varieties, Eldorado, Friar and Laroda, displayed average general health ratings, according to the above mentioned scale. However, there was no significant difference between varieties or trees of the same variety treated with either traditional or targeted copper programs.

Analysis of both cankers per metre, and cankers per cane, showed a significant variety effect, and a significant copper program by variety effect (Table 3.6). The differences within varieties are significant for the varieties Eldorado, Friar and Laroda, with disease levels significantly reduced in the trees treated with the targeted copper program. There was no significant difference between the copper program treated trees in the less susceptible varieties of Black Amber and Queen Rosa.

Discussion

Although there was no observable significant difference in general tree health over the four year period, the measurements made on one-year-old shoots show a significant reduction in cankers when the targeted treatment is used. The exact reason/s for this remains unclear. It is possible that more than four years of differences in annual shoot symptoms may be required to produce observable symptoms at a whole of tree level.

These results show that the use of the targeted copper program will result in a significant reduction in bacterial spot canker symptoms on annual shoots. The next step in this research is to define the benefits of annual shoot canker reduction in terms of reduced inoculum within the orchard during the growing season, and the potential reduction of overwintering bacteria in the orchard.

Table 3.6 Analysis of number of cankers per shoot and number of cankers per metre of cane for plum trees treated with the traditional and targeted copper programs for four consecutive seasons (2002/03 – 2005/06).

Variety	Treatment	Cankers/cane*	Cankers/m*
Black Amber	Traditional	0.2 a	0.4 i
Black Amber	Targeted	0.0 a	0.1 i
Queen Rosa	Traditional	0.6 a	0.5 i
Queen Rosa	Targeted	0.4 a	0.4 i
Eldorado	Traditional	4.0 cd	4.6 fg
Eldorado	Targeted	0.8 ab	1.2 hi
Friar	Traditional	2.4 bd	2.5 gh
Friar	Targeted	0.2 a	0.4 i
Laroda	Traditional	7.3 c	6.5 f
Laroda	Targeted	2.0 bd	1.9 gh

*Statistical analyses as described in Section 4.1.2.6. Values with the same letter are not significantly different ($p<0.001$). Results in shaded boxes were not significantly different ($p<0.001$) within that variety.

3.5 Development of spots in relation to physical damage such as hail

Christine Horlock and Duncan Cameron (DPI&F, Queensland)

The moderately damaging hail storm that occurred in the Applethorpe Research Station orchard during the 2003/04 season provided an opportunity to study the relationship between substantial physical damage to fruit and bacterial spot infection. Common sense thinking would indicate that substantial wounds, such as those caused by the impact of storm driven hail, would provide excellent entry points for bacterial infection.

However, recent anecdotal evidence (personal communication Prof David Ritchie) has suggested that often minimal bacterial infection of fruit and leaves occurs after storms with heavy rainfall. This is due to the heavy rainfall effectively washing the bacterial inoculum off of the leaves and fruit, and actually resulting in lower levels of bacterial spot inoculum within trees. As most hailstorms in the Applethorpe district are accompanied by heavy rain, it seemed possible that increased bacterial spot infection of fruit may not result from such a storm.

Aim

To determine if fruit injuries, such as those caused by hail damage, increase the number of bacterial spot lesions formed on the fruit of susceptible plum varieties.

Materials and methods

Fruit from the mature plum orchard at Applethorpe Research Station (Section 4.1) was severely affected by hail in the 2003/04 (Figures B.3 and B.6, Appendix B) season. Thinning of the fruit on trees occurred in mid-October. Fruit were removed on the basis of population density, and no fruit were removed due to hail damage.

Fruit were surveyed for the presence of bacterial spot symptoms on a weekly basis from the onset of disease symptoms in late October until harvest. During the fruit rating process (Section 4.1.2.4) hail damaged fruit was also assessed for the presence or absence of bacterial spot lesions on hail marks.

Results

Results from the three highly susceptible varieties, Eldorado, Laroda and Friar were combined for this analysis. Trees, of these three varieties, with less than ten fruit expressing bacterial spot symptoms were not included in the analysis. Of the remaining trees, hail marks were recorded on 67.1% of all fruit harvested. This level of damage was consistent throughout the orchard and similar for each variety. When the fruit with bacterial spot symptoms were examined, it was found that overall 53% of fruit infections were initiated on hail marks (Table 3.7), and 47% of fruit infections were not initiated on hail marks; indicating that fruit infection is not strongly influenced by the presence of physical injuries caused by hail. As previously mentioned this may be due to the heavy rain that accompanied the hail washing *Xap* cells off of fruit.

It should also be noted that the level of infection on fruit from trees treated with the traditional copper program was much higher (on susceptible varieties) than the level produced on targeted copper program treated trees (Table 4.4).

Table 3.7. Percentage of hail damaged sites on plum fruit also infected with bacterial spot.

Variety*	% of fruit with spots originating on a hail mark	% of fruit with spots not originating on a hail mark	% of fruit harvested with hail marks
Eldorado	52	48	64
Laroda	56	44	70
Friar	48	52	67
Mean	52	48	67

Discussion

The overall level of bacterial spot identified within the orchard during 2003/04 was significantly higher than in any of the other seasons, suggesting a seasonal influence on disease incidence. One factor that occurred during 2003/04 that did not occur in other seasons was a hail storm. However, the 2003/04 season was generally much more conducive to bacterial diseases due to higher rainfall totals, higher number of rainy days and increased days at high relative humidity (Figure B.3 and Table B.1, Appendix B).

3.6 Study of bacterial spot epidemiology on nectarines

Dr Chin Gouk (Plant Pathologist, DPI Victoria)

Aim

The incidence of bacterial spot on nectarines was studied in a Victorian orchard to provide information on bacterial spot development under climatic conditions differing from those of Queensland.

Materials and methods

Monitoring of bacterial spot incidence was conducted in a Swan Hill orchard during the 2003/04 season. Detailed inspections were conducted on six Artic Pride nectarine trees, each in a separate row, in an orchard block that had been infected with bacterial spot the previous season. The incidence of bacterial spot on all the fruit up to head height on three to five branches of each tree was monitored six times between October 2003 and February 2004. At each inspection, fruit with bacterial spot symptoms were tagged with a ribbon and dated. The number of diseased fruit on the ground was also recorded and added to the total count for the tree.

Bacterial spot incidence on the fruit was assessed on harvested fruit in February 2004. Isolation from fruit lesions was conducted to confirm the presence of *Xap.*

Tinytag weather recording equipment was installed within the monitored block to record weather conditions. The parameters recorded included temperature (maximum and minimum), rainfall, relative humidity, leaf wetness and wind speed.

Results

Bacterial spot was not detected in the 13 October inspection of newly set fruit. Symptoms were first observed on 20 November after shuck fall (Figure 3.5). Between November and February, the disease level was low and ranged between 2.0 – 5.2% for fruit on the tree and 0–3.9% for fruit on the ground. The combined disease incidence is shown in Figure 3.3.

Over 2000 fruitlets were assessed on 13 October 2003. The number of fruit was reduced by 80% in subsequent assessments, due to storm and frost events that occurred between 13 October and 20 November (Figures C.1-C.4, Appendix C). Sub-zero temperatures were recorded on 30 September and below 5°C on 1 November 2003 (Figure C.2, Appendix C). Wind speeds of over 60 kph were recorded between 16 October and 26 October 2003. Rainfall >0.5 mm was recorded on only three days in the three months between 13 September and 13 December 2003.

Discussion

Drought conditions were not conducive to the development of bacterial spot on nectarine fruit in Swan Hill during the 2003/04 season. Symptoms of bacterial spot were not observed on 13 October, but were first recorded after shuck fall on 20 November, following two rain events greater than 5 mm on the 1st and 5th October. It appears that between shuck split in October and shuck fall, nectarine fruit were susceptible to bacterial spot infection.

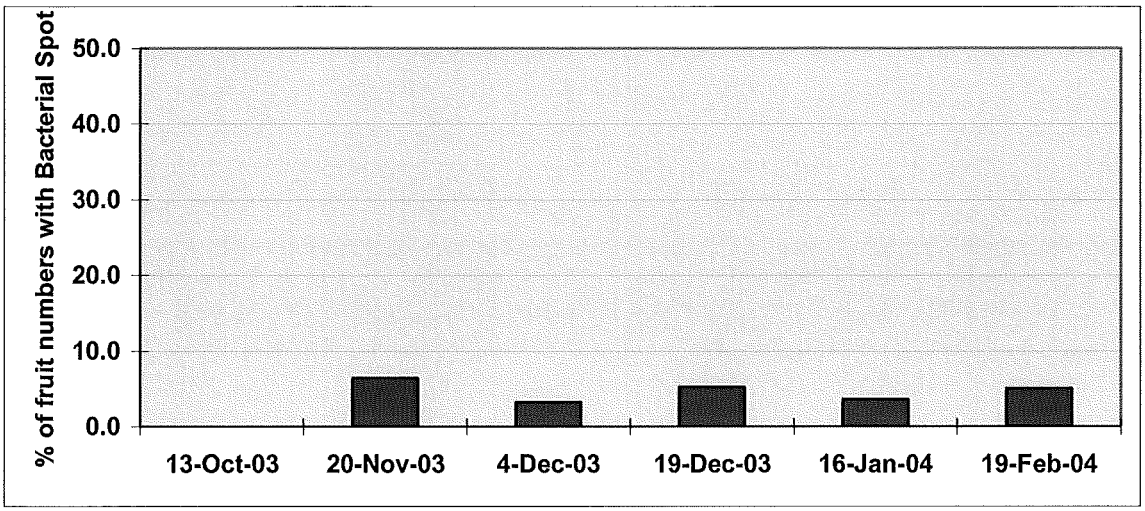


Figure 3.5. Percentage incidence of bacterial spot infection in an Artic Pride nectarine orchard in Swan Hill, October 2003-February 2004.

3.7 Study of Bacterial Spot Cankers on Plums

Dr Chin Gouk (Plant Pathologist, DPI Victoria)

Aim

A study of the development of bacterial spot cankers on plum branches in an orchard in the Goulburn Valley was undertaken in the spring of 2005 and 2006 in an attempt to gain greater understanding of the spread of the disease in a previously infected orchard.

Methods

A Pizzazz plum orchard with a history of hail damage during 2003/04 and heavy bacterial spot infection during previous seasons, was studied in the spring of 2005 and 2006. In October 2005, twigs (1-2 years old) with multiple canker lesions were randomly collected from a block of trees in the orchard for measurement of the number of cankers and the distance between cankers on each twig. Isolations were made from selected canker lesions to confirm the presence of bacterial spot.

In September 2006, 12 samples each consisting of 15 newly emerging leaves or flower clusters were collected from the orchard, washed with sterile phosphate buffered saline, then plated on King's Medium B for recovery of *Xap* populations. Yellow bacterial colonies were subcultured on Yeast-Dextrose-Chalk Agar, and tested for milk and starch utilisation to confirm the presence of *Xap*.

Results

Pizzazz plum twigs harbouring numerous cankers on both one and two-year-old wood were observed throughout the orchard. A total of 190 cankers were found on 17 twigs, with the number of cankers ranging from 5-17 per twig. All except one twig had cankers 0.6-2.5 cm apart. These closely spaced cankers accounted for 46% of the cankers and were found to be located between consecutive leaf nodes. All cankers resulted in darkening of the wood under the bark. Some cankers had internal lesions greater in length than the external lesions. *Xap* was recovered from some of the canker lesions on the one year-old wood in 2005.

Yellow bacteria were isolated from the flower and leaf washings in September 2006. However, no *Xap* were recovered, suggesting that the inoculum was absent on the surface of the flowers and leaves in early spring in 2006.

Discussion

The heavily infected and hail damaged plum trees presented an opportunity for study of the development and spread of bacterial spot cankers. It would have yielded more valuable data had the researcher had the opportunity to undertake additional studies in the previous 2003/04 season. Dry conditions in subsequent years may have arrested canker activity, thereby limiting development of new cankers and inoculum. Whilst it was not possible to recover *Xap* in each lesion, the isolation of *Xap* from internal bark tissues and the extended internal lesions nevertheless confirmed *Xap* infection. Further mapping of cankers and testing for *Xap* in canker lesions in new season's wood would provide useful information on *Xap* survival in twig cankers between seasons.

3.8 Outcomes from "Epidemiological studies of bacterial spot of stone fruit: developing and using a specific PCR detection system for Xanthomonas arboricola pv. pruni"

Emma Ballard (nee George), PhD Candidate, University of Queensland.

This section is brief summary of the experimental outcomes from the work of Emma Ballard (nee George), a PhD candidate sponsored by the project. Full details of experimental methods and results will be presented in Emma's thesis.

Summary of experimental chapters

- Chapter One: The aim of this chapter is to observe the development of Bacterial spot in plums at an orchard in Applethorpe, Queensland. This chapter is divided into four sections. The first section is the identification of disease symptoms, the second is to observe whether the host and copper treatment can influence disease onset and development, the third is to attempt to identify relationships between disease onset/development with weather factors and the fourth section is to determine if key factors influence disease onset and the disease incidence 28 days after disease onset for both leaves and fruit.
- Chapter Two: The aim of this chapter is to describe the development of a molecular diagnostic test specific to the detection of *Xap*. The chapter will be divided into two components the first being the development of the test and the second its comparison with the other molecular diagnostic tests available for *Xap* detection. This chapter has been largely reproduced in this report as Section 2.
- Chapter Three: The aim of this chapter is to determine is the Author's real time PCR test can detect *Xap* from symptomatic plant tissue. The chapter is divided into two components the first being the testing of symptomatic plant tissue and the second is confirmation of *Xap* identification using 16s sequencing, detached leaf assay and BIOLOG (an identification system for bacteria based on carbon utilisation).
- Chapter Four: The aim of this chapter is to study the epidemiology of *Xap* prior to disease onset with the used of the Author's real time PCR test. The chapter is divided into three components the first being the identification of *Xap* overwintering sites, the second being determining the numbers of *Xap* present at these sites and the fourth is the influence of weather conditions on *Xap* present at these sites.

Some Conclusions

The symptoms of bacterial spot present on plum trees in Applethorpe are typical of those seen in other parts of the world. Symptom onset occurs in a two month window between October and November each year. Leaves are always infected before or at the same time as fruit. The susceptibility level of a variety and copper treatments do not delay disease onset, but both play an important role in the resultant level of disease incidence. A molecular diagnostic test has been developed that is specific to the detection of *Xap*. Protocols have been developed for a gel based assay and a real time PCR test. The gel based test reliably detects *Xap* in suspensions with thirty colony forming units/ml or 0.02 ng/µl of *Xap* DNA. The real time test detects *Xap* in suspensions with only one colony forming unit/ml or 0.1 pg/µl of *Xap* DNA. The sensitivity of these tests compares very favourably against other tests, with Zuli's (2003) protocol only able to detect concentration of 10 000 colony forming units/ml and

Pagani's (2004) protocol 5000 colony forming units/ml. *Xap* can be detected from cell suspensions, extracted DNA and directly from crushed symptomatic tissue. *Xap* overwinters in all parts of the plant including debris but is in greatest numbers in the leaf scars. Moisture, in the form of rainfall or humidity, appears to be a highly significant factor in influencing bacterial spot disease onset.

4. Field management of bacterial spot in stone fruit orchards

This section describes the field testing of an extended copper spray program designed to reduce bacterial spot symptoms on plum fruit. This program is based upon the work of Prof David Ritchie and his group at the University of North Carolina, who have researched the epidemiology, detection and management of bacterial spot of peaches over the last 15 years (Pagani 2004; Pagani, Leoni *et al.* 2001; Pagani and Ritchie 2002; Pagani, Ritchie *et al.* 1995; Ritchie 2003; Ritchie 2005; Ritchie, Werner *et al.* 1993).

4.1 Using the Targeted Copper Spray Program to reduce bacterial spot infection in plums and nectarines – Granite Belt, Queensland

Christine Horlock and Duncan Cameron (DPI&F, Queensland).

This section details a series of experiments undertaken in experimental orchards at Applethorpe Research Station in the Granite Belt, Queensland, testing the efficacy of the targeted copper program on plums and nectarines.

Aims

To compare the Targeted Copper Spray Program with traditional spray control programs for the treatment of bacterial spot caused by *Xap*, in plums and nectarines in Queensland.

To evaluate the phytotoxic effects of a Targeted Copper Spray Program on plum and nectarine varieties in Queensland.

Materials and methods

4.1.1 Materials

4.1.1.1 Copper hydroxide products used

2002/03 and 2003/04 = Kocide Blue® (Griffin Pty Ltd) and Kocide Liquid Blue® (Griffin Pty Ltd)

2004/05 and 2005/06 = Kocide Blue Xtra® (DuPont Pty Ltd)

4.1.1.2 Experimental orchard – mature plums

This trial was conducted in an established orchard of mature plum trees at Applethorpe Research Station. The orchard originally consisted of thirteen blocks, with one tree each of Laroda, Friar, Eldorado, Queen Rosa and Black Amber per block. Despite tree losses in the orchard during the course of the project, all varieties were represented by at least five replicate trees for each treatment every season. The orchard consisted of a randomised pattern of all varieties over four rows, with three blocks per row, and a mixture of Black Amber and Queen Rosa blocks completing a fifth row.

4.1.1.3 Experimental orchard – young plums and nectarines

The targeted copper spray program was also trialled on a separate block of young plum trees and a block of nectarines. Four plum varieties (Autumn Giant, Durado, Pizzazz, and Tegan Blue) and two nectarine varieties (Fantasia and Harvest Sun) were included.

The treatment program and methods of disease assessment was the same for these trees as that described for the mature plum orchard.

4.1.2 Targeted Copper Spray Program

4.1.2.1 General orchard management

During the first two seasons of trials (2002/03 and 2003/04) copper was not applied to the control trees. Due to the onset of severe symptoms in the 2003/04 season, resulting in large numbers of cankers and significant losses of buds and twigs, it was decided to apply the industry standard of two early spring copper sprays (Table 4.1) to the control trees to prevent tree death.

Table 4.1. Traditional spring spray schedule.

Stage of tree/fruit development	Chemical* and rate
1. Early budswell	Copper hydroxide (150g/100L)
2. 7–10 days later	Copper hydroxide (150g/100L)

*Kocide Blue 2002- autumn 2004; Kocide Blue Xtra spring 2004-2006.

All trees received two autumn copper sprays as per recommended guidelines (Queensland Orchard Guide, 2001).

All trees were pruned in winter, and fruit thinned before stone hardening (approximately mid October) in spring. Thinning was restricted to removal of small fruit, without regard to russet or other fruit marks.

