Investigation of Host Cell Wall Degrading Strategies of *Pseudomonas syringae* pv. *actinidiae* *(Psa)*

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Rasika Subasinghe

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Abstract

Kiwifruit (*Actinidia deliciosa* and *Actinidia chinensis*) is cultivated in several countries including Italy, France, Chile, China, Japan, Korea and New Zealand and is an important income earner for a majority of these countries. In New Zealand, kiwifruit earns approximately USD 714 million per annum. In the recent past, the New Zealand kiwifruit industry was threatened by an outbreak of bacterial canker caused by *Pseudomonas syringae* pv. *actinidiae (Psa)*. The invasion strategies and the mobility of *Psa* pathogen in the kiwifruit plant have not been fully described. Recent comparative genomic studies of *Psa* strains and other *P. syringae* pathovars that also infect woody hosts indicate similarities in genetic makeup among these pathovars. Interestingly, these pathovars contain genes capable of producing cell wall degrading enzymes, thus suggesting that *Psa* possesses the same capability. However, no previous research demonstrated that any *P. syringae* pathovar degrades the host's cell walls or any cell wall component. This research investigated whether *Psa* produces xylanases to degrade cell wall material of kiwifruit stems and facilitate systemic mobility of the pathogen from the infection site. The research also investigates whether vessel anatomy and xylem architecture play a role in the systemic mobility of the pathogen within the plant.

Chapter Two of the thesis determines whether *Psa* is able to multiply and grow on kiwifruit wood-containing medium and whether *Psa* synthesises any xylanase to degrade cell wall material contained in the kiwifruit wood. For this purpose, the genome of *Psa* was analysed to ascertain whether sequences homologous to well-known cell wall degrading enzymes were present in the genome. The degree of similarity of the identified genes was compared to the cell wall degrading genes of other *Pseudomonas* woody host pathogens. Six genes encoding for well-known cell wall degrading enzymes were identified, with the degree of amino acid similarity varying between 58% and 98%. Patterns of *Psa* growth were examined in three media: standard nutrient broth, xylan and casein media and minimal media supplemented with kiwifruit cell wall material, called kiwifruit xylem medium. Growth of the bacterium was observed in the kiwifruit xylem medium, however, the increase in CFU (Colony Forming Units) counts in kiwifruit xylem medium was low compared to that in the other two media. A characteristic

bacterial growth pattern was observed in the standard nutrient broth media. In the kiwifruit xylem media, bacteria counts declined initially, then briefly recovered. *In vitro* enzyme assays were conducted for *Psa* in the kiwifruit xylem medium to determine the activity of xylanase enzyme. Two types of assays were conducted: 3,5-Dinitrosalicylic acid (DNSA) and Remazol Brilliant Blue (RBB). A significant xylanase activity was detected only in the *Psa* cultured in minimal medium supplemented with 0.5% kiwifruit xylem using the DNSA assay. However, no xylanase activity was detected using the RBB. It was concluded that *Psa* is capable of producing an active xylanase enzyme, and that synthesis of the enzyme is induced under low nutrient conditions and in the presence of kiwifruit xylem cell wall material.

Chapter Three describes investigations of xylanase activity *in planta*. Twenty mature *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' plants were used. Ten plants were left un-inoculated and ten plants were inoculated with *Psa.* Disease symptoms appeared in the inoculated plants after which both inoculated and non-inoculated shoots were harvested. *Psa* was re-isolated from infected plants and duplex PCRs were conducted to confirm that symptoms were due to *Psa* infection. RBB and DNSA assays were conducted on ground kiwifruit stem pieces to ascertain putative xylanase activity. The RBB assay indicated xylanase activity in inoculated kiwifruit stem pieces. RBB assay parameters were varied to ascertain whether the xylanase activity in infected tissues was consistent with enzymatic activity. Strength tests were conducted on infected and non-infected kiwifruit shoots to determine whether infection affected stem structural integrity. The average strength per mm thickness of non-infected kiwifruit xylem was significantly higher than that of infected xylem. RNA was extracted from both inoculated and non-inoculated stems and PCR conducted with primers specific to the xylanase genes identified in the Psa genome. One bacterial xylanase gene was expressed during infection of the kiwifruit stems. The observed reduction in stem strength was consistent with the xylanase activity detected using the RBB assay and the expression of the bacterial xylanase gene during infection.

Chapter Four investigated kiwifruit vessel lengths, the frequency of open vessel connections between kiwifruit stems and leaves, and the potential for movement of *Psa* from leaf inoculation sites into the supporting stem. Silicone injection was

used to document xylem vessel length distribution within mature one-year-old stems of three kiwifruit cultivars that differ in Psa susceptibility. There was no correlation between average vessel lengths and susceptibility. The air injection technique was used to estimate maximum vessel lengths in Hort16A seedlings and vessel connections from leaf to stem. It was observed that the maximum xylem vessel length of Hort16A kiwifruit seedlings was 345 mm, that 71% of leaves had an open vessel from the stem to the leaf blade, and that 79% of leaves had an open vessel from stem to the petiole. The average maximum vessel length and plant height were positively correlated. A negative correlation was observed between height of the plant and the number of nodes with vessels extending into leaves. *Psa* was observed to move in both basipetal and acropetal directions when the leaf blade of Hort16A seedlings was inoculated, and in the majority of leaves was detected in the petiole or stem when either the tip or base of the leaf lamina was inoculated. *Psa* movement exceeded the boundaries of known open vessel connections between stem and leaf, suggesting that if *Psa* was mobile in the xylem, then vessel end walls were not a significant barrier to movement. This finding supports the conclusion that *Psa* has the ability to overcome plant cell wall barriers to movement.