4.1.2.2 . Targeted Copper Spray Program

The targeted copper spray schedule (outlined in Table 4.2) involved five early spring copper sprays timed to the developmental stages of the fruit buds, and a further two to four sprays on the developing fruit (dependent on weather conditions) before the start of November. The latter sprays were applied before forecast rainfall or as soon possible after rainfall, once foliage was dry and relative humidity had returned to low levels during the 2002/03 and 2003/04 seasons. Rain dependent copper sprays for the 2004/05 and 2005/06 seasons were applied within 48 hours after rainfall. Temporary plastic barriers were used to avoid spray drift during spray applications.

This spray program was applied to mature plum, young plum and nectarine orchards over the four seasons of the project. Details of the rain dependent part of the program are provided for the mature plum orchard in Table 4.3. The young plum and nectarine blocks were sprayed in a similar manner, but as no significant data was obtained from these orchards the minor variations in spray program are not presented.

Table 4.2. Targeted Copper Spray Program schedule

Stage of tree/fruit development	Chemical* and rate
1. Early budswell	Copper hydroxide (150g/100L)
2. 7–10 days later	Copper hydroxide (150g/100L)
3. Pink to 10% bloom	Copper hydroxide (150g/100L)
4. Petal fall to 1% shuck split	Copper hydroxide (64g/100L)
5. 75% shuck split to 1% shuck off	Copper hydroxide (43g/100L)
6. Just prior to, or less than 48 hours after rainfall (2–4 applications)[#]	Copper hydroxide liquid (22ml/100L)

*Kocide Blue 2002- autumn 2004; Kocide Blue Xtra spring 2004-2006.
[#] In 2002/03 the aim was to apply sprays prior to rainfall; in subsequent seasons all applications were made after rainfall, unless rain occurred within three days of a previous post-rainfall application.

Table 4.3. Rain dependent copper spray applications made to mature plum trees in the 2002/03, 2003/04, 2004/05 and 2005/06 seasons.

Season	Number of rain dependent sprays applied per season*				
	B	E	F	L	QR
2002/03	4	4	4	3	4
2003/04	4	4	4	4	4
2004/05	2	2	1	1	2
2005/06	3	3	3	3	3

B = Black Amber; E = Eldorado; F = Friar; L = Laroda and QR = Queen Rosa.
*Rain dependent sprays were applied prior to rainfall in the 2002/03 season, and within 48 hours after rainfall in the subsequent seasons.

4.1.2.3 Fruit harvest

Harvest was conducted in one operation for each variety, when the majority of fruit had ripened to a stage where they would be picked on a commercial orchard. The fruit from each tree were picked in sequence from the bottom of the tree, with each zone 250 mm in height (as per Figure 4.1). The number of fruit in each height zone was recorded.

4.1.2.4 Fruit assessment after harvest

Assessment of bacterial spot infection

Each piece of fruit from each height zone was assessed for visual symptoms of bacterial spot. Fruit were recorded as being bacterial spot free, displaying typical bacterial spot symptoms or showing indeterminate symptoms. Fruit with indeterminate symptoms were stored at 4°C for up to seven days before undergoing *in vitro* testing (described in the following section) for the presence of *Xap*. Records of the distribution of the infected fruit were used for analysis in Section 3.2.

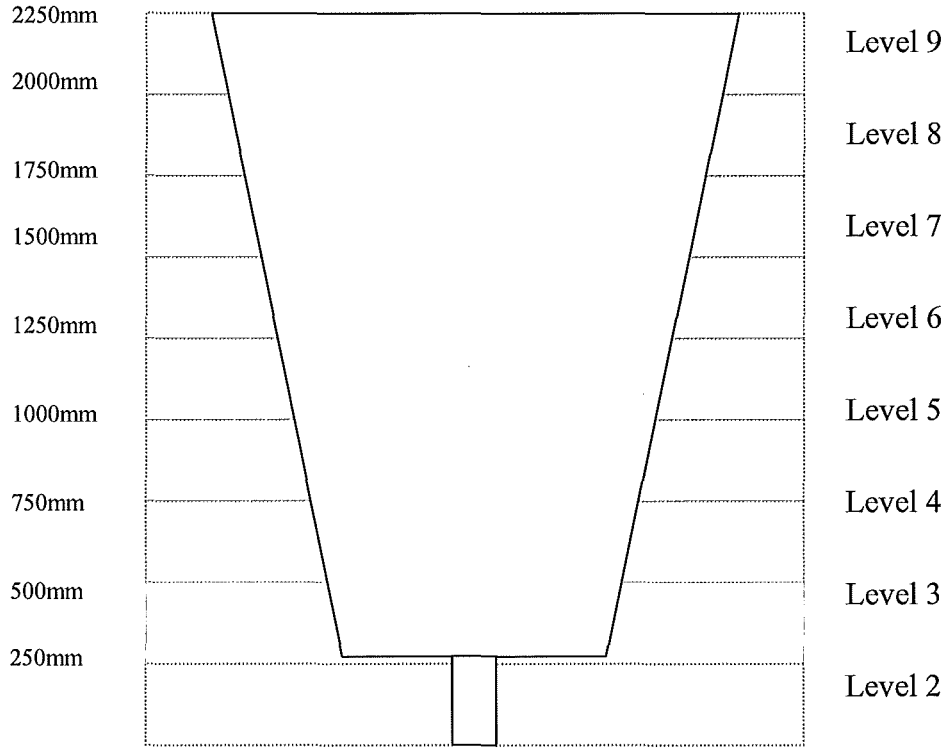


Figure 4.1. Fruit from each 250 mm interval was picked separately for pathogen population and disease symptom distribution analysis.

Assessment of fruit damage

Every fruit was rated visually for russeting and hail damage during each season. Russet levels were described by the following rating system: 0 = 0% of the fruit surface russetted; 1 = 1-10% of the fruit surface russetted; 2 = 11-20% of the fruit surface russetted; 3 = 21-30% of the fruit surface russetted; 4 = 31-50% of the fruit surface russetted and 5 = 51-100% of the fruit surface russetted. Hail marks were rated as present or absent on each fruit, except for 2003/04, when significant hail damage occurred, and fruit were rated independently for hail damage as described in Section 3.3.

Assessment of fruit quality parameters

A representative sample, comprising of 15 randomly selected pieces of fruit per tree, was assessed for several fruit quality parameters, including size, weight and sugar content. These fruit were weighed and had their diameters measured perpendicular to the suture using digital callipers (Figure 4.2). Juice was then extracted from three cores taken from around the centre of the fruit, and tested for sugar content (Brix) using a Schmidt and Haensch automatic refractometer.

4.1.2.5 In vitro testing of indeterminate fruit symptoms

All fruit with marks suspected of being bacterial spot symptoms were tested in the laboratory. The area was surface sterilized with 70% alcohol and dried under sterile conditions. A small section of the suspect mark was removed and crushed with a minimal amount of sterile distilled water in a sterile mortar and pestle. The resulting

suspension was then streaked onto a nutrient agar plate containing 100 ug/ml cycloheximide. Plates were incubated for 3-5 days at 28°C. The identity of colonies displaying the phenotypical characteristics of *Xap* were confirmed by Adgen Express identification kit for *Xap* (ADGEN Ltd, UK) or Ballard's *Xap* specific PCR identification test described in Section 1.

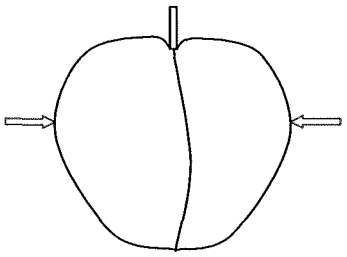


Figure 4.2. Fruit diameter was measured at the widest point perpendicular to the suture. Three ca. 10 mm square cores were taken from around the centre of the fruit; juiced and analysed for Brix analysis.

4.1.2.6 Statistical analyses

The experiment was analysed as a split plot (unbalanced) design, with spray treatment applied to whole plots, and varieties analysed as the subplot treatment. The whole plots were considered to be completely randomised in their layout. Not every variety occurred in each whole plot, hence the design was unbalanced and not able to be analysed using ANOVA. Instead a linear mixed model was fitted, and Residual maximum likelihood was used for estimation. This analysis allows varieties to be compared, as well as comparing the effectiveness of the spray treatment and any spray by variety interaction.

Variables analysed were: percentage of fruit numbers with bacterial spot infection, percentage of fruit numbers with russet, russet severity based on individual fruit ratings, average fruit weight, average fruit diameter, average fruit sugar content (Brix) and fruit number per tree. Height was used as a co-variate to analyse percent bacterial spot infection.

The statistical analyses for the 2002/03 and 2003/04 seasons are only valid to compare the targeted copper program treatment with the traditional copper program for each variety separately. Statistical analyses for the 2004/05 and 2005/06 seasons are valid within and between varieties, i.e. targeted and traditional copper treatments can be compared between all varieties.

Results

4.1.3 Targeted Copper Spray Program – mature plums

Pollination and yield were generally poor during the 2004/05 season. The total number of fruit produced by the Friar trees was very low in 2004/05, especially on the trees receiving the targeted copper program. Fruit size was also smaller in 2004/05 for the Friar trees receiving the targeted copper program (data not shown). Friar trees had large crops the previous (2003/04) season, which also may have affected fruit size and yield (Rettke and Dahlenburg 1999).

Assessment of bacterial spot fruit infection

Of the five plum varieties tested, consistently high levels of disease were detected each year in the more susceptible varieties of Eldorado, Friar and Laroda. Levels of disease in the less susceptible varieties Queen Rosa and Black Amber were much lower, as expected, and there were no significant differences between treatments for these two varieties. The targeted copper spray program (Table 4.2) significantly reduced the levels of visible bacterial spot symptoms on fruit for Eldorado, Friar and Laroda in all four seasons of trials, except for Friar in the 2004/05 season (Table 4.4).

Infection levels in Friar were not significant in 2004/05. This was due to a combination of lower disease levels caused by dry weather (Table B.4, Appendix B) and reduced fruit set.

Table 4.4. Percentage of fruit from mature plum trees treated with (a) traditional or (b) targeted copper programs, which showed bacterial spot symptoms in 2002/03, 2003/04, 2004/05 and 2005/06 seasons.

Variety	Treatment	2002/03*^	2003/04*^	2004/05*v	2005/06*v
Eldorado	Traditional	11.8 e	25.9 k	21.4 m	20.0 o
	Targeted	1.7 f	3.0 l	1.6 n	0.1 p
Friar	Traditional	7.6 c	24.6 i	3.7	13.3 o
	Targeted	1.4 d	9.0 j	0.4	2.6 p
Laroda	Traditional	9.7 a	50.3 g	7.8 m	11.5 o
	Targeted	0.4 b	13.6 h	2.7 n	0.7 p
Black Amber	Traditional	1.5	1.7	0.4	0.5
	Targeted	0.3	0.6	0.0	0.1
Queen Rosa	Traditional	0.1	1.0	0.4	0.5
	Targeted	0.1	0.4	0.1	0.1

*Statistical analyses as described in Section 4.1.2.6. Values with the same letter are not significantly different ($p<0.05$). Results in shaded boxes were not significantly different ($p<0.05$) within that variety.
^Statistical analyses for the 2002/03 and 2003/04 seasons are only valid within varieties.
vStatistical analyses for the 2004/05 and 2005/06 seasons are valid within and between varieties.

Assessment of fruit damage

There were no statistically significant differences between the targeted and traditional copper programs with regard to fruit russet. Neither the total number of individual fruit with russet symptoms, nor the severity of russet damage on individual fruit was significantly different between the targeted or traditional copper treatments. The extra sprays used in the targeted program did not therefore significantly increase fruit damage due to russeting.

Significant levels of hail damage were only recorded in the 2003/04 season. There was no statistically significant difference in the levels of hail damage recorded on fruit from either treatment for any variety in 2003/04.

Assessment of fruit quality

There was no significant difference between targeted or traditional copper programs on fruit quality of Black Amber, Eldorado, Laroda or Queen Rosa varieties during the four seasons of trials. There was also no significant difference between the copper program treatments on Friar fruit quality in the 2002/03 season. In subsequent seasons, fruit from Friar trees treated with the targeted copper program had significantly lower weight (2003/04 and 2005/06), diameter (2003/04, 2004/05 and 2005/06) and sugar content (2003/04). The reduced fruit size of Friar in 2003/04 was most likely caused by overcropping. Very high crop loads can affect fruit quality parameters, typically resulting in smaller fruit with reduced flavour (Rettke and Dahlenburg 1999).

4.1.4 Targeted copper spray program – young plums and nectarines

Due to the immaturity of the young plum trees, and very low disease levels in the nectarines, no significant data was obtained from these blocks on the efficacy of the targeted copper program. Data was collected on copper phytotoxicity effects, with no significant differences observed between the targeted and traditional copper programs for any of the fruit quality parameters measured (russet, size, weight or sugar content).

Discussion

The targeted copper spray program significantly reduced bacterial spot fruit disease symptoms on three highly susceptible plum varieties over several seasons of field trials, without significantly increasing fruit russeting or reducing fruit quality (size, weight and sugar content). The use of low concentration copper sprays after rain during the highly susceptible period just prior to and for three weeks following shuck off (Pagani and Ritchie 2002) effectively reduced disease to acceptable levels for commercial production.

It should be remembered however that these results were obtained during relatively dry seasons, with the exception of 2003/04 which was a moderately wet season, so the ability of the program to reduce disease levels in very high spring/summer rainfall seasons was not able to be assessed.

4.2 Using the Targeted Copper Spray Program to reduce bacterial spot infection on commercial orchards - Granite Belt, Queensland

Christine Horlock and Duncan Cameron (DPI&F, Queensland).

This section details a series of experiments undertaken on commercial orchards in the Granite Belt, Queensland. Trials of the targeted copper spray program were undertaken by growers on their own orchards to determine the 'ease of use' of the targeted program and to increase the number of varieties evaluated.

Nine trials were conducted on five commercial orchards within a 20 km radius of each other in the Granite Belt production area of south-east Queensland. Each trial was conducted over a single season. All orchards had a history of consistent bacterial spot infection in previous seasons, used trickle irrigation and were planted on light sandy loam soils.

Aim

To trial the Targeted Copper Spray Program on a wider range of commercially grown stone fruit varieties that are severely affected by bacterial spot.

To determine the 'ease of use' of the Targeted Copper Spray Program from a grower's perspective.

Materials and Methods

4.2.1 General orchard management

Growers used their normal orchard management practices in the experimental blocks, except for copper applications.

4.2.2 Copper applications

Copper was applied in the 2002/03 season by project staff using DPI&F spray equipment; and in the 2004/05 and 2005/06 seasons by the cooperating orchardists using their own spray equipment, following project staff instructions.

All trees received two autumn copper sprays recommended as the industry standard, in 2001 Queensland Orchard Management Guide.

The purpose of the 2004/05 and 2005/06 season trials was to test the efficacy of the targeted copper spray program when applied by growers, using their own commercial spray equipment. As well as applying the copper sprays required during the treatment phase, growers were also asked to rate the ease of use and practicality of the targeted copper spray program.

4.2.3 Commercial orchard trials - 2002/03 Season

Trial location

In 2002/03, trials were conducted on an orchard near Glen Aplin, 10 km to the south of Applethorpe Research Station. Varieties used were Autumn Giant (plum), Zee Lady (peach) and Summer Fire (nectarine).

Trial design

Each trial was conducted within a single row of trees on the eastern edge of individual variety plantings. Tree rows were oriented north-south, and were not covered by hail netting. The Zee Lady peaches bordered onto an internal farm road on the eastern side. All treatments were applied to six replicates, with one tree per replicate and a minimum of two guard trees between datum trees. All plantings were sufficiently separated from one another so as not to require dedicated guard trees between blocks.

Treatments

Trees were treated according to the schedule set out in Table 4.5.

Table 4.5. Treatment application timetable for 2002/03 commercial orchard trial

Variety	Treatment		Copper applications* from Table 4.2.							
			Stage dependent sprays (Steps 1-5)					Rain dependent sprays (Step 6)		
	Spray #		1	2	3	4	5	6	7	8
Autumn Giant (plum)	1	Untreated								
	2	Traditional	KB	KB						
	3	Targeted	KB	KB	KB	KB	KB	KB	KB	
	4	Targeted using LB after rain	KB	KB	KB	KB	KB	LB	LB	
Zee Lady (peach)	1	Untreated								
	2	No BM prior to flowering	KB	KB	KB	KB	KB	LB	LB	LB
	3	BM prior to flowering	BM	BM	BM	KB	KB	LB	LB	LB
Summer Fire (nectarine)	1	Untreated								
	2	Targeted	KB	KB	KB	KB	KB	LB	LB	LB

* Copper applications consisted of: Blank space = no application; KB = Kocide Blue®; LB = Kocide Liquid Blue®; BM = Bordeaux mixture (1 kg hydrated lime: 1 kg copper sulphate: 100 L water).

Leaf and fruit assessment

Trees were monitored for signs of bacterial spot infection on the fruit and leaves from early November 2002.

Final rating of the fruit occurred prior to the harvest of the earliest maturing fruit. The total crop on each tree was counted and a final inspection for bacterial spot infection undertaken. Fifteen fruit were randomly sampled from each replicate (one tree) for fruit quality assessments.

4.2.4 Commercial orchard trials - 2004/05

Trial locations

In 2004/05, four identical trials were conducted on three varieties of stone fruit at two different locations on the Granite Belt, Queensland. May Grand (nectarine) and Summer Fire (nectarine) trees were used on a Cottonvale orchard, while May Grand (peach) and Queen Rosa (plum) trees were used from an orchard near Ballandean.

Trial design

All trial designs consisted of randomised pattern of treatment blocks, usually spread over several rows of a larger planting of the same variety.

May Grand (Cottonvale): This trial was conducted on a block of trees, in the centre of a mixed orchard without hail netting. All datum trees were in one row running north to south, with unsprayed guard rows on either side. There were four replicates of each treatment, and six, seven or eight trees per replicate. Data was collected from two trees in the centre of each replicate.

Summer Fire (Cottonvale): These trees were within a hail-netted block, near the edge of the orchard. Rows ran north to south. Datum trees were in two rows, the second and forth rows from the east, and separated by a guard row. Each row had two replicates of each treatment and five or six trees per replicate. Data was collected from two trees in the centre of each replicate.

May Grand (Ballandean): This block was relatively isolated from other stone fruit, with natural scrub boundaries on all sides. It was not hail-netted and was planted in a wider spaced (4 m x 5 m) layout than the other orchards. Four treatment blocks were present within the orchard; each block consisting of two adjacent rows of three treated trees, with at least one guard row between blocks. Data was collected from the centre tree of each row, i.e. two trees per replicate.