The overall findings of this thesis are that *Psa* does actively produce at least one cell wall degrading enzyme during infection of kiwifruit tissue, and that cell wall degrading enzymes contribute to an active invasion mechanism by this pathogen. For this reason *Psa* movement within the plant is not limited by cell wall barriers or the architecture of the xylem vascular system.

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List of Abbreviations

aa	Amino Acid
AZCL	Azurine-Crosslinked Polysaccharides
BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Units
CWDE	Cell wWall dDegrading Enzymes
DNSA	3,5-Dinitrosalicylic aAcid
EPS	Extracellular pPolysaccharides
EPS	Exopolysaccharides
GFP-*Psa*	Green Fluorescence Protien *Psa*
GM	Genetically Modified
ICMP	International Collection of Microorganisms from Plants
kb	kilobases
KVH	Kiwifruit Vine Health Incorporation
LMEs	Lignin Modifying Enzymes
NCPPB	National Collection of Plant Pathogenic Bacteria
nt	Nucleotides
ORFs	Open rReading fFrames
PG	Polygalacturonase

PME	Pectin Methyl Esterase
Psa	*Pseudomonas syringae* pv. *actinidiae*
Psm	*Pseudomonas syringae* pv. *morsprunorum*
Pss	*Pseudomonas syringae* pv. *syringae*
PVPP	Polyvinylpolypyrrolidone
RBB	Remazol Brilliant Blue
T2S	Type II Secretion
XETs	Xyloglucan EndoTransglycosylases
Xf	*Xylella fastidiosa*

CHAPTER 1

1.1 Introduction

The kiwifruit vine is a member of the family Actinidiaceae; genus *Actinidia* comprising approximately 60 species, including the commercially important *A. deliciosa* (Green kiwifruit) and *A. chinensis* (Gold kiwifruit). Plants are perennial, vigorous and deciduous and grow to a height of about 20 metres in the wild. *A. deliciosa* and *A. chinensis* have large and round leaves of about 7 cm to 12 cm in diameter, and bear male and female flowers on separate plants (dioecious). These woody climbers produce berries, which are known to consumers as the kiwifruit berry. The vines need fertile soil, shelter from the wind and protection from frost (Ewers *et al.*, 1990). Diseases commonly affecting commercially grown kiwifruit include root rots, which can develop from infection by the soil borne oomycete *Phytophthora* usually in association with poor drainage conditions. Another pathogen, *Armillaria novaezelandiae* (bootlace fungus) spreads to kiwifruit from the infected remnants of dead plants. When the climate is humid, *Botrytis cinerea* (grey mould rot) infects flowers and young fruit, and can cause serious post-harvest losses (Ferguson, 1990). However, the New Zealand kiwifruit industry has developed horticultural practises that have reduced the losses caused by these pathogens to an acceptable level. In contrast, the recently emerged bacteria *Pseudomonas syringae* pv. *actinidiae* (*Psa*) is causing severe economic losses, with no effective control strategies developed to date (Everett *et al.*, 2011).

Despite the previous occurrences of *Psa* infections being recorded in a number of countries, the discovery of the virulent strain *Pseudomonas syringae* pv. *actinidiae* (*Psa*-V) on a New Zealand kiwifruit orchard dates back to only November 2010 (Everett *et al.*, 2011). Prior to the discovery of *Psa*-V in New Zealand, there were reports of a similar strain causing severe damages to the kiwifruit industry in the Latino region of Italy. Despites its discovery in Italy in 1992, reports indicate a low incidence of *Psa*-V until 2007/08. However, since 2008, the infection has been reported to have caused serious damage in the Latina region (Balestra *et al.*, 2009; Scortichini *et al.*, 2012). The damage reached its peak in February 2012 when an estimated 650 hectares out of the total cultivation of 680 hectares of gold kiwifruit in the Latina Region were destroyed by the

infection. Lower levels of vine removal are reported in the other growing regions in Italy. The infected plants in all growing regions in France, South Korea, Portugal and Chile have tested positive for the bacterium during this period (Scortichini *et al.*, 2012). Unconfirmed reports suggest that the disease has also affected Chinese orchards during the same period (KVH, 2012b). It has now been established that the infection described in most of the growing regions prior to 2008 was caused by a less virulent strain of *Psa*.

The risk of *Psa*-V infection across kiwifruit orchards in New Zealand has been estimated to be quite significant. There was a sharp increase of the number of orchards tested positive for *Psa*-V through autumn and winter of 2011. Further increase in the number of infected orchards was recorded during the spring of 2011 possibly aided by the rise of sap that occurs in spring. In August 2011 *Psa*-V symptoms were discovered on a Hayward orchard. Until spring 2011, the infected orchards were reported from the Te Puke area only. However, from September to December 2011, a sharp increase of the disease was recorded throughout the Bay of Plenty (Source KVH). Severe damage to vines and orchards was reported in the Te Puke area and the infection spread across the kiwifruit growing region of Bay of Plenty. Furthermore, a small number of orchards in the South Auckland and Waikato regions have also been infected. According to kiwifruit Vine Health Incorporation (KVH) currently *Psa*-V present in orchards in all regions except far North, Whangarei and South Island (Nelson, Motueka, Golden Bay area).

According to KVH, the response of the kiwifruit industry to the *Psa* outbreak was immediate. The primary objective of the industry-wide response to counter the threat of *Psa*-V was to contain and eradicate the disease. However, strategies implemented by the industry to achieve this objective were far from effective. When the eradication efforts failed, the industry focussed on minimising the damage and identification of a pathway for recovery.

Studies conducted to investigate the actual invasion strategies and mobility of *Psa* pathogen in the plant remain inconclusive. However the outcome of the research conducted into the host cell wall degrading activity and invasion strategies of a number of other pathogens and also histological analysis of *Psa* infected host plants shed some light on the possible cell wall degrading mechanism of the pathogen (Lionetti *et al.*, 2007; Mazzaglia *et al.*, 2012a; Perez-Donoso *et al.*,

2010; Raiola *et al.*, 2011; Renzi *et al.*, 2012; Roper *et al.*, 2007; Sun *et al.*, 2011; Vasse *et al.*, 1995). In addition, recent comparative genomic studies of *Psa* strains and other *P.syringae* pathovars that also infect woody hosts point towards marked similarities in the genetic makeup among these pathovars. Interestingly, these pathovars have been found to possess genes capable of producing cell wall degrading enzymes, thus indicating a strong possibility that *Psa* possesses this capability (Marcelletti *et al.*, 2011; McCann *et al.*, 2013 ; Scortichini *et al.*, 2012). However, none of the existing studies have actually demonstrated that any *P. syringae* pathovar has the capability to degrade cell walls or any cell wall component.

As indicated by (Serizawa & Ichikawa, 1993 c), it is important to study the mobility of bacterium within the host plant and to investigate whether the movement is taking place via the vascular system. The pathogen's putative capability to degrade cell walls of the host plant and the ability to enter and move within the vascular system, particularly the xylem, are assumed to be facilitated by the structure of this tissue. In the strict theoretical sense, it is a prerequisite for the bacterium to possess cell wall degrading activity to enable it to move along the xylem tissue. The pathogen's perceived capability of cell wall degradation and vascular movement necessitates an in-depth understanding of the vascular anatomy and cell wall composition of the host. It will also be interesting to ascertain the specific features of the plant's anatomy and vascular structure that might facilitate pathogen movement, since there have been investigations carried out into this aspect of the disease. So far the actual cause of the characteristic wilting and dying back of young shoots caused by the pathogen have not been identified, although anecdotal evidence suggests possible blocking of the xylem by the colonising bacteria.

In this analysis, an attempt has been made to extract and document the evidence that suggests a possible cell wall degrading activity by *Psa* and to investigate the strategies adopted by *Psa* to infect host plants through degradation of the host cell wall material, to move within the plant, and ultimately cause tissue wilt and cane die back.

1.2 Literature review

1.2.1 Background

In this section of the literature review the general characteristics of the host plant and the pathogen are described.

Kiwifruit is categorized in the **Family – Actinidiaceae**. Actinidiaceae consists of woody vines, shrubs and trees that are native to Asia, Central America and South America. These plants have a simple, spiral arrangement of leaves.

Kiwifruit is classified under the **Genus – *Actinidia*.** This name is given to plants that are tough and hardy. The word "actinidia" derives from a Greek word meaning "difficult" or "hard" and therefore it describes a physical characteristic of kiwifruit. The vine and skin of the kiwifruit are extremely tough, resistant, strong and hardy.

Kiwifruit's final classification is based on the features of its fruit. The species name, "deliciosa", is derived from the Greek word, which means "luxury" or "luxurious" and refers to the luscious taste of the green, fleshy fruit. *Actinidia deliciosa* (A. Chev.) C.F. Liang *et* A.R. Ferguson var. *deliciosa*, is considered as green or Hayward kiwifruit, and recently released *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A', is considered as the gold-fleshed kiwifruit, an alternative cultivar (Ferguson, 1990; Fraser *et al.*, 2009).