Queen Rosa (Ballandean): This block of trees was not hail-netted and trees were spaced 4 m apart within rows. Experimental trees were in two adjacent rows. Treatment blocks consisted of two rows of three trees, with data collected from the centre tree of each row, as described above for the May Grand trees.

Treatments

All varieties received the same treatments (Table 4.6). Spray applications were made by the grower using their own spray equipment.

Table 4.6. Treatment application timetable for the 2004/05 commercial orchard trials.

Variety	Treatment		Copper applications* from Table 4.2.								
			Stage dependent sprays (Steps 1-5)					Rain dependent sprays (Step 6)			
	Spray #		1	2	3	4	5	6	7	8	9
May Grand (Nectarine)	1	Traditional	KB	KB							
	2	Targeted	KB	KB				LB	LB		
Summer Fire (nectarine)	1	Traditional	KB	KB							
	2	Targeted	KB	KB				LB	LB		
May Grand (Nectarine)	1	Traditional	KB	KB							
	2	Targeted	KB	KB				LB	LB		
Queen Rosa (Plum)	1	Traditional	KB	KB							
	2	Targeted	KB	KB				LB	LB		

* Copper applications consisted of: blank = no application; KB = Kocide Blue®; LB = Kocide Liquid Blue®.

Leaf and fruit assessment

All datum trees were monitored for visual signs of bacterial spot from November until harvest. Phytotoxic effects on leaves and fruit were also monitored.

Fruit rating and sampling was undertaken just before harvest on all but the Queen Rosa trees, where a first pick of mature fruit by the grower had already taken place. Fifteen fruit were randomly selected from 1 m above the ground on each side of the datum trees, and were rated for russet as described for mature plum fruit in the previous experiment. Ten of the most mature fruit from the top half of the datum trees were harvested and tested for fruit quality parameters as described for mature plum fruit in the previous experiment.

4.2.5 Commercial orchard trials - 2005/06

In 2005/06, trials were conducted on Black Amber plum trees near Ballandean, and on selection 109-85 (a highly bacterial spot susceptible plum) trees on an orchard close to Stanthorpe.

Black Amber

The Black Amber orchard had heavy bacterial spot infection in 2004/05. Rows of four-year-old trees ran north to south and no hail-netting was present. The northern ends of the rows were visibly affected by disease, with branch loss and tree death evident. Trees used in the trial were from the southern end of the rows, which were much less

severely affected and had not lost any branches. Four replicates of each treatment, with three trees per replicate were used. Trees were in two blocks, three rows apart. Each replicate was separated by at least two guard trees.

Selection 109-85

This orchard had a strong history of bacterial spot infection and selection 109-85 is highly susceptible to the disease. Two rows that ran north to south were used for this experiment. Selection 109-85 had been top-worked onto the branches of pre-existing/established trees. Treatment blocks consisted of two rows of 3-6 trees, with guard trees in between, and were randomly distributed over 5 rows. Data was collected from a total of 18 trees for each treatment, with trees on the outside edge of the treatment blocks not assessed.

Treatments

The same treatments were applied to both Black Amber and 109-85 trees (Table 4.7).

Table 4.7. Treatment application timetable for the 2005/06 commercial orchard trials.

Variety	Treatment		Copper applications* from Table 4.2.						
			Stage dependent sprays (Steps 1-5)					Stage dependent sprays (Steps 1-5)	
	Spray #		1	2	3	4	5	6	7
Black Amber (plum)	1	Traditional	BM	BM			BM		
	2	Targeted	BM	BM			BM	LB	LB
109-85 (plum)	1	Traditional	KB	KB			KB		
	2	Targeted	KB	KB			KB	LB	LB

* Copper applications consisted of: blank = no application; KB = Kocide Blue®; LB = Kocide Liquid Blue®; BM – Bordeaux Mixture (1 kg CuSO4:1 kg Hydrated Lime: 100 l water).

Leaf and fruit assessment

Phytotoxic effects on leaves and fruit were monitored. Due to existing phytotoxic effects a third copper spray was not applied to the targeted treatment trees; despite a rainfall event occurring on 28 October (the targeted program is usually continued until 1 November).

Fruit were visually inspected for bacterial spot infection in late November. Fifteen mature Black Amber fruit were sampled from the top half of the trees for fruit quality analysis. The trees had been picked by the grower once before sampling. Fruit quality analysis was performed on the harvested fruit as described in the previous section.

The 109-85 trial had to be abandoned because of a hail storm that struck the orchard in mid-November 2005.

Results

4.2.6 Commercial orchard trials – bacterial spot fruit infection

There were no significant levels of bacterial spot fruit symptoms detected on the commercial orchard trials undertaken 2004/05 (data not shown). In the 2005/06 season, some disease was recorded on the Black Amber trial. There was no significant difference between the levels of disease recorded on fruit from trees treated with targeted and traditional spray programs. However, disease distribution over the trial site was very uneven, with several severely affected trees significantly altering treatment averages for both the targeted and traditional programs (data not provided).

4.2.7 Commercial orchard trials – russet and fruit quality

Fruit surface damage (russeting) and quality parameters (size, weight and sugar content) were not significantly altered by the application of either the targeted or traditional copper programs in any of the stone fruit varieties used in these trials (data not shown). This indicates that the extra copper sprays applied in the targeted treatment did not have a detectable influence on the fruit quality parameters measured.

4.2.8 Commercial orchard trials – copper phytotoxicity

There were no significant levels of leaf or fruit phytotoxicity caused by the use of copper in any of the commercial orchard trials (data not shown).

4.2.9 Commercial orchard trials – 'Ease of use' evaluation

All of the cooperating orchardists indicated they would continue using the targeted copper spray program as standard management practise for bacterial spot disease in future years.

Discussion

Although no further data on the capacity of the targeted copper spray program to reduce bacterial spot fruit symptoms was gained from these trials, important information on the user friendliness of the program was collected and the number of varieties assessed for copper phytotoxicity increased.

These experiments also acted as informal demonstration blocks for the program with neighbouring growers taking a keen interest in the trials. The opportunity to familiarise growers with the program will result in a more timely and widespread uptake of the program once registration for this use pattern of copper is approved in Queensland.

4.3 Targeted Copper Spray Program – Swan Hill, Victoria

Dr Chin Gouk (Plant pathologist, DPI Victoria)

A targeted copper spray program was previously shown to be effective in reducing bacterial spot incidence on plums in trials conducted in Queensland. Additional trials were conducted in Victoria to gain efficacy and phytotoxicity data on the effects of the targeted copper spray program on other stone fruit cultivars.

Aim

Trials were conducted in two Swan Hill orchards during the 2003/4 and 2004/05 seasons to evaluate the efficacy of dilute copper sprays applied after blossom for control of bacterial spot.

Materials and methods

Trials were conducted on Artic Pride nectarines in the 2003/04 season and on Ryan Sun peaches in the 2004/05 season. In each trial the targeted copper spray program was compared against a water (untreated control) treatment. It should be noted that a water treatment was used as a control in these trials as copper is currently not registered for use against bacterial spot in Victoria.

The treatments were applied to single tree plots, replicated six times in a randomised block design. In the 2003/04 season, copper sprays were applied where possible 24-48 hours before an anticipated rain event of more than 5 mm of rainfall. In the 2004/05 season, copper sprays were applied within 48 hours following a rain event with more than 5 mm of rainfall. The post-rain spray timing was found to be more manageable. For both seasons, spray applications ceased after 30 October.

The timing of the targeted copper spray schedule and the rates of copper used are included in Table 4.8.

Table 4.8: Rates and timing of copper applications for Victorian spray trials 2003/04 and 2004/05

Spray timing	Copper spray rates
Early budswell	BM
7-10 days after early budswell	BM
5% petal-fall	BM
75 to 95% petal-fall to 1% shuck split	KB 64g / 100L
75% shuck-split to 1% shuck-off	KB 43g / 100L
24-48 hours before or up to 48 hours after each predicted rain event from 1% shuck off until 30 October each year.	LB 22 ml / 100L

*Copper applications consisted of: KB = Kocide Blue®; LB = Kocide Liquid Blue®; BM = Bordeaux mixture (1 kg hydrated lime: 1 kg copper sulphate: 100 L water).

4.3.1 Assessment of Disease and Phytotoxicity

In the 2003/04 season, the incidence of bacterial spot on tagged branch sections up to head height on trees in each plot was assessed on 13 October, 20 November, 4 December, 19 December, 16 January and at harvest on 19 February. In the 2004/05 season, a preliminary assessment of bacterial spot symptoms was conducted in November (using a subset of the trial trees), followed by assessments in December and at harvest in February. The incidence of bacterial spot and russet on the fruit were recorded on all three occasions. The presence of phytotoxicity on the leaves was also noted.

4.3.2 Harvest Assessment

The total number of fruit, fruit weight and number of fruit with bacterial spot was recorded from each plot at harvest. The number of fruit with russet was also recorded. Isolations were conducted on fruit with bacterial spot lesions to confirm the presence of the disease.

The effect of copper sprays on fruit quality and maturity was assessed by measuring the level of soluble solids and fruit firmness. The diameter of 12 fruit taken at random from each plot was measured using a vernier calliper. Brix (sugar content) levels of seven fruit, taken randomly from each plot, were measured by extracting the juice from three pieces of fruit which were sliced from around the circumference of each fruit. Fruit firmness was measured by taking two penetrometer readings on each of seven fruit taken randomly from each tree.

Results

4.3.3 Targeted Copper Treatments on Nectarines, 2003/04 season

Over one thousand Artic Pride nectarine fruit per treatment were assessed shortly after fruit set in October 2003. By November, the fruit numbers for both treatments had decreased by 70–80% from those at fruit set. There was little difference in the total fruit numbers between the unsprayed and copper treatments at each assessment date (Figure 4.3). Frost and storm events occurring after fruit set (Figures C.1-C.4, Appendix C) were the cause of large reduction in fruit numbers in the plots.

The incidence of bacterial spot in the 2003/04 season was very low in the trial block. Only 1.1% of nectarines were affected at the beginning of the season, and 1.8% at harvest. There were no differences in the levels of bacterial spot between the water (untreated) and copper treatments (Figure 4.4).

Between October and harvest, the background level of russet on untreated fruit varied from 19-28%, whilst the level of russet on the treated fruit in this period was 1.7-2.6 times higher (Figure 4.5). At harvest, the levels of russet were both high at 56.4% and 66.1% respectively for the untreated and treated plots (Figure 4.5). It should be noted from Figure 4.5 that on the 13 October russet was recorded on treated fruit.

Phytotoxic symptoms were observed on the newly emerging leaves in October. Leaves developed reddish-brown spots, some of which coalesced along the edges and tips. Some leaves had a reddish purple tinge and were slightly curled. Some of the necrotic tissue within the spots dropped out resulting in a 'shot-hole' appearance. Leaves that emerged after the cessation of copper sprays did not appear to develop symptoms of

phytotoxicity. Phytotoxicity became less noticeable with further development of the leaf canopy later in the summer.

The fruit quality data for the harvested nectarines are presented in Table 4.9. There was little difference in the mean fruit diameter, brix level and fruit firmness between treatments.

4.3.4 Targeted Copper Treatment on Peaches, 2004-05 Season

A preliminary assessment in November 2004 (using a subset of the trial trees) showed that there were fewer fruit on peach trees treated with the targeted copper schedule (Figure 4.6). The difference in fruit numbers between treatments became more noticeable by December 2004, with the copper treatment resulting in 40% less fruit than the untreated (water) treatment.

The incidence of bacterial spot was very low for both treatments, with less than 1% of fruit affected (Figure 4.7).

In November, the incidence of russet on fruit from trees receiving the targeted copper treatment was nearly three times that of the water treatment (Figure 4.8). The level of russet on the copper treated fruit increased to 8% in December, which was nearly double that of the water treatment. Even greater levels of russet were recorded on fruit harvested in February for both copper and water treatments.

Symptoms of phytotoxicity similar to those described for the copper treatment on the nectarine trees were observed on the peach trees sprayed with copper post blossom.

A storm with high winds, cold temperatures and rain (Figures C.5-C.8, Appendix C) that occurred just prior to harvest, and resulted in loss of fruit across the Swan Hill district. Fruit numbers and yield in the trial trees were also affected. Bruising of fruit across both treatments in the trial resulted in low brix levels and low fruit firmness (Table 4.10).

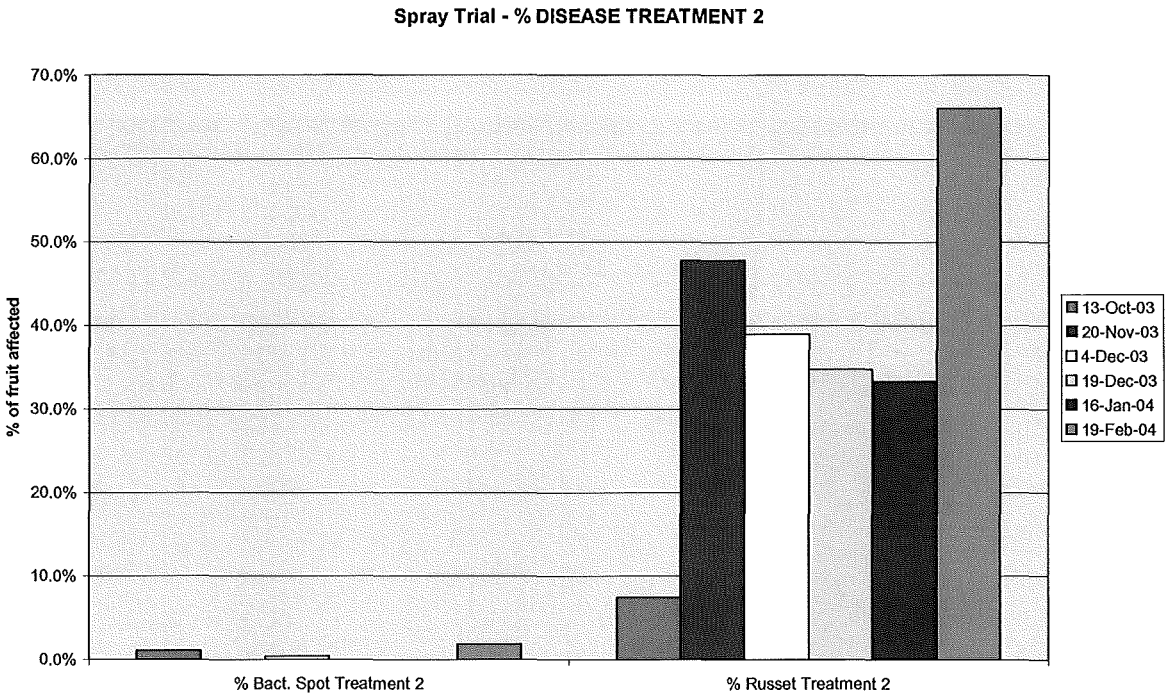


Figure 4.3. The total number of Artic Pride nectarine fruit assessed from the water and targeted copper spray treatments in a Swan Hill orchard during October 2003 to February 2004.

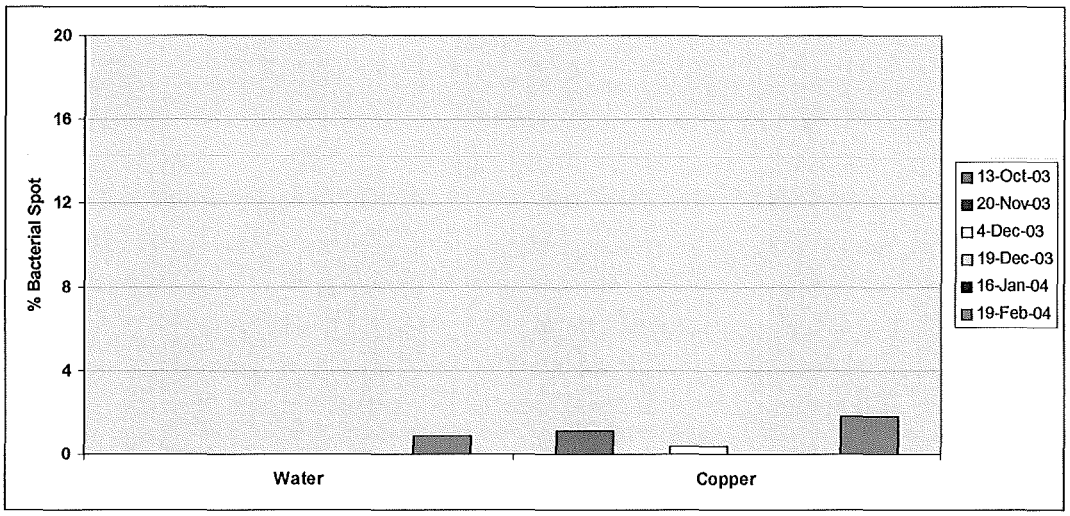


Figure 4.4. The percentage incidence of bacterial spot on Artic Pride nectarine fruit from the water and targeted copper spray treatments, Swan Hill, 2003/04 season.

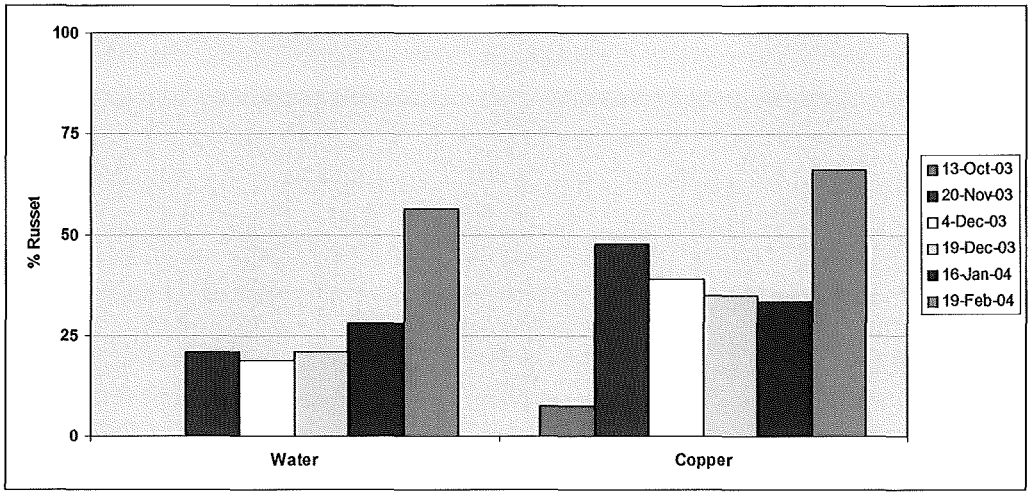


Figure 4.5. The percentage incidence of russet on Artic Pride nectarine fruit from the water and targeted copper spray treatments, Swan Hill, 2003/04 season.