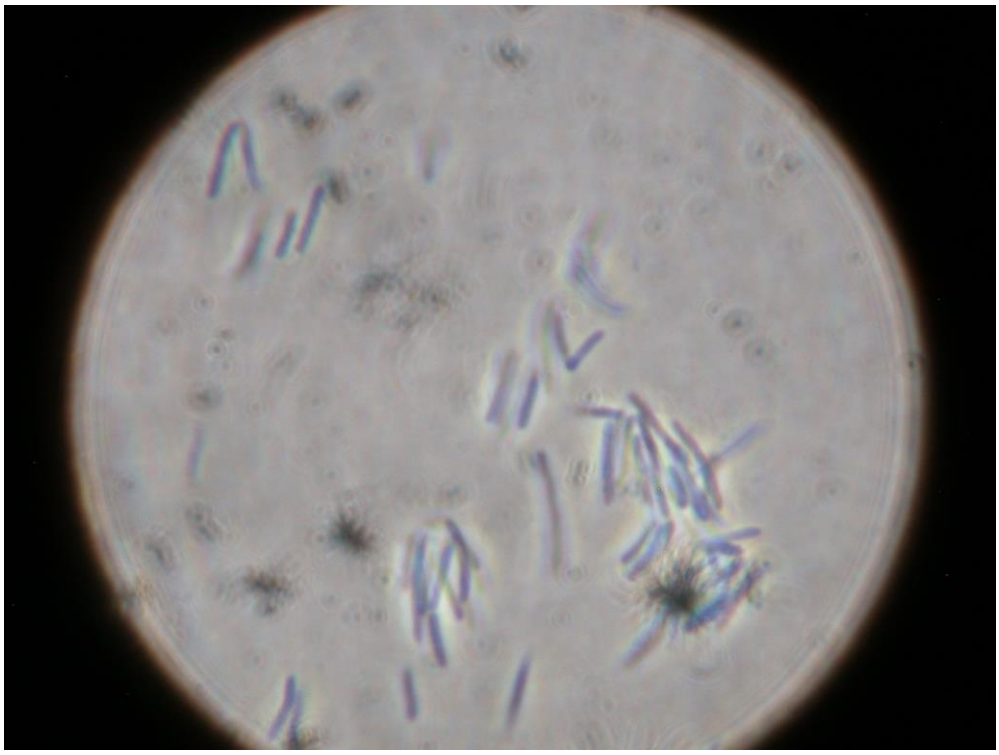

Figure 1-1 Light microscopic view of *Pseudomonas syringae* pv. *actinidiae* (*Psa*) cells. The photograph was taken when the specimen was under oil immersion. 100X enlargement.

The pathogen ***Pseudomonas syringae*** is a Gram-negative bacterium. The bacterium is rod shaped (Fig 1.1) and is equipped with polar flagella. The bacterium is pathogenic on a wide range of plant species and exists as more than 50 different pathovars (Anzai *et al.*, 2000). All the named pathovars are available via international culture collections such as the National Collection of Plant Pathogenic Bacteria (NCPPB) and the International Collection of Microorganisms from Plants (ICMP). It is unclear whether these pathovars represent a single species or a collection of related species (Anzai *et al.*, 2000).

Pseudomonas syringae belongs to the genus *Pseudomonas sensu stricto.* This genus is included in the γ subclass of the Proteobacteria (Kersters *et al.*, 1996). At present the genus *Pseudomonas sensu stricto* includes only the species of the previous *Pseudomonas* rRNA group I (Palleroni, 1984). The group consists of the fluorescent species that produce the pigmented iron-chelating siderophore pyoverdines that are fluorescent under UV light. The pathogenic species included in this group are harmful to human, plants and some fungi. *P. syringae* is an aerobic and motile plant pathogenic fluorescent *Pseudomonas* bacterium with a

straight or slightly curved rod shape equipped with one or several polar flagella (Holt J.G. *et al.*, 1994; Palleroni, 2005).

At present, over 50 pathovars of this bacterium that are pathogenic to more than 180 plant species, including fruit plants, vegetables, ornamentals, and other annual and perennial species have been identified (Bradbury, 1986 ; Young J.M. *et al.*, 1996). *Psa* is one of the 50 pathovars. Chapman *et al.* (2012), described the relationships between the various strains of the *Psa* pathogen in the following manner:

Psa 1 – P. syringae pv *actinidiae* population discovered from Japan and Italy in 1992 outbreak

Psa 2 - P. syringae pv *actinidiae* population present in Korea.

Psa 3 - P. syringae pv *actinidiae* population present in the Ravenna and Latina regions of Italy (2008-09 outbreak), Te Puke region of New Zealand, Chile and the Shaanxi province of China.

Psa 4 - P. syringae pv *actinidiae* population present across New Zealand and in Australia.

Psa 4 is clearly genetically distinct from *Psa 1*, *Psa* 2 and *Psa 3* and is less closely related to these strains than is the pathovar *P. syringae* pv. *theae. Psa 4* causes only minor symptoms of disease in kiwifruit. It was not detected in New Zealand until the outbreak of *Psa 3* began, and was originally referred to *Psa* 'LV' (low virulence). However, for the first time, strains of pathovar (biovar) 4 have been isolated outside New Zealand or Australia. In France, *Psa* 4 has been identified and it is proposed that *Psa* biovar 4 be renamed *Pseudomonas syringae* pv. *actinidifoliorum* pv. nov. Strain CFBP 8039 is designated as the pathotype strain (Cunty et al., 2015).

Unless otherwise stated, the use of the term *Psa* in this thesis refers to *Psa* 3, also referred to by other authors as *Psa*-V (virulent), I2-Psa (Italy outbreak 2) or the 'outbreak strain'.

1.3 Other *Pseudomonas syringae* canker forming bacteria

1.3.1 *Pseudomonas syringae* pv. *syringae* (*Pss*)

Pss is the pathogen that causes canker and blast in stone fruit trees. The pathogen affects all commercially grown *Prunus* species, including peach *(Prunus persica)*, prune and plum *(Prunus domestica)*, sweet cherry *(Prunus avium)*, almond *(Prunus dulcis)* and apricot *(Prunus armeniaca)* (Abbasi *et al.*, 2013). *Pseudomonas syringae* pv. *syringae* causes disease in over 180 species of plants in several unrelated genera (Bradbury, 1986). This disease causing ability is unique among most *P. syringae* pathovars.

Canker development on shoots and at the base of spurs are the first disease symptoms. Then the canker development progresses upwards supplemented with gum exudation beginning in the growing season (Hattingh & Roos, 1995). *Pss* attacks twigs, buds, flowers, leaves and fruits. In the beginning of the spring, dark brown submerged wounds appear on small branches underneath the infected spurs. Shoot blight and death of the infected branches with gums frequently appearing from cankered regions on the large branches in severe infection of twigs (Goto, 1992).

The outcome of the bacterial infection is manifested in a reduction in yield due to one or more of the infection related symptoms such as death of buds and flowers, tree decline and death due to the development of cankers in branches and major scaffold limbs (Ogawa & English, 1991). *Pss* grows on leaf surfaces and enters the apoplast through stomata and wounds. When the pathogen enters the leaf apoplast, it initially propagates biotrophically, but subsequently causes necrotic lesions (Misas-Villamil *et al.*, 2011). Colonization from wound sites on *Nicotiana benthamiana* is common for *P. syringae* strains representing major branches of the *P. syringae* phylogenetic tree (phylogroups). Two of the strains that efficiently colonize tissues from wound sites are *P.syringae* pv. *syringae* B728a (*Pss*B728a) and B301D-R (*Pss*B301D), both from phylogroup II. An aggressive strain of *Pss* inoculated into plum petioles, spreads to the xylem and other elements of leaf veins suggesting *Pss* has the ability to systemically spread in leaves (Hattingh *et al.*, 1989). In a scanning electron microscope study, it was observed that *Pss* colonises the surface of pear leaves and enters leaf tissue following bacteria

multiplication on the leaf surface, particularly on trichomes and in depressions of the cuticular layer. Lumps of bacteria were accompanied with leaf spots, which begin to exist 9 days after inoculation when conditions are favourable (Mansvelt & Hattingh, 1987). *Pss* frequently multiplies on apple leaves and blossoms during the growing season without causing lesions. Scanning electron microscopy indicated that *Pss* entered leaves through stomata and multiplied in the substomatal chambers. Another *Pss* strain applied to blossoms colonized stigmas and also occurred in intercellular spaces of styles (Mansvelt & Hattingh, 1989). Whether *Pss* has the ability to degrade the cell walls of *Prunus* has not been recorded in the literature.

1.3.2 *Pseudomonas syringae* pv. *morsprunorum* (*Psm*)

Psm causes bacterial canker of stone fruits. This disease has been known to growers for more than a century. The characteristic symptoms of the infection includes cankers and necroses developing on branches and trunk, often located around spurs, wounds and branch junctions. In early infections on branches, the tissue is sunken, water-soaked and slightly discoloured brown. Later, it becomes darker and finally reddish-brown black (Bultreys & Kaluzna, 2010).

During the infection, the infected parts of the plant produce orange-brown gummy substances and this is thought to be associated with cankers and necroses. The symptoms on the leaves appear as small round wounds of various sizes. At the initial stages of the infection, the wounds appear light brown in colour and with time they can become dark brown. The wounds can be surrounded by a halo which may appear yellow. The infected tissue often separates from the leaf and this symptom is well known among the farmers as shot-hole symptoms. The pathogen may also infect blossoms, resulting in a brown colour and ultimately resulting in abscission before full opening. The immature fruits of the infected sweet and sour cherry plants display sunken necroses that are brown or black in colour (Bultreys & Kaluzna, 2010).

The pathovars *syringae* and *morsprunorum* race 1 and race 2 were the first described *P. syringae* pathovars. They can be found more frequently in worldwide stone fruit orchards (Crosse, 1966; Kennelly *et al.*, 2007a). *Pss*, *Psm* race 1 and

Psm race 2 are genetically distant and distinct pathogens adapted to the same hosts (Gardan *et al.*, 1999; Ménard *et al.*, 2003).

In a study of sweet cherry leaves, *Psm* entered through the stomata, multiplied in the cavities and survived in a lump for 6 days post inoculation (Roos & Hattingh, 1983). Scanning Electron Microscopy indicated that *Psm* applied by spraying most probably entered through stomata and then dispersed intercellularly from the mesophyll through the parenchyma of the bundle sheath into the vascular system of a minor vein. When a vein had been invaded, movement occurred to other regions in the leaf blade and petiole (Roos & Hattingh, 1987).

1.3.3 *Pseudomonas syringae* pv. a*esculi*

P. syringae pv. *aesculi* is responsible for causing bleeding canker in trunks and branches of *Aesculus* spp. (horse chestnuts). The disease was first discovered in Northwest Europe (de Keijzer *et al.*, 2012). During the last 10 years, *Pseudomonas syringae* pv. *aesculi* has been identified as a major threat to horse chestnut. The infection is manifested as extensive necrosis of phloem and cambium resulting in dieback. The details of the interaction between the host and the pathogen from the infection to extensive necroses have not been well documented (de Keijzer *et al.*, 2012).

When the infection reaches its peak, its severity is displayed by bleeding symptoms across large populations of the host (Green *et al.*, 2009). The infection is manifested on trunks and branches by way of necrosis of bark tissues and bleeding of an amber coloured sap. The vascular cambium is the most commonly infected host tissue leading to irregular secondary growth. When necrosis of the phloem is extensive, tree vitality is reduced and girdling lesions ultimately lead to shoot dieback.

This pathogen is known to enter and infect the host through its lenticels. Other than *Psa* this is the only Pseudomonad pathogen that infects through lenticels (Steele *et al.*, 2010).

1.3.4 *Pseudomonas avellanae*

The pathogen causes bacterial canker in hazelnut (*Corylus avellana*). *P. avellanae* has been placed in the *P. syringae* group on the basis of 16S rRNA analysis (Anzai *et al.*, 2000). This species was previously identified as a pathovar of *Pseudomonas syringae.* However, DNA-DNA hybridization analyses (A technique that measures the degree of genetic similarity between pools of DNA sequences) lead to categorization of the pathogen as a separate species (Janse *et al.*, 1996). In addition to *P. avellanae, Pseudomonas syringae* pv. *theae* was also incorporated into this group on the basis of ribotypical analysis (Gardan *et al.*, 1999).