Table 4.9. Artic Pride nectarine fruit quality parameters from water and targeted copper treatments, February 2004.

Fruit quality parameters	Water treatment	Copper treatment
Mean fruit diameter (mm)	67.3	65.6
Mean Brix (%)	16.2	18.1
Mean penetrometer reading (kg)	8.8	7.6

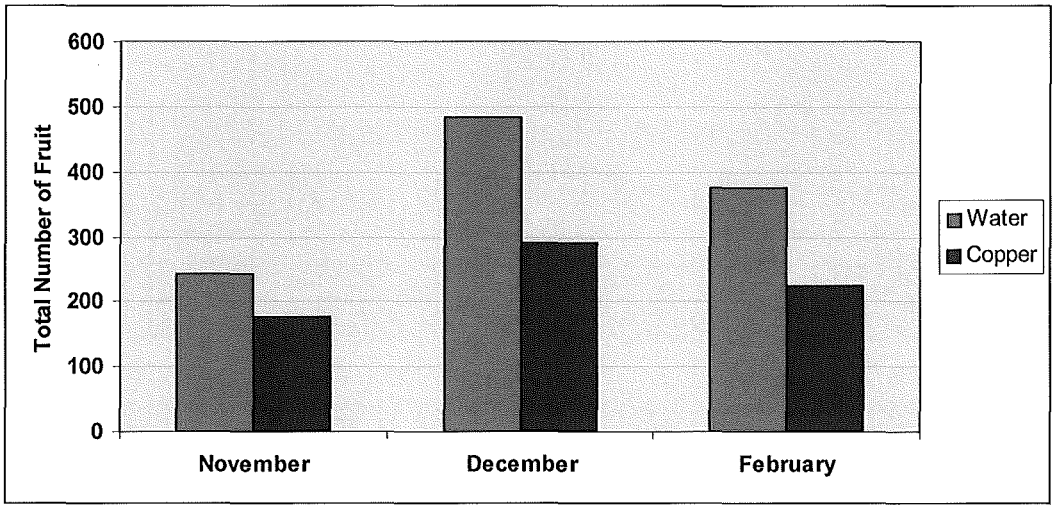


Figure 4.6. The total number of Ryan Sun peach fruit assessed from the water and targeted copper spray treatments in a Swan Hill orchard, October 2004 to February 2005.

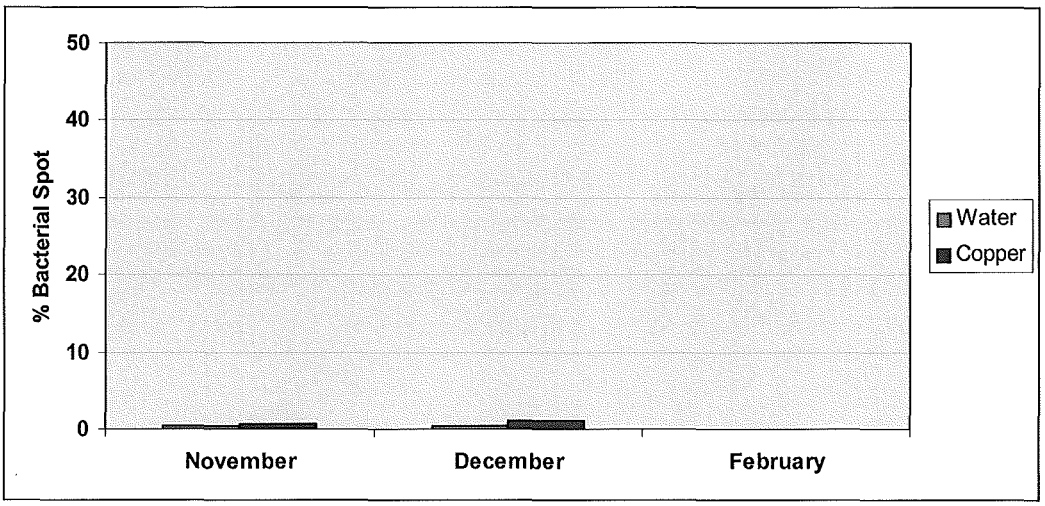


Figure 4.7. The incidence of bacterial spot (% of fruit numbers) on Ryan Sun peach fruit from the water and targeted copper spray treatments, Swan Hill, 2004/05 season.

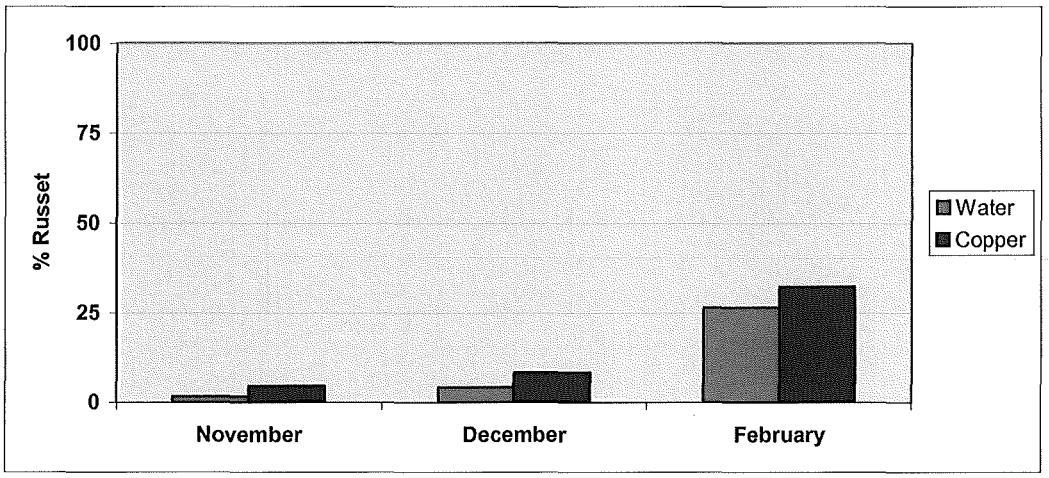


Figure 4.8. The incidence of russet (% of fruit numbers) on Ryan Sun peach fruit from the water and targeted copper spray treatments, Swan Hill, 2004/05 season.

Table 4.10. Ryan Sun peach fruit quality parameters from the water and targeted copper treatments, February 2005.

Fruit quality parameters	Water treatment	Copper treatment
Mean fruit diameter (mm)	65.5	68.7
Mean Brix (%)	11.9	11.6
Mean penetrometer reading (kg)	1.2	1.3

Discussion

4.3.5 Targeted Copper Treatments on Nectarines, 2003/04

Swan Hill orchards experienced drought conditions during both the 2003/04 and 2004/05 seasons. Weather conditions were not conducive to development of bacterial spot; hence a low incidence of disease was recorded. The onset of frost and storm events after fruit set in 2003 resulted in loss of fruit from monitored trees. The level of fruit russet at harvest on both the untreated (water) and copper treatments was high, with a sharp increase in the level of russet between January and February. It is not known what factors contributed to the increased levels of russet on fruit close to harvest, but further trials are recommended to see if copper is causal factor.

4.3.6 Targeted Copper Treatment on Peaches, 2004/05

The extended copper spray schedule resulted in lower fruit numbers and a higher level of russet than in the untreated (water) control. There was little difference in the quality of fruit (fruit size, soluble solids and firmness), between the treatments. However, the storm prior to harvest had most likely resulted in the increased fruit softness, and reduced levels of soluble solids in the fruit.

As in the 2003/04 season, the dry conditions in the following season (2004/05) were not conducive to the development of bacterial spot. In the three months following 9 September (considered to be critical period for bacterial spot infection and symptom development), only four days had more than 5 mm rainfall. Only two of these days had over 10 mm of rainfall. In essence, these seasons were not conducive to disease development.

The loss of fruit and the bruising of remaining fruit on the tree, most likely due to the storm near harvest, has precluded meaningful comparison of fruit numbers and weights at harvest.

Conclusion

The data collected highlighted the importance of conducting evaluations of any new copper spray schedules over multiple seasons, on a range of different stone fruit cultivars and under different climatic conditions. The lack of bacterial spot infection due to drought conditions in both seasons has precluded meaningful comparison of the efficacy of the targeted copper spray program with the (water) control treatment for disease control. Despite reducing copper concentration to a low level in the sprays, russet occurred on fruit and phytotoxic symptoms were evident on leaves. In both trials, fruit size, soluble solids and firmness were unaffected by the extended copper treatments.

4.4 Management of bacterial spot using cultural methods

Bacterial spot of stonefruit, caused by *Xap*, causes significant economic losses in Australia. Chemical control has been limited, consisting of judicious use of copper products. This can lead to hypersensitive reactions, including russeting of fruit skin and localised leaf burns that reduce leaf area and induce defoliation in severe cases. Prolonged use of copper can have negative environmental impacts, especially on soils. Many nations are considering banning copper products for this reason.

Aim

To examine two cultural methods of disease control:

- the addition of hydrophilic polymers (water crystals) to the soil at the manufacturers recommended rate (equivalent to 8% bentonite)
- the use of a stonefruit variety less susceptible to bacterial spot as an inter-stem between susceptible scion and rootstock.

Materials and Methods

Treatments

In August 2003, cuttings from the plum varieties Friar and Eldorado were grafted onto Golden Queen peach rootstocks. Friar was also grafted onto Segundo plum inter-stems on Golden Queen rootstocks. Bare-rooted plants were established in potting mix and then re-potted into Granite Belt soil (a granitic sand with less than 2% organic matter), Granite Belt soil plus water crystals, or Granite Belt soil plus 8.67% bentonite (same water holding capacity as the water crystals). The top graft of some of the inter-scion trees died, and in these situations, the Segundo inter-stem was retained and trained to become the scion.

Inoculation

Xap cells were grown overnight (18 h) in nutrient broth, centrifuged then re-suspended in sterile distilled water to a concentration of 3.8×10^8 culturable bacteria /ml.

Potted trees were watered to field capacity. Shoot tip growth and vigour were rated as nil, low or high, and the trees sprayed to the point of run-off with the *Xap* suspension. Trees were maintained in a humidity tent at 22-29°C with 100% relative humidity for ca. 30 h, after which they were removed from the humidity tent and kept at 22-29°C and ambient humidity in a glasshouse. After three and eight weeks, growing leaves and stems were rated for the presence or absence of bacterial spot symptoms.

Results

All trees of the susceptible varieties developed leaf lesions and green stem cankers within 20 days of inoculation. A high level of infection was observed in the susceptible varieties Eldorado and Friar (Figure 4.9), while the resistant Segundo variety showed little sign of infection. The severity of symptoms present at the three week rating increased at the eight week rating, but no new points of infection were observed.

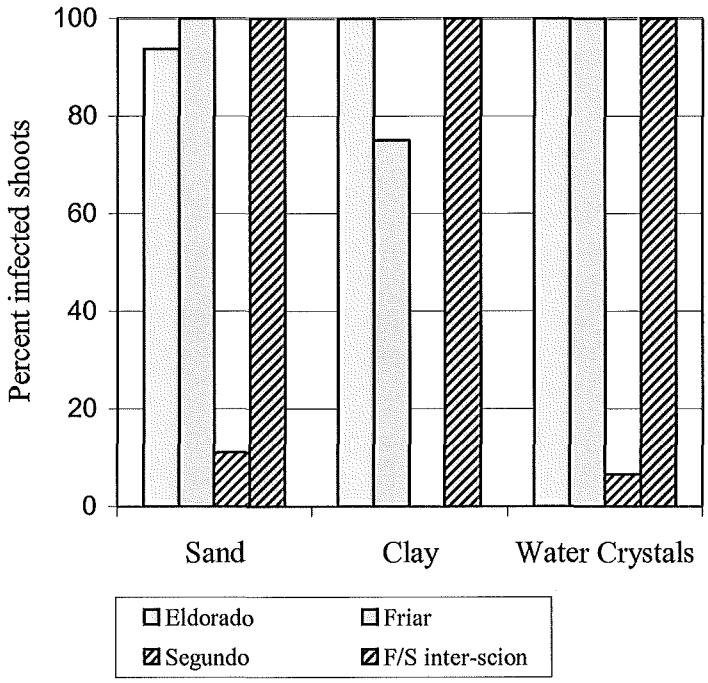


Figure 4.9. Effect of soil amendments and a *Xap*-resistant inter-scion on symptom development in bacterial spot susceptible plum varieties.

Discussion

Soil type can influence susceptibility of trees to bacterial spot (Zehr, Shepard *et al.* 1996). Stonefruit trees grown in soils with low clay content have been reported to be more severely affected by bacterial spot than trees grown in soils with higher clay content (Peter Stephens, personal communication).

Sunhigh peach trees grown in sandy soils had a lower water diffusion pressure deficit at field capacity than trees grown in heavier soils (Zehr, Shepard *et al.* 1996). This was linked to higher susceptibility to bacterial spot.

Our experiment showed no evidence of soil clay content influencing bacterial spot infection. Increasing soil water holding capacity and reducing water stress did not significantly reduce susceptibility to bacterial spot. Similarly, the bacterial spot resistant Segundo variety used as an inter-stem did not significantly reduce bacterial spot symptoms on the susceptible scions. Two main factors may have contributed to this result: (i) a very high infection pressure, with even Segundo (a resistant variety) developing some symptoms; and (ii) the susceptibility of the Eldorado and Friar varieties was simply too high to be overcome by either reduced water stress or inter-stem interactions. Finally, the influence of soil clay content on bacterial spot symptom severity may not be the result of interactions with soil water holding capacity, but clay's interaction with other unknown physical properties of the soil.

Conclusions

Bacterial spot remains the most serious bacterial disease of stone fruit in high spring/summer rainfall areas in Australia. Although this project was undertaken during one of the lowest rainfall periods of Australian history, some significant progress in the understanding and management of bacterial spot disease in Australia was made. This progress occurred in three main areas:

- A **molecular diagnostic test for the specific detection and identification of *Xap*** from diseased stone fruit tissues has been developed and is ready for use in further studies.

- **Important disease cycle information for Queensland orchards** has been determined:
    - The locations of overwintering sites for *Xap* are infected mummified fruit and leaves.
    - Epiphytic survival of *Xap* was demonstrated on leaves, fruit and twigs.
    - The developmental stage at which *Xap* infection caused the most severe bacterial spot symptoms on fruit was confirmed to be immediately prior to three weeks after shuck off. In Queensland, this is generally late September to the end of October.
    - Wet weather and the growing of highly susceptible varieties are the most important factors increasing the levels of bacterial spot development in Queensland orchards.

- **Effective management of bacterial spot symptoms on plum fruit** was achieved through the application of the targeted copper spray program. This program, as described in Section 4.1, significantly reduces the percentage of bacterial spot affected fruit on highly susceptible plum varieties, without significantly increasing russet or reducing fruit quality (fruit diameter, weight or sugar content).

Technology Transfer

Grower meetings

- September 2004. Dr Chin Gouk, DPI Victoria, presented a seminar to Summerfruit growers at Swan Hill, 'Bacterial Spot of Stonefruit', coordinated by Steven Lorimer, FruitCheque, DPI, Victoria.

- August 2005. Dr Chin Gouk, DPI Victoria, presented a seminar to Summerfruit growers at Swan Hill, 'Control of Bacterial Spot of in Stonefruit Orchards', coordinated by Steven Lorimer, FruitCheque, DPI, Victoria.

- October 2005. 'Spot, Rot and Scab'. Prof David Ritchie, University of North Carolina, presented seminars on control of bacterial spot and brown rot of stonefruit. Dr Chin Gouk presented an update on the Bacterial Spot project. The three seminars were held at Swan Hill, Tatura and Cobram, in coordination with C. Mansfield, S. Lorimer, H. Schneider, FruitCheque, DPI, Victoria.

- October 6, 2005. Prof David Ritchie, University of North Carolina, presented details of his work on bacterial spot of peach in the USA, Christine Horlock and Duncan Cameron presented information on research into bacterial spot of plum in Australia, including details of the targeted copper spray program.

- June 20, 2006. A combined meeting with Dr Shane Hetherington to launch the IPDM manual for Australian Summerfruit. Christine Horlock presented information on management of bacterial spot in stone fruit using the targeted copper spray program.

Industry journal articles

Lorimer, S. (2003). What's Hot in Bacterial Spot. Swan Hill Summerfruit. October 2003.

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Other Industry Publications

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Horlock, C.M. (2006). Control of bacterial spot in stone fruit orchards. HAL Summerfruit Annual Industry report 2005/06, page 1.

Scientific publications

Cameron, D.A. and C.M. Horlock. (2005). The use of hydrophylic polymers or an inter-scion to reduce susceptibility to bacterial spot in plums. *The 15th Biennial Australasian Plant Pathology Society Conference Proceedings*, 100.

Recommendations – Industry

Bacterial spot of stone fruit – points for growers to remember

- Bacteria are only affected by bactericides. Copper is the only effective bactericide currently registered in Australia.
- The use of Dodine® and Captan® to manage bacterial spot infections is no longer recommended in Australia or the USA. The use of Dodine® during the growing season can result in severe russeting of plum, peach, apricot and nectarine fruit.
- Not all coppers are the same. The active copper is the only portion of copper in a product which will have an effect on bacteria. Whereas, all of the copper in a product will cause fruit and leaf phytotoxicity. Therefore to reduce phytotoxicity, use a copper formulation where the proportion of active copper is very high, relative to the total amount of copper in the product.
- Finally and most importantly do not confuse bacterial spot symptoms with phytotoxicity, see next page.

Management suggestions

General management techniques

- Once it is registered use the targeted copper spray program to reduce bacterial spot infection on plum fruit during the season. Further work is required to determine the phytotoxic effects of copper on nectarines and peaches.
- Remove infected plant tissues from the orchard, especially leaves with lesions and fruit mummies with lesions
- Cut out new branch cankers, if possible with minimal disruption to tree structure, growth and fruiting potential.
- In high spring/summer rainfall areas, apply copper after harvest; especially for very early maturing varieties.

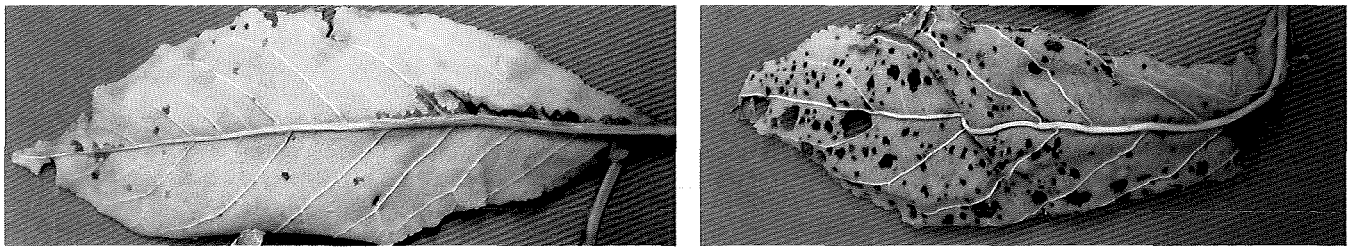
Targeted Copper Spray Program

Copper application timings and rates

Stage of tree/fruit development	Chemical and rate*
1. Early budswell	Kocide Blue Xtra (150g/100L)
2. 7–10 days later	Kocide Blue Xtra (150g/100L)
3. Pink to 10% bloom	Kocide Blue Xtra (150g/100L)
4. Petal fall to 1% shuck split	Kocide Blue Xtra (64g/100L)
5. 75% shuck split to 1% shuck off	Kocide Blue Xtra (43g/100L)
6. Less than 48 hours after rainfall (2–4 applications after 1% shuck off)	Kocide Liquid Blue (22ml/100L)

*It is important to remember that these spray concentrations may be different to those listed on the Kocide Blue and Kocide Liquid Blue labels for stone fruit. So if you are attempting to trial our regime, please check the concentrations carefully, and contact us if you have any questions.

Do not confuse bacterial spot with phytotoxicity



Left: bacterial spot of stone fruit symptoms on a plum leaf. Right: symptoms caused by copper phytotoxicity on a plum leaf.

- Bacterial spot symptoms are angular, and often have a wet or greasy appearance.
- Bacterial spot symptoms often occur along the veins, or on the leaf tip – places where water has collected.
- Formation of new bacterial spot symptoms should slow down or stop after the application of copper.
- Phytotoxicity symptoms are almost always circular, and appear either randomly over the leaf or on the part of the leaf most exposed to copper sprays.
- Phytotoxicity symptoms are cumulative; so that the more you spray the more symptoms will appear.
- Stone fruit varieties vary in their sensitivity to copper. If testing the application of copper during the growing season, caution is warranted. All varieties not previously tested, should be evaluated for copper phytotoxic reactions by applying copper to a few trees or rows; prior to widespread application.

Recommendations – Scientific

Bacterial spot (caused by *Xanthomonas arboricola* pv. *pruni; Xap)* is currently the most devastating bacterial disease of Australian stone fruit. The disease causes significant reductions in marketable fruit (up to 80% in wet seasons), and limb and tree death of high value susceptible varieties.

The aims of future research in bacterial spot of stone fruit in Australia should be to:

1. Deliver the Targeted Copper Spray Program to growers

The targeted copper spray program works well at Applethorpe Research Station, but further testing, and perhaps optimisation, is required to ensure effective use by growers in all areas of Australia. In particular,

- the ability of growers to make orchard wide applications within 48 hours of rainfall is vital to the success of the Program.
- the training of growers to use the program. This includes determining how much rain warrants an application of copper, and the difference between bacterial spot and phytotoxicity symptoms.
- the assessment of a wider range of varieties for phytotoxic reactions.

As with all systems, there is always the potential for improvement, and we have identified some areas where we think there is potential for further refinement of the Program. Namely, could we further reduce the number of the early spring sprays? Should we continue to apply copper after October to later maturing varieties? Can we reduce phytotoxic effects, and maintain control by using even lower concentrations of copper? In short, can the current Program be even better?

2. Evaluate alternatives for disease management

Traditionally, growers have used copper to control bacterial diseases and fungicides to control fungal diseases, but what if there were other products that could increase the natural defence systems of plants to combat a number of diseases at once? These chemicals, broadly termed as plant defence elicitors, work by switching on the plant's natural defence systems before the pathogen arrives. Plant defence elicitors do not act against a pathogen directly, so are not subject to the development of pathogen resistance in the same way as copper and fungicides.

The evaluation of a broad range of these products is warranted, initially by literature searches and seeking expert advice, followed by glasshouse tests and then small plot trials at Applethorpe Research Station. These products are unlikely to replace bactericides or fungicides entirely, but may lead to a reduction in the number of copper and fungicide sprays needed over a season.

The economic value of cultural methods of inoculum reduction also need to be evaluated. We know from previous work presented in this report that a large amount of inoculum can be carried over from one season to the next on infected leaves, twigs and fruit mummies. Now we need to determine whether the cost of removing or reducing these inoculum sources results in increased profits for growers.

3. Undertake further Xap epidemiological and population studies

Current work has identified the location of *Xap* (on the surface of leaves and twigs) within the orchard in early spring, and its subsequent movement into leaves and fruit. It is still not clear how these populations are affected by the application of copper, how long the copper on the leaf/fruit surface remains effective and what is the influence of rainfall. There is some evidence to suggest that high intensity rainfall actually washes bacterial cells off the leaves and fruit, reducing the need for control measures. On his visit to Australia in 2005, Professor David Ritchie suggested that the success of the post-rain applications of copper used in the targeted copper spray program may be due to induction of the plant's defence system, as well as direct effects on bacterial cell numbers. We would like to investigate this area further, by examining the relationship between *Xap* populations and copper on the leaf surface.

Also of interest is the question of what constitutes a rainfall event requiring application of a copper spray? Anecdotally we have evidence to suggest that 5 mm of rain is sufficient, but we have no specific science to back this up. It may be that only rainfall events of 10 mm or more necessitate spraying. This sort of distinction will be very important for growers using the program in a wet spring, with multiple rainfall events during the weeks after shuck off. Conversely, high rainfall events over a short period of time may wash the bacteria off the tree, negating the need for copper application. The role of prolonged overnight leaf wetness, such as heavy dews, also remains unclear..

At this time copper is the only effective chemical for bacterial spot control available for use in Australia. There have been no reports of copper resistance in *Xap* populations anywhere in the world, however copper resistance is present amongst other *Xanthomonas* species, in particular the closely related *Xanthomonas arboricola* pv. *juglandis* (causal agent of walnut blight; Lee, Hendson *et al.* 1994). Copper resistance genes are often found on plasmids, small pieces of extra chromosomal nucleic acid, which can be easily transferred between populations of bacteria. The likelihood of the transfer of copper resistance genes from another *Xanthomonas* species to *Xap* is unclear, but it is possible.

If copper resistance does occur, it is likely to significantly reduce the efficacy of copper against bacterial spot in a very short period of time. Particularly important is the potential for widespread copper resistance. If this occurs growers will find copper sprays to be essentially useless for control and can expect close to 100% losses in some areas during wet seasons. The Australian stone fruit industry needs to assess its level of risk by monitoring for copper resistance in Australian *Xap* populations, and examining *Xap* isolates from around the world for the presence of copper resistance genes.

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- Project development - Sze Flett and David Williams.
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University of Queensland

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Appendix A: Extra data accompanying Section 2 on the development of a *Xap* specific PCR test.

Table A.1 The panel of bacterial isolates used for specificity testing of newly developed primer sets specific to the detection of *Xap* (Section 2.1.4.1).

#	ID#	Strain ID	Origin	Host	Cultivar	Date Collected	Pathovar
1	Xp1	QDPI - JNG 1	QLD, Australia	Plum	Purple Majority	9/9/1999	*pruni*
2	Xp2	QDPI - JNG 2	QLD, Australia	Plum	Red Beaut	22/9/1999	*pruni*
3	Xp3	QDPI - JNG 3	QLD, Australia	Plum	Eldorado	Dec. 2001	*pruni*
4	Xp4	QDPI - JNG 4	QLD, Australia	Plum	Eldorado	Dec. 2001	*pruni*
5	Xp5	QDPI - JNG 5	QLD, Australia	Plum	Laroda	Dec. 2001	*pruni*
6	Xp6	QDPI - JNG 6	QLD, Australia	Plum	Friar		*pruni*
7	Xp7	QDPI - JNG 7	VIC, Australia	Nectarine		14/1/2000	*pruni*
8	Xp8	QDPI - JNG 8	NSW, Australia	Plum	Autumn Giant	27/8/1999	*pruni*
9	Xp9	QDPI - JNG 9	QLD, Australia	Plum	Red Ace	27/9/1999	*pruni*
10	Xp10	QDPI - JNG 10	QLD, Australia	Plum	Red Ace	3/11/1999	*pruni*
11	Xp11	QDPI - JNG 11	WA, Australia	Plum	Pizzaz	20/9	*pruni*
12	Xp12	QDPI - JNG 12	QLD, Australia	Plum	Friar	Dec. 2001	*pruni*
13	Xp13	QDPI - JNG 13	QLD, Australia	Plum	Friar	Dec. 2001	*pruni*
14	Xp14	QDPI - JNG 14	QLD, Australia	Plum	Red Ace	22/9/1999	*pruni*
15	Xp15	QDPI - JNG 15	VIC, Australia	Plum	Betty Anne	27/9/1999	*pruni*
16	Xp16	QDPI - JNG 16	VIC, Australia	Plum	Betty Anne	27/9/1999	*pruni*
17	Xp17	QDPI - JNG 17	QLD, Australia	Plum	Purple Majority		*pruni*
18	Xp18	QDPI - JNG 18	VIC, Australia	Nectarine		14/1/2000	*pruni*
19	Xp19	QDPI - JNG 19	QLD, Australia	Plum	Laroda	12/2001	*pruni*
20	Xp20	QDPI - JNG 20	QLD, Australia	Plum	Red Beaut	22/9/1999	*pruni*
21	Xp22	ICMP 51 a	Mt Albert, New Zealand	Japanese Plum	*Prunus salicina* Lindl.	1/1/1953	*pruni*
22	Xp23	ICMP 59 aQ	United Kingdom				pruni
23	Xp24	ICMP 60 aQ	Argentina	Plum	*Prunus domestica* L.		*pruni*

#	ID#	Strain ID	Origin	Host	Cultivar	Date Collected	Pathovar
24	Xp25	ICMP 62 aQ	USA	Peach	*Prunus persica* (L.) Batsch	1/1/1944	*pruni*
25	Xp26	ICMP 4288 Q	Ontario, Canada	Peach	*Prunus persica* (L.) Batsch	1/1/1972	*pruni*
26	Xp27	ICMP 6677	South Africa	Apricot	*Prunus armeniaca* L.	1/1/1979	*pruni*
27	Xp28	ICMP 7492	Sao Paulo, Brazil	Peach	*Prunus persica* (L.) Batsch	1/1/1981	*pruni*
28	Xp29	QDPI - 16	Australia	Plum	Laroda		*pruni*
29	Xp30	QDPI - 18	Australia	Plum	Elution		*pruni*
30	Xp31	QDPI - 19	Australia	Plum	Eldorado		*pruni*
31	Xp32	QDPI - 20	Australia	Plum	Friar		*pruni*
32	Xp33	B0003	Thulimbah, QLD	Plum		21/11/1968	*pruni*
33	Xp34	B0028	Thulimbah, QLD	Plum		1969	*pruni*
34	Xp35	B0030	The Summit, QLD	Plum		1969	*pruni*
35	Xp36	B0109	Stanthorpe, QLD	Plum		26/1/1971	*pruni*
36	Xp37	B0144	Thulimbah, QLD	Plum		7/12/1971	*pruni*
37	Xp38	B1539	Ballandean, QLD	Plum		21/11/1984	*pruni*
38	Xp39	B1540	Ballandean, QLD	Plum		21/11/1984	*pruni*
39	Xp40	B1541	Ballandean, QLD	Plum		21/11/1984	*pruni*
40	Xp41	B1542	Ballandean, QLD	Plum		21/11/1984	*pruni*
41	Xp42	B1825	Applethorpe, QLD	Plum		8/1/1986	*pruni*
42	Xp45	CFBP 5565	France	Peach	*Prunus persica*	1998	*pruni*
43	Xp48	CFBP 5577	France	Peach	*Prunus persica*	1996	*pruni*
44	Xp49	CFBP 5580	France	Korean Cherry	*Prunus japonica*	2000	*pruni*
45	Xc 1	ACM 2135	VIC	Hazelnut	*Corylus avellana* L.	1980	*corylina*
46	Xc 2	ICMP 449 Q	Oregon, USA	Hazelnut	*Corylus avellana* L.		*corylina*
47	Xc 3	ICMP 5726 aQ	USA	Filbert	*Corylus maxima*	1/1/1939	*corylina*
48	Xc 4	ICMP 7081 a	United Kingdom	Hazelnut	*Corylus avellana* L.	1/1/1978	*corylina*
49	Xc 5	ICMP 11956	France	Hazelnut	*Corylus avellana* L.	1/1/1985	*corylina*

#	ID#	Strain ID	Origin	Host	Cultivar	Date Collected	Pathovar
50	Xj 2	ICMP 34 aQ	England, United Kindom	Walnut	*Juglans regia* L.	1/1/1955	*juglandis*
51	Xj 3	ICMP 35 a	Mt Albert, New Zealand	Walnut	*Juglans regia* L.	1/1/1956	*juglandis*
52	Xj 4	ICMP 10865	Valencia, Spain	Walnut	*Juglans regia* L.	1/1/1988	*juglandis*
53	Xj 5	ICMP 11829	France	Walnut	*Juglans regia* L.	1/1/1967	*juglandis*
54	Xj 6	ICMP 11955	Italy	Walnut	*Juglans regia* L.		*juglandis*
55	Xcel 1	ICMP 1488 a	Auckland, New Zealand	Banana	*Musa acuminata* Colla	1/2/1960	*celebensis*
56	Xpoi 1	ICMP 6274	Mt Albert, New Zealand	Poinsettia	*Euphorbia pulcherrima* Klotzsch	1/6/1972	*poinsettiicola*
57	Xpoi 2	ICMP 7180	Mt Albert, New Zealand	Poinsettia	*Euphorbia pulcherrima* Klotzsch	1/4/1980	*poinsettiicola*
58	Xpop 1	ICMP 8923 a	Roggebotslusis, Netherlands	Euramerican poplars	*Populus x euramericana*	1/8/1979	*populi*
59	Xpop 2	ICMP 9367	New Zealand	Interamerican poplars	*Populus x generosa* A. Henry	1/3/1986	*populi*
60	Xpop 3	ICMP 11965	France	Necklace poplar	*Populus deltoides* Marshall	1/1/1987	*populi*
61	Xpop 4	ICMP 11974	Italy	Euramerican poplars	*Populus x euramericana*	1/1/1989	*populi*
62	XAF	LMG 19145	Cesena, Italy	Strawberry	*Fragaria (x) ananassa*		*fragariae*

Table A.2: BLASTn analysis of clones containing potential Xp22 specific sequences

Clone No.	Insert length (bp)	Position of aligned sequence		No. of bases aligned	NCBI BLASTn Results	Score (bits)	E Value	% Identity
		Begin	End					
7	457	19	454	429	*Xanthomonas campestris* pv. *campestris* str. ATCC 33913, section 335 of 460 of the complete genome (AE012427)	809	0	98
14	576	164	317	140	*Xanthomonas campestris* pv. *campestris* str. ATCC 33913, section 226 of 460 of the complete genome (AE012318)	194	2.E-46	90
15	553				No similarity			
28	540				No similarity			
29	564				No similarity			
34	555	414	535	117	*Xanthomonas campestris* pv. *campestris* str. ATCC 33913, section 25 of 460 of the complete genome (AE012117)	202	9.E-49	95
41	498	18	430	333	*Xanthomonas campestris* pv. *campestris* str. ATCC 33913, section 269 of 460 of the complete genome (AE012361)	184	2.E-43	80
44	611				No similarity			
45	502				No similarity			
47	594				No similarity			

Clone No.	Insert length (bp)	Position of aligned sequence		No. of bases aligned	NCBI BLASTn Results	Score (bits)	E Value	% Identity
		Begin	End					
56	629	19	629	604	*Xanthomonas campestris* pv. *vesicatoria* complete genome (AM039952)	1085	0	98
57	558	19	558	535	*Xanthomonas campestris* pv. *campestris* str. ATCC 33913, section 335 of 460 of the complete genome (AE012427)	1031	0	99
69	597				No similarity			
70	580				No similarity			
73	534	19	464	439	*Xanthomonas campestris* pv. *campestris* str. ATCC 33913, section 335 of 460 of the complete genome (AE012427)	829	0	98
78	534				No similarity			
83	513	24	218	169	*Xanthomonas campestris* pv. *vesicatoria* complete genome (AM039952)	180	3.E-42	86
86	574				No similarity			
96	556	17	555	514	*Xanthomonas campestris* pv. *campestris* str. ATCC 33913, section 337 of 460 of the complete genome (AE012429)	835	0	95
99	579				No similarity			
106	582				No similarity			
115	563	133	504	296	*Xanthomonas campestris* pv. *vesicatoria* complete genome (AM039952)	135	2.E-28	79

Clone No.	Insert length (bp)	Position of aligned sequence		No. of bases aligned	NCBI BLASTn Results	Score (bits)	E Value	% Identity
		Begin	End					
120	573	19	558	551	*Xanthomonas campestris* pv. *campestris* str. ATCC 33913, section 335 of 460 of the complete genome (AE012427)	1068	0	99
150	558				No similarity			
153	611	19	611	590	*Xanthomonas campestris* pv. *campestris* str. ATCC 33913, section 335 of 460 of the complete genome (AE012427)	1152	0	99
158	612	21	612	586	*Xanthomonas campestris* pv. *vesicatoria* complete genome (AM039952)	1104	0	98
160	640	252	575	299	*Xanthomonas oryzae* pv. *oryzae* KACC10331, complete genome (AE013598)	436	4.E-119	92
161	531				No similarity			
162	575	19	575	556	*Xanthomonas oryzae* pv. *oryzae* MAFF 311018 DNA, complete genome (AP008229)	1096	0	99
13f1	494	19	349	321	*Xanthomonas campestris* pv. *campestris* str. ATCC 33913, section 96 of 460 of the complete genome (AE012188)	577	1.E-161	96
2f1	584	24	568	500	*Xanthomonas campestris* pv. *vesicatoria* complete genome (AM039952)	724	0	91

Table A.3: NCBI BLASTx analysis of clones containing potential Xp22 specific sequences