1.3.5 Similarities between *Pseudomonas syringae* canker forming bacteria

Cankers on the trunk and leader are common to all five *Pseudomonas syringae* canker forming species including *Psa*. Leaf spots and necrosis are the first symptoms common to both *Psa* and *Psm* infection. In *Psa* infection black colour, angular leaf spots with or without a chlorotic halo are prominent (Scortichini *et al.*, 2012). Small round lesions of various sizes, initially light brown then changing to dark brown surrounded by a yellowish halo are prominent with *Psm* infection (Latorre & Jones, 1979). Twig die-back occurs in both *Pss* and *Psa* infection. *Pss* produces a gum-like exudate on cankered regions, while *Psa* produces whitish to orange ooze or reddish brown exudates. With *Psm* infection, the exudates are represented by orange-brown gummosis (Garrett *et al.*, 1966). Bleeding of an amber coloured sap which turns black in later stages occurs with *P. srringae* pv. *aesculi* attack. When infection becomes severe, all the external woody tissues of the plant are destroyed and ultimate die back of the plant occurs in both the *Psa* and pv. *aesculi* infection (Kennelly *et al.*, 2007b).

Although *Pseudomonas avellanae* and *Psa* are in different taxonomic groups, both pathogens cause similar symptoms in their respective woody hosts, hazelnut and kiwifruit as explained above (Psallidas, 1993). The infections caused by the pathogens described above are manifested in a range of symptoms that are remarkably similar irrespective of the host plant that they infect. The common name of the disease caused by these pathogens is bacterial canker.

1.4 Plant cell wall

1.4.1 Structure of the plant cell wall

There are various features that are common to both plant and animal cells. One such feature is the cytoplasm which is bounded by a plasma membrane. However, plant cells are surrounded by a 'wall' which is uniquely structured with cellulose (Raven *et al.*, 1999) which is exterior to the plasma membrane and itself is a part of the apoplast. The apoplast, which is a largely self-contiguous component, contains all the structures located between the plasma membrane and the cuticle. Hence, the apoplast is made up of the primary wall, the middle lamella (a polysaccharide-rich region between primary walls of adjacent cells), intercellular air spaces and water and solutes. Another significant feature that differentiates plant cells from animal cells is the symplast. Symplast is a self-contiguous phase that exists due to the tube-like structures known as plasmodesmata that connect the cytoplasm of adjacent plant cells (Fisher, 2000). In growing plant tissues the primary wall and middle lamella account for most of the apoplast. Thus, in the broadest sense the wall corresponds to the contents of the apoplast. In some tissues there are significant volumes of air space in the apoplast (e.g. in the mesophyll of leaves, in lenticels of the bark etc). The air spaces are significant in relation to *Psa* as the pathogen tends to fill air spaces as it multiplies in the host plant tissues.

The cell wall is tough and usually flexible while in certain situations it can be fairly rigid. The cell wall provides the cells with structural support and protection. The cell wall also performs filtering mechanisms and acts as a pressure vessel, preventing overexpansion when water enters the cell. Furthermore the cell wall is involved in a number of metabolic and resistance activities of the plant cell. Many cells are highly differentiated to an extent that there are more than 35 cell types in plants with different sizes, shapes, positions and wall characteristics. The cell walls of growing cells are typically thin (0.1–1 μm) and flexible and made up primarily of complex polysaccharides and a small amount of structural proteins. Under the electron microscope at nanometre scale, the cell wall's fibrous character can be observed. The wall forms a strong network capable of supporting contours of the plant cell, compressing and providing shape to the protoplast that it encloses. The cell walls are expanded through a process of controlled polymer

creep during the growth of the plant cell. The cells are tightly bound to each other through their cell walls making cell migration impossible. Therefore morphogenesis of the plant is confined to localized cell division and selective cell enlargement. Some of the cells expands to such extents that the largest plant cells like xylem vessels can increase by more than 30,000-fold in volume from their meristematic initials (Cosgrove, 2005).

One of the most important functions of the plant cell wall is the defence against invading pathogens. The cell wall acts as a physical barrier against pathogens. Due to this reason, plant pathogens have evolved a number of adaptations to invade host plant cell walls. As part of their invasion strategies against the host cell walls, plant pathogens produce a range of glucanhydrolases including polygalacturonase (PG), pectinlyase, pectin methyl esterase and xylanase. These enzymes help the pathogens to invade the host tissues by dismantling the host cell wall (Stahl & Bishop, 2000).

In response to pathogen's cell wall degrading enzymes, plant cells also produce a range of inhibitor proteins that are secreted through the cell wall. These inhibitors include xylanase inhibitor proteins and polygalacturonase inhibitor proteins (PGIP). It has been found that PGIP directly interferes with the host cell wall degradation (Stahl & Bishop, 2000).

1.4.2 The composition of the primary cell wall

The main component of the primary cell walls of the angiosperms is polysaccharides (up to 90% of the dry weight). The other components include structural glycoproteins (2–10%), phenolic esters (<2%), ionically and covalently bound minerals (1–5%) and enzymes. The primary cell wall of living tissues contains water up to 70% of its composition (Monro *et al.*, 1976). Some of the plant cells that require high mechanical strength and structural reinforcement also produce a secondary cell wall. The secondary cell wall is fortified mainly with lignin.

The cell walls of growing cells consist of cellulose microfibrils that are embedded in a matrix of complex polysaccharides forming a fibreglass-like structure (Carpita & Gibeaut, 1993; O'Neill & York, 2003). These matrix polysaccharides are divided into two classes, hemicelluloses and pectin. Pectin can be dissolved in

aqueous buffers and dilute acidic solutions or calcium chelators. Hemicelluloses are polysaccharides in plant cell walls that have β-(1→4)-linked backbones with an equatorial configuration. Hemicelluloses include xyloglucans, xylans, mannans and glucomannans, and β-(1→3,1→4)-glucans (Scheller & Ulvskov, 2010). Hemicelluloses are cellulose-binding polysaccharides. Together with cellulose they form a network that is both strong and yet resilient.

The cell walls of both angiosperms and gymnosperms combine cellulose, hemicelluloses (xyloglucan, glucomannan or arabinoxylan) and pectic polysaccharides (homogalacturonan, rhamnogalacturonans and substituted galacturonans) in different amounts (Cosgrove, 2005). The cell walls are categorised under two general types based on the relative amounts of pectic polysaccharides and the structure and amounts of hemicellulosic polysaccharides. Type I walls (Carpita & Gibeaut, 1993) characteristically contain xyloglucan and/or glucomannan and 20%–35% pectin. This type of cell walls is found in all dicotyledons, the non-graminaceous monocotyledons (e.g. Liliidae) and gymnosperms (e.g. Douglas fir). Type II walls are rich in arabinoxylan, and contain <10% pectin (Carpita, 1996). These types of cell walls are found in the graminaceous monocotyledons (Poaceae, e.g. rice and barley).

Pectins are highly complex polysaccharides and have several functions (Vincken *et al.*, 2003; Willats *et al.*, 2001a). When hydrated, pectin forms gels that maintain the separation of cell wall microfibrils. The dispersion of microfibrils facilitates lateral slippage during cell growth. Once the growth ceases, pectin gel helps lock the microfibrils in place. Pectin also controls wall porosity and wall thickness. Pectin is a component of the middle lamella, an adhesive layer that binds cells together in (Iwai *et al.*, 2002). Pectins are often primary targets of attack of plant pathogens. When the pectin is disintegrated by the pathogens, the resulting components of pectin can function as plant-defence response elicitors (Rose, 2003).

Cellulose and matrix polysaccharides are synthesised in two different pathways. Cellulose is synthesized by large membrane complexes. Matrix polysaccharides are synthesized in the Golgi apparatus and are stored in small vesicles. Subsequently, the vesicles fuse with the plasma membrane and the polysaccharides are incorporated to the cell wall. Matrix polysaccharides then

become a component of the wall network by physical interactions, enzymatic ligations and cross linking reactions. Newly secreted matrix polysaccharides can penetrate into the cell wall (Ray, 1967) . This is facilitated by cell turgor pressure, which creates an outward pressure on the cell wall leading to increase of its porosity and provides an energy gradient to drive polymers into the wall (Proseus & Boyer, 2005).

Primary cell walls may contain hydrophobic molecules such as waxes. In addition, ions and other inorganic molecules such as silicates may also be present (Epstein, 1999). These quantitatively minor components are often more abundant in specific plants or cell types. For example, silicates are abundant in grasses and seedless vascular plants such as horsetails (*Equisetum*) (Epstein, 1999).

1.4.3 Structures of the no cellulosic components of primary plant cell walls

Since cellulose is the lastly attacked polysaccharide in the plant cell wall by the microbes and they have to degrade other components of the cell wall first, before they attack cellulose (Roberts & Boothroyd, 1972), the structure of non-cellulosic polysaccharides are here considered in detail.

1.4.3.1 Hemicellulosic polysaccharides

Hemicelluloses are defined as the cell wall polysaccharides that cannot be dissolved in hot water or chelating agents, but are soluble in aqueous alkali. Based on the above differentiation, we can identify xyloglucan, xylans (including glucuronoxylan, arabinoxylan, glucuronoarabinoxylan), mannans (including glucomannan, galactomannan, galactoglucomannan), and arabinogalactan as hemicelluloses. Hemicelluloses are usually branched and are structurally homologous to cellulose. Similar to cellulose, hemicelluloses contains a backbone composed of 1,4-linked β-D-pyranosyl residues such as glucose, mannose and xylose, where O4 is in the equatorial orientation. Xyloglucan, xylans and mannans are included under this chemical definition of hemicelluloses with arabinogalactan excluded from this group. There is a significant structural similarity between hemicellulose and cellulose. This similarity results in a strong, noncovalent association between the hemicellulose and cellulose microfibrils (O'Neill & York, 2003).

1.4.3.1.1 Xyloglucan

Xyloglucan is the predominant hemicellulosic polysaccharide in the primary cell walls of non-graminaceous plants. It contributes to 20% of the dry mass of the wall. Xyloglucan has a 'cellulosic' backbone consisting of β-1,4-linked -D-Glc*p* residues. Bearing β-1,4-D-Xyl*p* residues at O6, up to 75% of the backbone residues are branched. Many of the Xyl*p* residues bear glycosyl substituents at O2, thereby extending the side chain. Only a limited number of xyloglucan side chain structures have been described and the cellulosic backbone itself does not vary among xyloglucans from different plant species and tissues.