Clone No.	Position of aligned sequence		No of bases aligned	NCBI BLASTx Results	Score (bits)	E Value	% Identity
	Begin	End					
15	88	546	42	Transmembrane hypothetical protein [*Ralstonia solanacearum* UW551] (ZP_00945271)	38.1	0.19	27
28	22	525	53	unnamed protein product [*Photorhabdus luminescens* subsp. *laumondii* TTO1] (CAE12421)	86.7	4.E-16	30
29	215	520	32	Cytochrome-c oxidase [Mycobacterium sp. MCS] (YP_639017.1)	36.6	0.58	29
34	21	521	91	DNA methylase N-4/N-6 [*Pelodictyon phaeoclathratiforme* BU-1] (ZP_00589971)	189	7.E-47	54
44	521	592	26	manganese superoxide dismutase [*Taiwanofungus camphorata*] (CAD42938)	52.4	1.E-05	92
45				No similarity			
47	152	478	40	single-domain response regulator [*Xanthomonas campestris* pv. *campestris* str. ATCC 33913] (AAM41342)	74.7	2.E-12	36
69	20	595	98	Rhs family protein [*Xanthomonas campestris* pv. *vesicatoria* str. 85-10] (YP_365898)	189	6.E-47	50
70	21	407	52	Putative glycosyltransferase [*Gluconobacter oxydans* 621H] (AAW60373)	102	9.E-21	40
78	26	442	73	hemolysin-type calcium binding protein [*Xylella fastidiosa* 9a5c] (AAF83478)	154	2.E-36	52

Clone No.	Position of aligned sequence		No of bases aligned	NCBI BLASTx Results	Score (bits)	E Value	% Identity
	Begin	End					
86	31	375	39	hydrolase, alpha/beta fold family [*Burkholderia pseudomallei* 1710b] (ABA50220)	38.5	2.E-01	31
99	198	578	84	Avirulence A protein (P11437)	177	3.E-43	66
106	23	370	79	two-component system sensor protein [*Xanthomonas axonopodis* pv. *citri* str. 306] (AAM38811)	173	3.E-42	68
150	22	558	87	Rhs family protein [*Xanthomonas campestris* pv. *vesicatoria* str. 85-10] (YP_365898)	170	3.E-41	48
161	1	174	22	ankyrin-related protein [*Cryptosporidium hominis* TU502] (XP_667229)	34.7	2.E+00	34

Appendix B: Weather Data from Applethorpe Research Station, Granite Belt, Queensland Trial Site for 2002/03, 2003/04, 2004/05 and 2005/06 Seasons

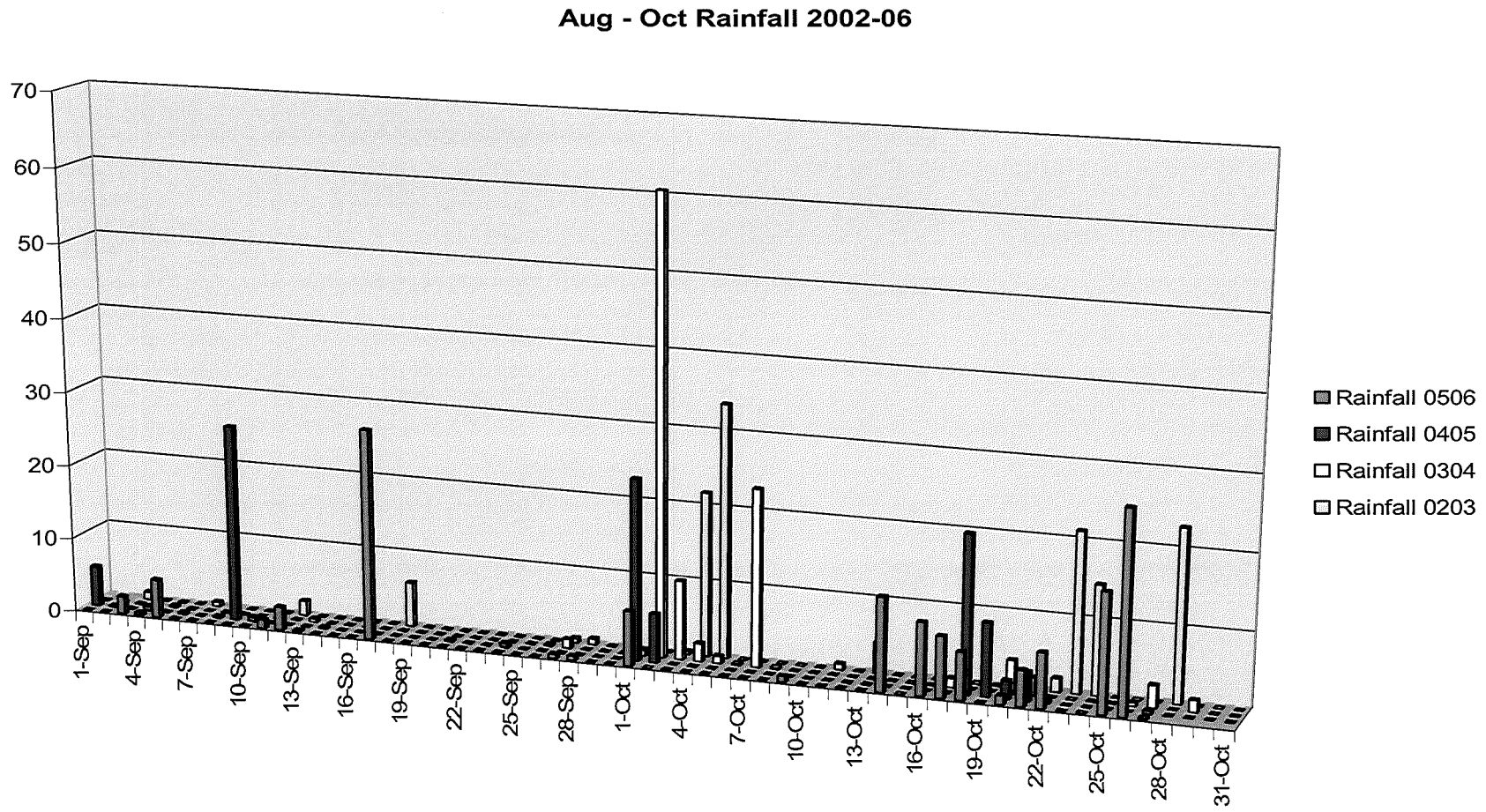

Figure B.1. Daily rainfall totals from the Applethorpe Research Station, Granite Belt, Queensland research orchards for the 2002/03, 2003/04, 2004/05 and 2005/06 stone fruit production seasons.

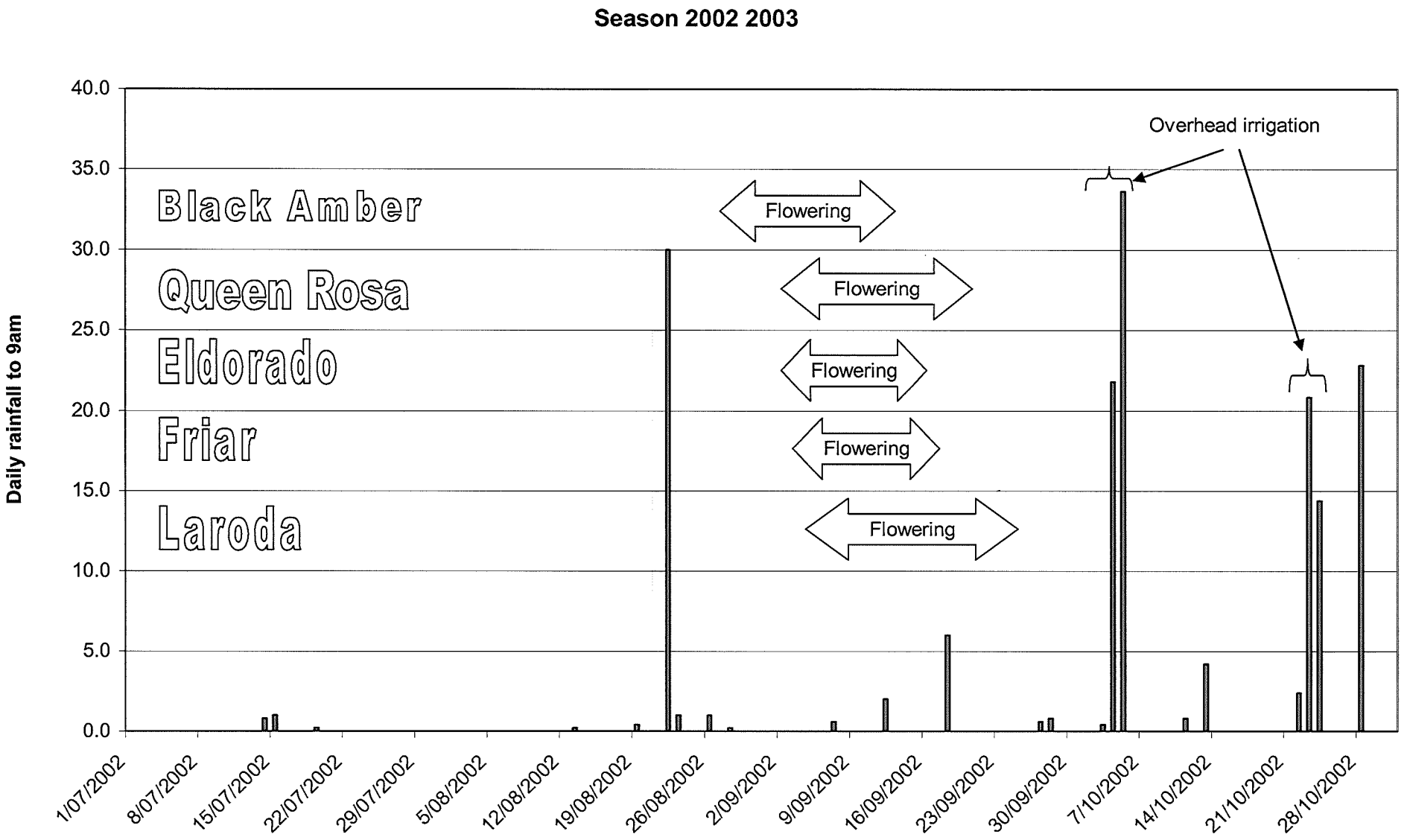

Figure B.2. Daily rainfall total and period of flowering for each plum variety studied at the Applethorpe Research Station, Granite Belt, Queensland research orchards during the 2002/03 stone fruit production season.

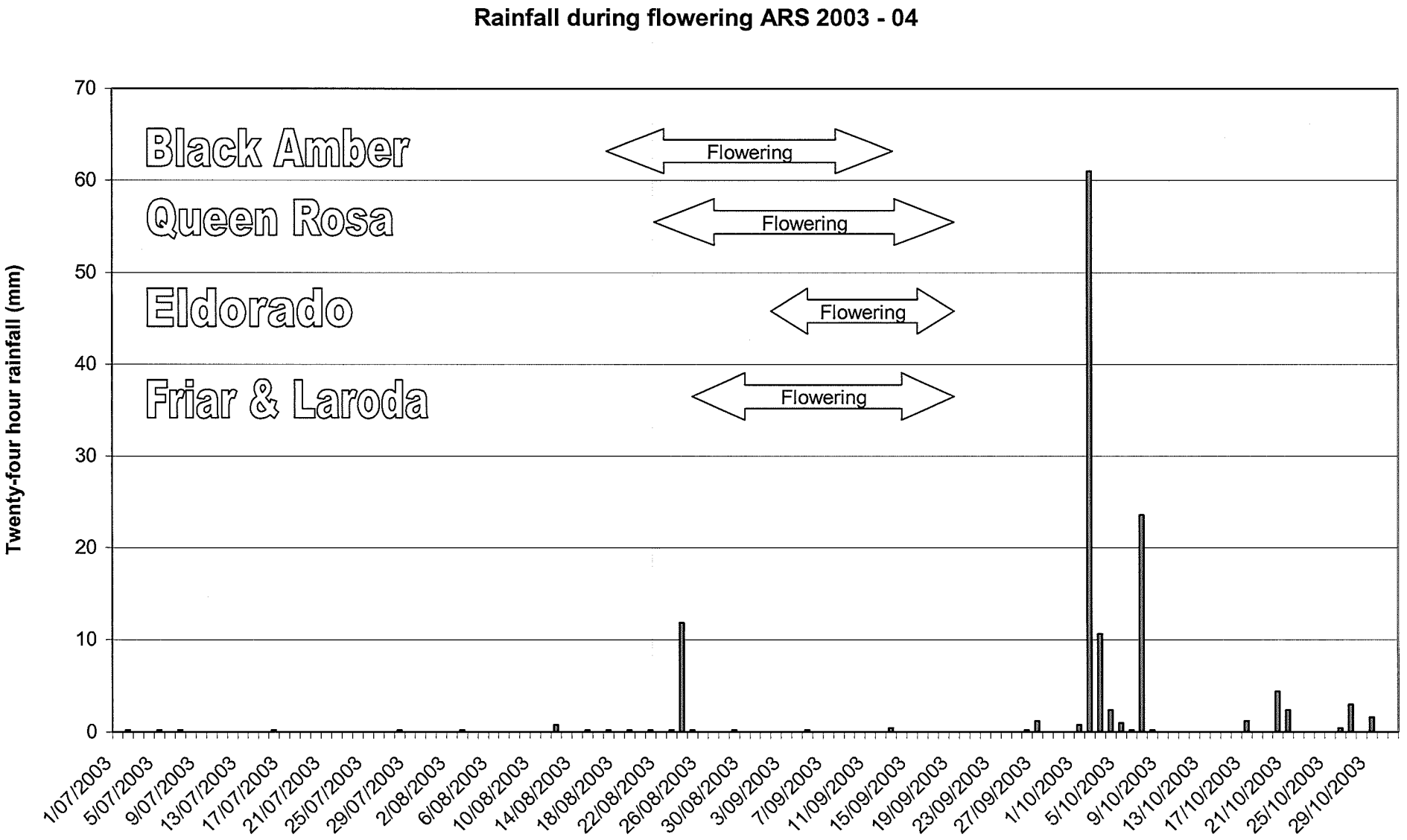

Figure B.3. Daily rainfall total and period of flowering for each plum variety studied at the Applethorpe Research Station, Granite Belt, Queensland research orchards during the 2003/04 stone fruit production season.

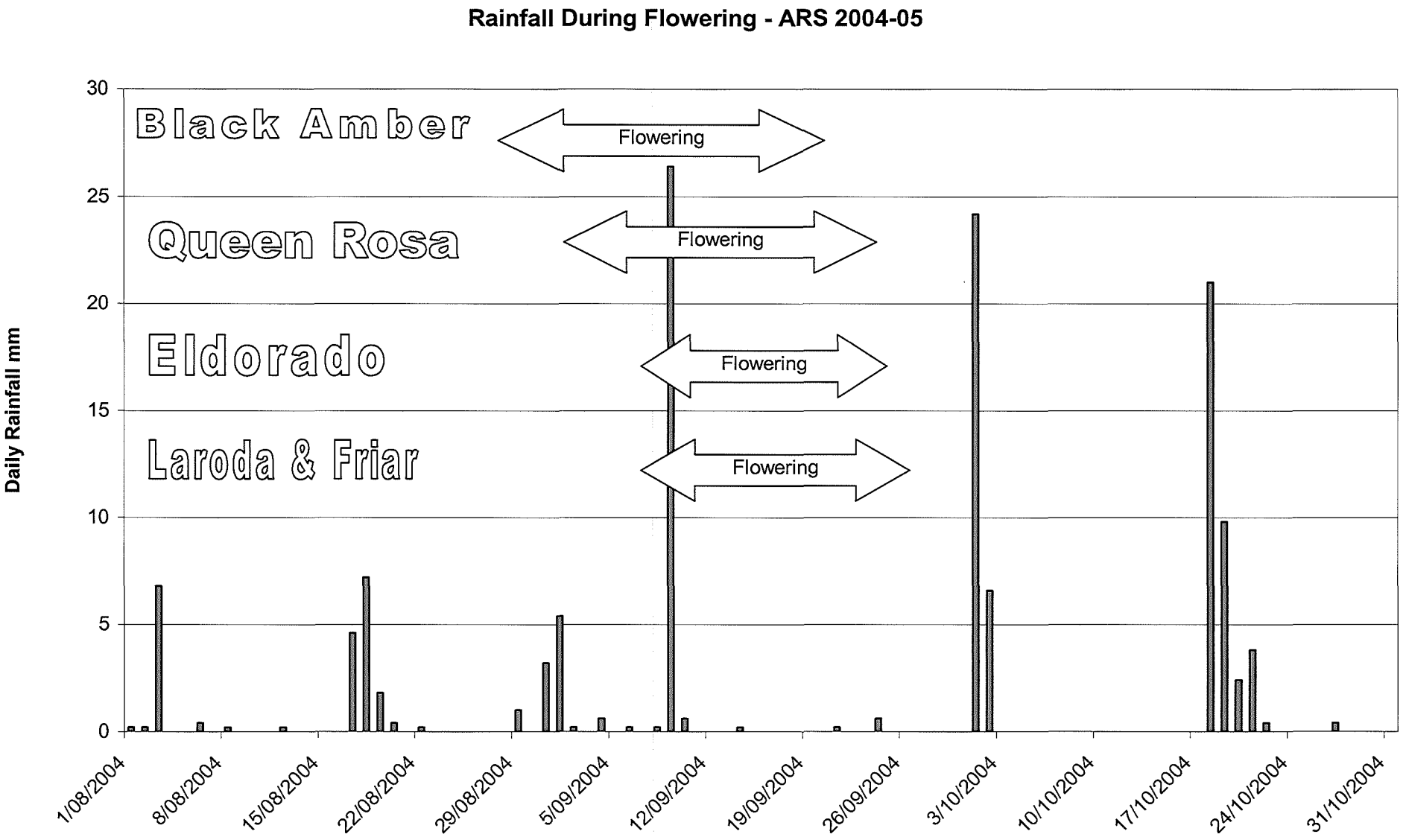

Figure B.4. Daily rainfall total and period of flowering for each plum variety studied at the Applethorpe Research Station, Granite Belt, Queensland research orchards during the 2004/05 stone fruit production season.

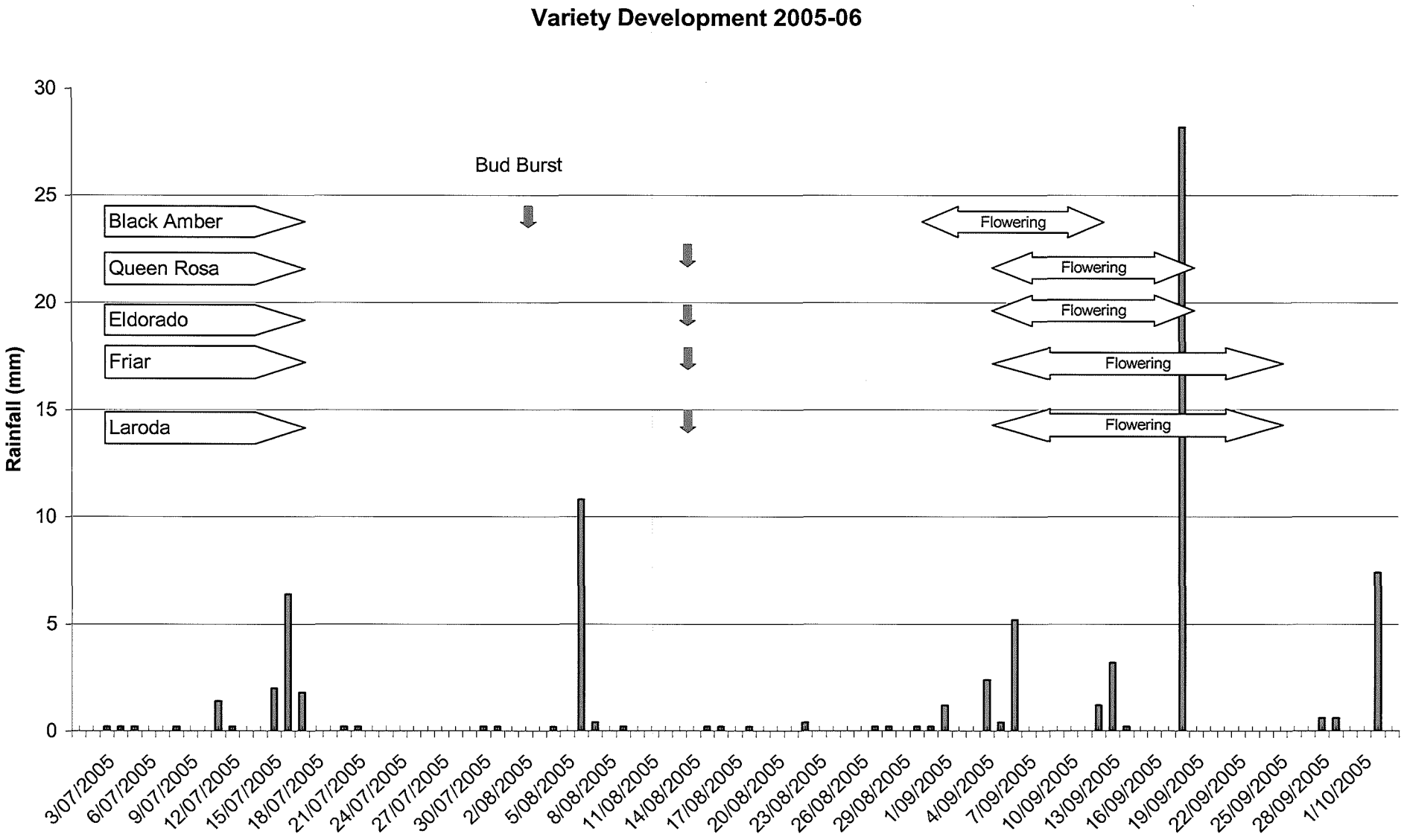

Figure B.5. Daily rainfall total and period of flowering for each plum variety studied at the Applethorpe Research Station, Granite Belt, Queensland research orchards during the 2005/06 stone fruit production season.

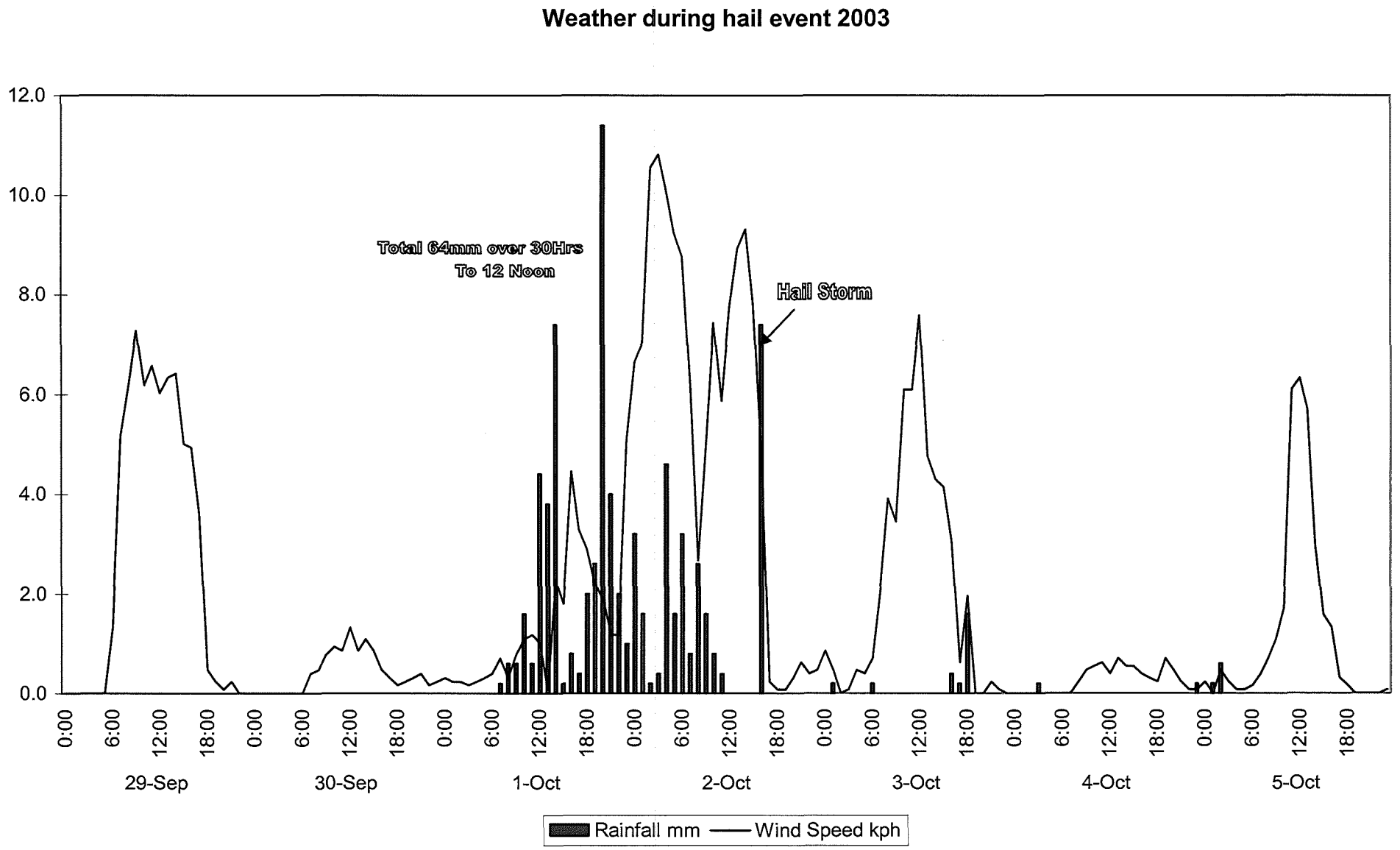

Figure B.6. Daily rainfall total and wind speed at the Applethorpe Research Station, Granite Belt, Queensland research orchards during the 2003 .

Table B.1. Actual daily minimum and maximum temperatures, relative humidity, total daily rainfall, leaf wetness and wind speed for the Applethorpe Research Station trial orchards for the 2003/04 stone fruit season.

Date	Average daily temperature		Relative humidity	Rainfall mm	Leaf Wetness % of day > 90%%	Wind Speed Max kph
	Max °C	Min °C	% of day > 90%			
1/08/03	11.27	-4.99	35.42	0	0.00	10
2/08/03	15.22	-3.32	47.92	0	4.17	6.57
3/08/03	17	-1.37	52.08	0.2	9.38	4.07
4/08/03	16.67	-1.24	62.50	0	13.54	2.19
5/08/03	16.76	1.92	57.29	0	12.50	8.14
6/08/03	17.83	9.91	37.50	0	0.00	13.5
7/08/03	19.86	5.28	4.17	0	0.00	9.39
8/08/03	17.65	6.82	55.21	0	40.63	4.7
9/08/03	15.25	1.47	7.29	0	0.00	5.95
10/08/03	14.7	-3.98	39.58	0	0.00	8.76
11/08/03	17.3	1.92	48.96	0	0.00	2.5
12/08/03	16.34	4.91	55.21	0.8	53.13	6.57
13/08/03	23.35	6.57	64.58	0	29.17	5.32
14/08/03	24.12	12.31	17.71	0	5.21	9.7
15/08/03	13.89	0.27	78.13	0.2	46.88	7.83
16/08/03	15.25	8.16	66.67	0	27.08	1.88
17/08/03	11.2	5.69	97.92	0.2	15.63	2.5
18/08/03	18.01	3.54	60.42	0	51.04	1.57
19/08/03	17.15	-1.33	27.08	0.2	2.08	6.26
20/08/03	17.42	-1.95	48.96	0	22.92	3.76
21/08/03	15.43	0.23	48.96	0.2	29.17	4.7
22/08/03	12.47	1.31	46.88	0	0.00	6.26
23/08/03	19.09	6.46	58.33	0.2	33.33	10.6
24/08/03	19.52	7.88	78.13	11.8	23.96	16
25/08/03	14.05	7.14	31.25	0.2	2.08	9.08
26/08/03	15.4	2.44	7.29	0	0.00	7.2
27/08/03	16.19	0.14	34.38	0	0.00	9.08
28/08/03	13.89	-2.13	36.46	0	4.17	11.3
29/08/03	17.21	-0.03	44.79	0.2	28.13	2.82
30/08/03	18.15	3.31	54.17	0	33.33	9.7
31/08/03	18.42	6.78	16.67	0	0.00	9.7
1/09/03	20.26	0.23	5.21	0	0.00	10.6
2/09/03	14.88	-1.24	38.54	0	16.67	11
3/09/03	13.51	-1.68	18.75	0	0.00	5.01
4/09/03	17.48	1.51	28.13	0	17.71	10
5/09/03	19.55	-1.33	45.83	0.2	37.50	3.13
6/09/03	19.75	-0.46	28.13	0	0.00	8.14
7/09/03	22.3	0.35	21.88	0	0.00	6.89
8/09/03	22.94	8.82	51.04	0	11.46	7.51
9/09/03	18.71	7.07	43.75	0	0.00	4.07
10/09/03	20.58	3.62	39.58	0	33.33	8.76
11/09/03	26.03	7.03	0.00	0	0.00	14.1
12/09/03	24.89	6.78	2.08	0	0.00	10.3
13/09/03	27.11	13.55	0.00	0.4	5.21	11

Date	Average daily temperature		Relative humidity % of day > 90%	Rainfall mm	Leaf Wetness % of day > 90%%	Wind Speed Max kph
	Max °C	Min °C				
14/09/03	23.49	2.8	10.42	0	0.00	9.08
15/09/03	21.82	8.4	0.00	0	0.00	12.5
16/09/03	16.28	6.28	0.00	0	0.00	14.7
17/09/03	15.37	9.02	0.00	0	0.00	14.4
18/09/03	20.04	4.57	8.33	0	0.00	9.7
19/09/03	22.94	2.2	9.38	0	0.00	8.76
20/09/03	24.7	3.42	0.00	0	0.00	7.83
21/09/03	25.41	5.36	51.04	0	38.54	9.39
22/09/03	26.38	9.94	21.88	0	0.00	7.2
23/09/03	28.9	17.15	0.00	0	0.00	8.14
24/09/03	30.99	12.59	0.00	0	0.00	8.76
25/09/03	29.16	14.01	0.00	0	0.00	9.39
26/09/03	30.25	11.23	6.25	0.2	5.21	8.76
27/09/03	27.14	10.61	0.00	1.2	6.25	6.57
28/09/03	25.22	-1.24	0.00	0	0.00	12.2
29/09/03	22.07	0.9	27.08	0	5.21	8.14
30/09/03	23.3	7.46	0.00	0	0.00	8.45
1/10/03	19.75	9.74	58.33	0.8	12.50	1.88
2/10/03	15.22	11.04	100.00	61	96.88	13.8
3/10/03	17.65	8.65	87.50	10.6	73.96	9.7
4/10/03	16.85	5.69	63.54	2.4	63.54	8.76
5/10/03	18.39	8.72	62.50	1	33.33	1.25
6/10/03	18.85	10.67	59.38	0.2	39.58	10.6
7/10/03	16.28	10.34	89.58	23.6	80.21	11.3
8/10/03	18.56	6.17	69.79	0.2	5.21	5.32
9/10/03	21.23	4.42	46.88	0	0.00	4.07
10/10/03	21.48	7.71	44.79	0	0.00	5.32
11/10/03	18.45	0.23	16.67	0	0.00	6.57
12/10/03	16.82	1.06	41.67	0	0.00	7.2
13/10/03	18.88	4.04	41.67	0	0.00	3.44
14/10/03	19.89	7.99	44.79	0	0.00	2.5
15/10/03	20.86	6.78	51.04	0	2.08	1.88
16/10/03	26.89	7.39	42.71	0	3.13	4.07
17/10/03	20.35	10.48	65.63	1.2	56.25	5.01
18/10/03	19.81	9.26	61.46	0	0.00	2.19
19/10/03	22.88	9.97	55.21	0	0.00	2.19
20/10/03	22.8	12.4	52.08	4.4	26.04	8.14
21/10/03	20.78	7.67	63.54	2.4	8.33	5.63
22/10/03	23.88	5.87	25.00	0	0.00	4.07
23/10/03	25.22	7.03	28.13	0	0.00	6.89
24/10/03	26.84	10.31	36.46	0	4.17	5.01
25/10/03	26.68	8.85	53.13	0	34.38	7.2
26/10/03	26.41	12.82	48.96	0.4	43.75	12.5
27/10/03	25.52	7.35	47.92	3	18.75	13.1
28/10/03	23.82	5.25	23.96	0	0.00	5.95
29/10/03	25.3	16.76	20.83	1.6	20.83	11.3
30/10/03	23.71	5.43	6.25	0	0.00	9.7

Date	Average daily temperature		Relative humidity % of day > 90%	Rainfall mm	Leaf Wetness % of day > 90%%	Wind Speed Max kph
	Max °C	Min °C				
31/10/03	20.41	5.65	14.58	0	1.04	6.26
1/11/03	25.03	10.84	0.00	0.4	10.42	7.51
2/11/03	20.69	1.23	11.46	0	0.00	9.7
3/11/03	20.09	3.38	17.71	0	12.50	10.3
4/11/03	21.63	4.31	44.79	0	27.08	2.19
5/11/03	23.63	4	35.42	0	27.08	1.88
6/11/03	26.03	5.17	36.46	0	31.25	5.63
7/11/03	26.54	9.4	38.54	0	30.21	3.13
8/11/03	25.9	11.98	35.42	0	4.17	4.38
9/11/03	22.74	9.2	58.33	0.8	42.71	1.88
10/11/03	22.94	9.74	64.58	3.6	29.17	4.07
11/11/03	23.68	12.37	65.63	16	22.92	5.01
12/11/03	25.3	9.16	48.96	0	3.13	5.01
13/11/03	27.89	13.36	53.13	0	1.04	5.63
14/11/03	18.53	11.69	75.00	0	7.29	1.57
15/11/03	23.79	9.94	46.88	0	32.29	1.25
16/11/03	29.43	7.81	37.50	0	30.21	2.5
17/11/03	29.59	14.45	19.79	0	3.13	5.32
18/11/03	27.94	11.98	62.50	0	40.63	2.5
19/11/03	24.89	15.07	64.58	0	34.38	2.5
20/11/03	28.9	15.98	46.88	0.4	4.17	6.57
21/11/03	29.61	16.28	36.46	0	0.00	7.2
22/11/03	30.2	17.59	41.67	2.4	31.25	7.51
23/11/03	22.35	14.08	88.54	5.2	71.88	6.89
24/11/03	24.86	6.46	13.54	0	0.00	6.26
25/11/03	21.46	3.85	37.50	0	15.63	11.6
26/11/03	23.3	6.82	42.71	0	12.50	4.38
27/11/03	22.66	5.65	36.46	0	21.88	3.44
28/11/03	24.89	7.25	26.04	0	1.04	2.5
29/11/03	25.41	11.17	36.46	0	0.00	3.13
30/11/03	26.65	8.44	38.54	0	31.25	2.5
1/12/03	28.79	10.21	33.33	0	26.04	4.07
2/12/03	30.04	11.4	33.33	0	19.79	4.7
3/12/03	28.47	13.92	38.54	0.6	28.13	3.13
4/12/03	23.71	15.43	70.83	2.6	25.00	5.01
5/12/03	27.62	17.59	62.50	41.6	44.79	6.89
6/12/03	20.55	13.89	100.00	15.8	70.83	5.63
7/12/03	15.13	10.77	95.83	10.8	20.83	4.38
8/12/03	19.03	10.01	56.25	0	5.21	3.44
9/12/03	25.08	15.01	48.96	0.2	0.00	3.44
10/12/03	28.31	15.62	50.00	0	1.04	2.19
11/12/03	29.8	14.97	41.67	0	1.04	2.5
12/12/03	27.73	16.1	28.13	0	6.25	4.7
13/12/03	30.2	15.62	40.63	8	39.58	5.95
14/12/03	30.43	17.59	46.88	0	21.88	4.38
15/12/03	29.11	15.89	73.96	10.2	22.92	4.38
16/12/03	27.46	16.31	64.58	0	3.13	2.5

Date	Average daily temperature		Relative humidity % of day > 90%	Rainfall mm	Leaf Wetness % of day > 90%%	Wind Speed Max kph
	Max °C	Min °C				
17/12/03	23.79	12.4	58.33	0	2.08	1.88
18/12/03	24.81	13.32	40.63	0	1.04	1.25
19/12/03	25.65	9.97	46.88	0	43.75	2.19
20/12/03	29.88	12.21	38.54	0	30.21	3.13
21/12/03	29.83	15.68	6.25	0	0.00	4.38
22/12/03	31.36	20.24	0.00	0	0.00	4.07
23/12/03	34.16	18.82	0.00	0	0.00	5.95
24/12/03	33.54	16.4	41.67	0	0.00	4.07
25/12/03	28.42	18.71	51.04	0	4.17	2.19
26/12/03	32.41	17.77	26.04	0	9.38	3.76
27/12/03	33.85	18.24	38.54	1.6	22.92	7.83
28/12/03	27.32	16.34	58.33	0.4	39.58	3.44
29/12/03	23.24	13.76	64.58	0	16.67	1.57
30/12/03	25.82	12.47	44.79	0	0.00	1.57
31/12/03	27.81	10.17	41.67	0	22.92	0.939
1/01/04	30.7	11.85	40.63	0	38.54	0.939
2/01/04	32.15	14.33	35.42	0	0.00	2.5
3/01/04	29.35	15.13	44.79	0	21.88	1.57
4/01/04	30.57	17.48	48.96	0	12.50	1.57
5/01/04	29.8	18.82	52.08	0	19.79	1.88
6/01/04	33.88	18.42	37.50	0	12.50	2.82
7/01/04	35.24	22.41	0.00	0	0.00	5.63
8/01/04	36.57	19.49	21.88	2.2	13.54	9.39
9/01/04	31.01	15.01	27.08	0	4.17	7.51
10/01/04	29.69	14.54	41.67	0	19.79	2.5
11/01/04	28.95	20.09	75.00	25.4	61.46	5.32
12/01/04	26.92	18.82	72.92	9.8	65.63	1.57
13/01/04	26.87	18.18	73.96	11.6	51.04	1.25
14/01/04	28.79	19.29	50.00	1.8	40.63	4.07
15/01/04	22.66	17.86	97.92	31	80.21	3.13
16/01/04	20.49	17.39	100.00	42.6	54.17	0.626
17/01/04	26.17	18.09	91.67	39.4	62.50	9.39
18/01/04	24.7	14.79	70.83	3	16.67	6.89
19/01/04	30.04	15.86	59.38	0.2	12.50	1.25
20/01/04	21.93	11.69	56.25	0	0.00	1.57
21/01/04	22.35	10.51	52.08	0.2	39.58	0.939
22/01/04	28.26	12.37	48.96	0	33.33	1.57
23/01/04	28.47	14.39	46.88	1.2	47.92	2.82
24/01/04	28.23	14.17	56.25	15.2	44.79	2.82
25/01/04	30.04	16.7	34.38	16	23.96	3.44
26/01/04	29.14	14.94	53.13	20.8	47.92	4.38
27/01/04	30.09	15.28	39.58	0	0.00	4.07
28/01/04	29.85	16.07	45.83	0	28.13	2.5
29/01/04	30.33	17.54	9.38	0.2	2.08	6.89
30/01/04	27.38	15.49	47.92	2.4	41.67	6.57
31/01/04	26.65	10.48	30.21	2.2	5.21	6.57
1/02/04	26.95	9.02	29.17	0	10.42	6.26

Date	Average daily temperature		Relative humidity % of day > 90%	Rainfall mm	Leaf Wetness % of day > 90%%	Wind Speed Max kph
	Max °C	Min °C				
2/02/04	27.43	12.91	33.33	0	3.13	6.57
3/02/04	25.65	12.75	71.88	45	41.67	3.13
4/02/04	19.66	12.5	67.71	0.6	10.42	0.939
5/02/04	24.23	10.84	47.92	0.2	0.00	0.939
6/02/04	25.79	11.56	51.04	0	19.79	0.626
7/02/04	27.08	13.58	53.13	0.2	12.50	0.626
8/02/04	28.34	14.05	50.00	0	17.71	0.626
9/02/04	29.48	13.32	51.04	0	28.13	2.19
10/02/04	29.35	14.85	42.71	0	20.83	2.19
11/02/04	33.38	15.53	50.00	0	36.46	1.57
12/02/04	31.28	18.27	44.79	0	14.58	2.19
13/02/04	32.3	19.43	50.00	0	17.71	3.44
14/02/04	34.19	17.83	73.96	16.4	0.00	4.07
15/02/04	30.86	18.82	68.75	1.8	0.00	1.88
16/02/04	32.01	19.55	54.17	3.6	0.00	4.07
17/02/04	33.25	17.54	65.63	4.2	0.00	1.57
18/02/04	26.41	17.18	59.38	0	0.00	0.939
19/02/04	30.3	18.18	47.92	0	0.00	3.13
20/02/04	31.88	20.69	52.08	0	0.00	4.07
21/02/04	33.56	22.85	1.04	0	0.00	4.38
22/02/04	35.39	20.78	0.00	0	0.00	4.7
23/02/04	34.77	18.85	47.92	0.2	0.00	3.76
24/02/04	31.8	18.24	66.67	6.4	0.00	4.38
25/02/04	22.02	17.83	89.58	0.4	0.00	1.88
26/02/04	24.51	11.53	72.92	0.2	0.00	3.76
27/02/04	25.44	15.43	52.08	0	0.00	3.13
28/02/04	27.32	14.01	52.08	0	0.00	1.88

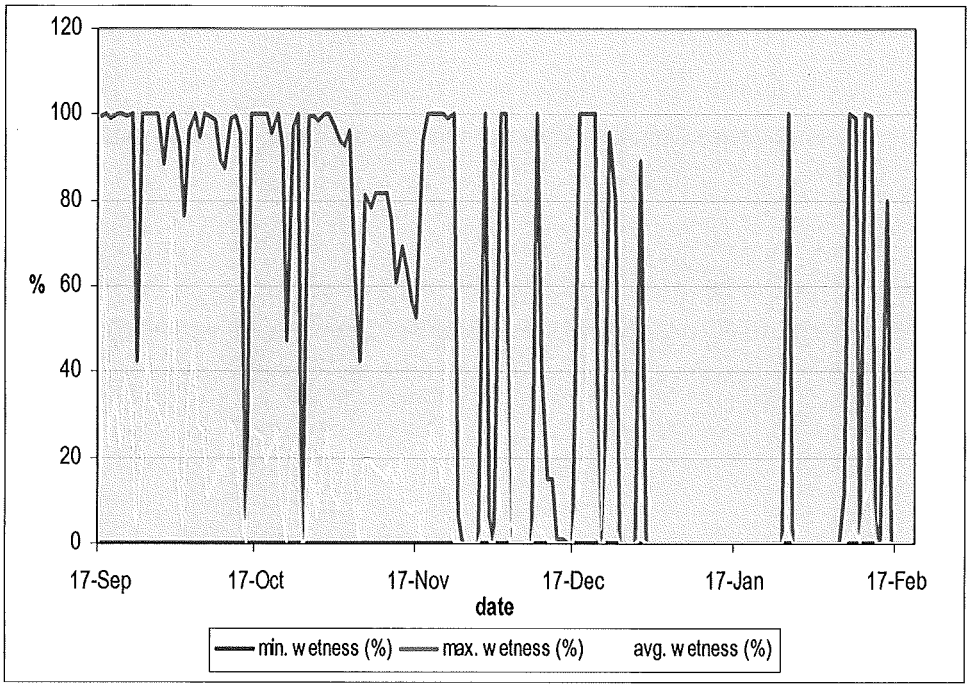

Figure C.3: Daily leaf wetness in a Swan Hill nectarine orchard, September 2003 – February 2004.

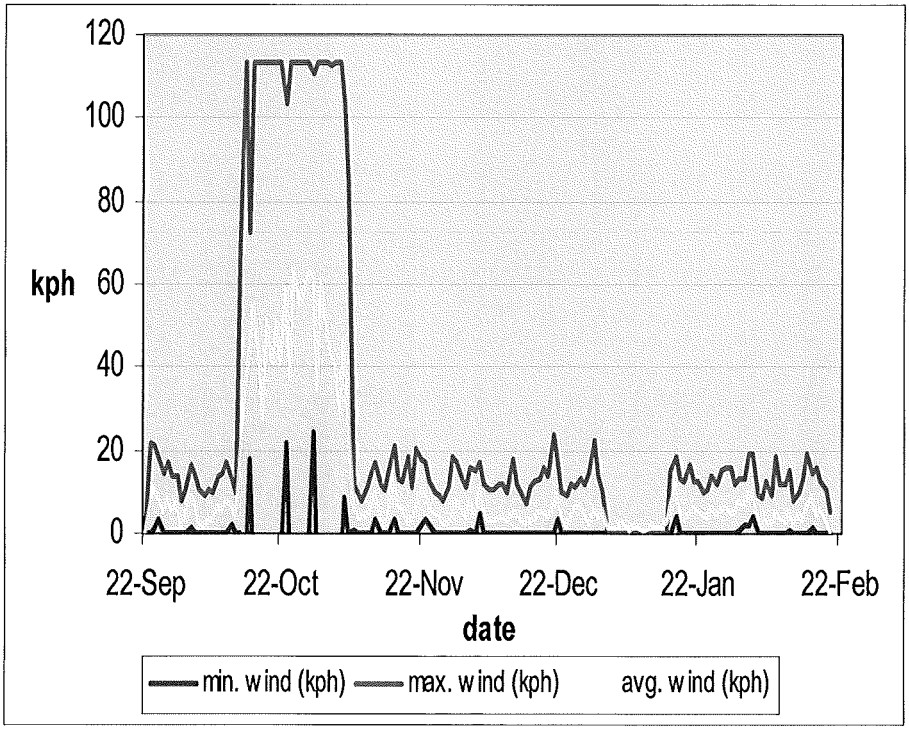

Figure C.4: Daily wind speed in a Swan Hill nectarine orchard, September 2003– February 2004.

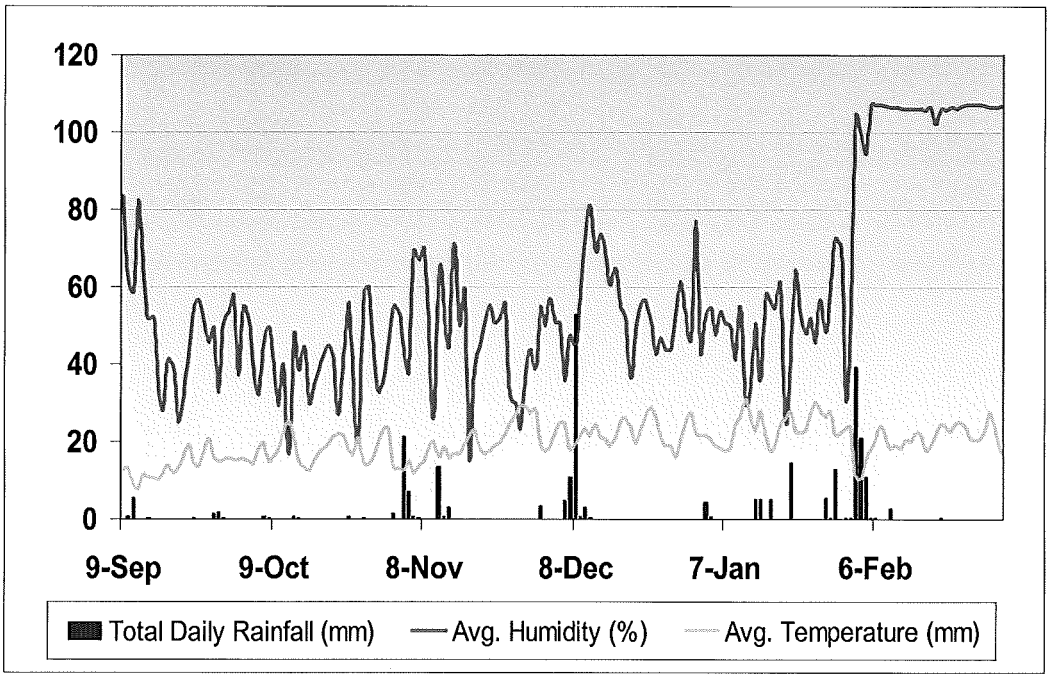

Figure C.5: Average daily temperatures, relative humidity and total daily rainfall in a Swan Hill peach orchard, September 2004 – February 2005.

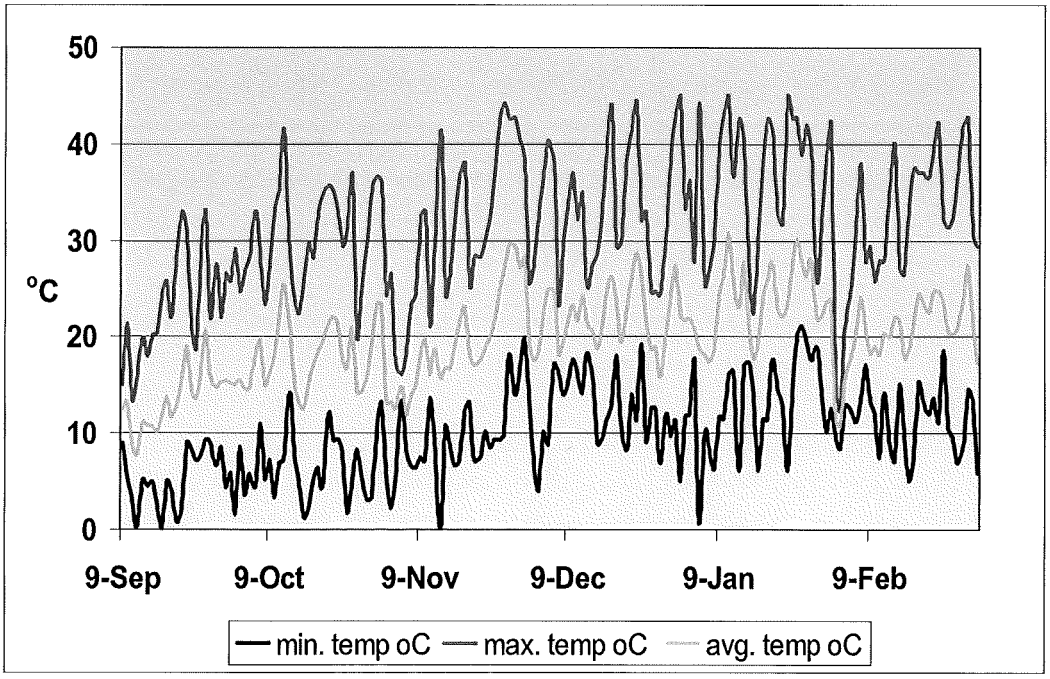

Figure C.6: Daily maximum, minimum and average temperatures in a Swan Hill peach orchard, September 2004 – February 2005.

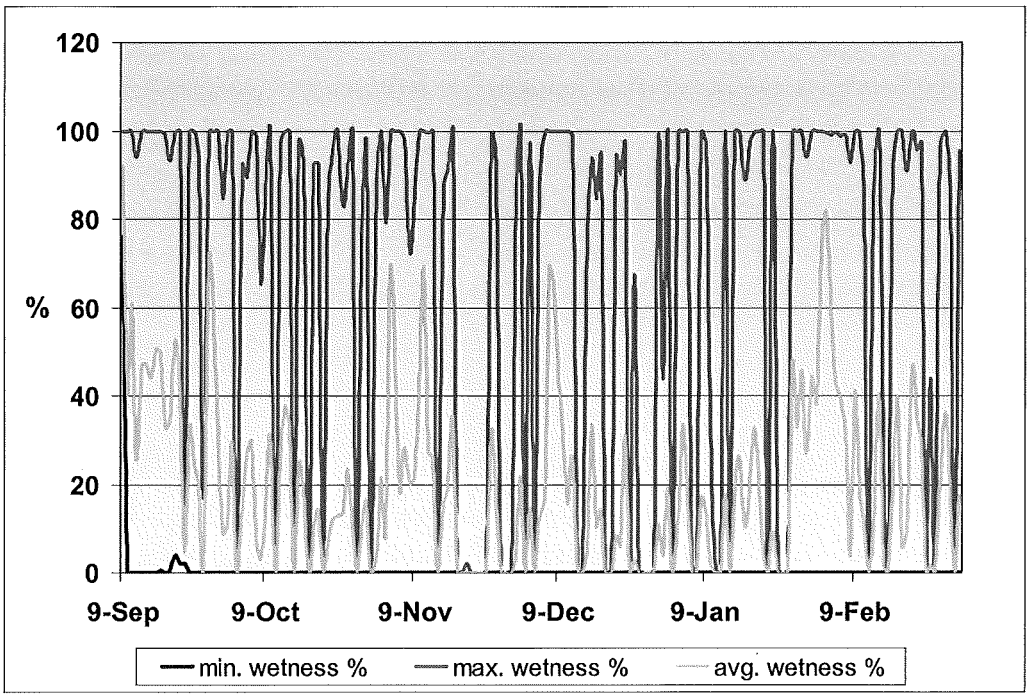

Figure C.7: Daily leaf wetness in a Swan Hill peach orchard, September 2004 – February 2005.

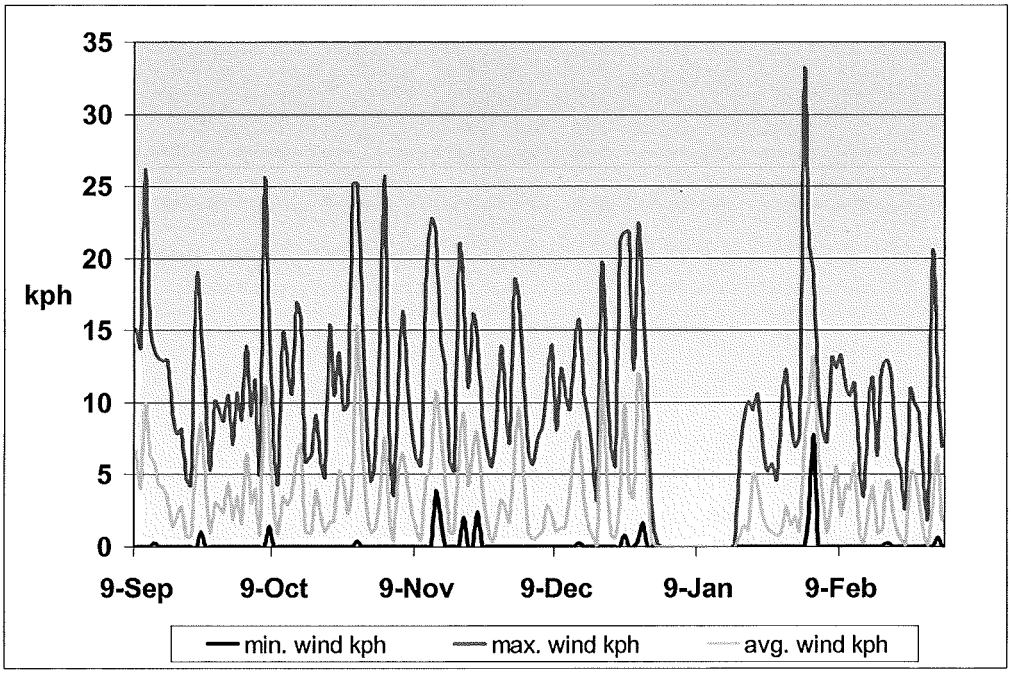

Figure C.8: Daily wind speed in a Swan Hill peach orchard, September 2004– February 2005.