Despite the major structural similarities of primary wall polymers in higher plants, the xyloglucan structure in dicotyledons and monocotyledons can have significant variations. These variations in structure are visible in different plant species, tissues, cell-types and occasionally even in different parts of the wall surrounding an individual cell (Freshour *et al.*, 1996). Apart from cellulose, xyloglucans are the most thoroughly characterized cell wall polysaccharides, and their general structure can be found among most of the higher plants.

1.4.3.1.2 Xylans

Xylans include cell wall components such as arabinoxylans, glucuronoxylans and glucuronoarabinoxylans (Darvill *et al.*, 1980). These components are found in lesser quantities in the primary cell walls of dicotyledons and non-graminaceous monocotyledons. However, xylans are found in larger quantities in the primary cell walls of the Gramineae and in the secondary cell walls of woody plants (Ebringerova & Heinze, 2000). Xylans consists of a backbone made up of 1,4-linked β-D-Xylp residues. Many of these residues are branched.

It can be deduced that the structural diversity of xylans is related to their functionality in plants and may explain the distribution of certain xylan types in the plant kingdom. The occurrence of xylans can be traced to the botanically oldest plant families. Xylans of all higher plants possess linked Xylp units as the backbone, usually substituted with sugar units and O-acetyl groups and are called heteroxylans. The only exception of heteroxylans with mixed linkages in the main chain, until now reported, have been isolated from the seeds of Plantago species. The occurrence of homoxylans in higher plants is rather rare (Vleeshouwers *et al.*,

2000). However, a neutral linear xylan has been isolated from guar seed husks as the hemicellulose A fraction. Lower plants (green algae and red seaweeds) cell wall components are comprised of homoxylans (Ebringerova & Heinze, 2000).

1.4.3.1.3 Mannose-containing hemicelluloses

Mannose-containing polysaccharides include mannans, galactomannans and galactoglucomannans. Homopolymers of 1,4-linked β-D-Manp are found in the endosperm of several plant species including, for example, ivory nut. Galactomannans, which are abundant in the seeds of many legume species, have a 1,4-linked β-D-Manp backbone that is substituted to different degrees at O6 with α-D-Galp residues. Glucomannans are found in larger quantities in the secondary cell walls of woody species. They have a backbone made up of both 1,4-linked β-D-Manp and 1,4-linked β-D-Glcp residues. Galactoglucomannans that are found in both primary and secondary cell walls, have a similar backbone as well (Stephen, 1982).

1.4.3.2 Pectic polysaccharides

The primary cell wall of higher plants can include three structurally different pectic polysaccharides, identified as homogalacturonan, substituted galacturonans and rhamnogalacturonans.

1.4.3.2.1 Homogalacturonan

Homogalacturonan (HG) is a linear chain of 1,4-linked α-D-galactopyranosyluronic acid (GalpA) residues. Some of the carboxyl groups of this linear chain are methyl esterified. When HG polymers are in highly methyl esterified form, they are referred to as ‘pectin’. On the other hand, when the polymers are methyl esterified to a lesser degree or are not esterified, they are called ‘pectic acid’. HGs may be partially O-acetylated in certain plant species (Ishii, 1997; Perrone *et al.*, 2002). The degree of methyl esterification of HG determines its ability to form gels (Goldberg *et al.*, 1996; Willats *et al.*, 2001b). When the HGs polymer is highly methyl esterified, it does not gel in the presence of Ca^{2+}. However, highly methyl esterified HG has been found to form gel at low pH in the presence of high concentrations of sucrose. As cells mature, the degree of methyl esterification of HG decreases which in turn leads to an increase in Ca^{2+}

cross-linking. The increase of Ca^{2+} cross-linking leads to increases in wall strength (Goldberg *et al.*, 1996; Willats *et al.*, 2001b).

Polygalacturonases (PG) are pectin degrading enzymes. PGs are expressed in a wide range of plant tissues and at various stages during plant development (Hadfield & Bennett, 1998). These PGs are likely to be involved in modifying the structure and properties of wall-bound pectin during normal plant growth and development. It is imperative to note that plant pathogens also commonly produce polygalacturonases. The main function of polygalacturonases secreted by plant pathogens is to facilitate invasion by dismantling the host cell walls.

1.4.3.2.2 Rhamnogalacturonans

Lau *et al.* (1985) stated that Rhamnogalacturonans (RGs) are a group of cell wall pectic polysaccharides that are closely related. They are made up of a backbone of the repeating disaccharide 4)-α-D-GalpA-(1,2)-α-L-Rhap. Rhap residues are substituted at C-4 with neutral and acidic oligosaccharides. Depending on the plant source and the method of isolation between 20% to 80% of Rhap s are found to be at C-4 (Ishii *et al.*, 1989; Lau *et al.*, 1987; McNeil *et al.*, 1982 b). The function of rhamnogalacturonans is believed to be associated with cell and tissue development (Orfila & Knox, 2000; Willats *et al.*, 1999; Willats *et al.*, 2001a).

1.4.3.2.3 Substituted galacturonans

Substituted galacturonans are a group of polysaccharides with a backbone of linear 1,4-linked α-D-Gal*p*A residues. Major components of this group include apiogalacturonans and xylogalacturonans (O'Neill & York, 2003).

Rhamnogalacturonan II (RG-II) is also a substituted galacturonan found in the walls of all higher plants (O'Neill *et al.*, 1990 ; Stevenson *et al.*, 1988). It is a pectic polysaccharide with a low molecular mass (~5–10 kDa). RG-II can be dissolved from the cell wall by treatment with endopolygalacturonase. RG-II contains eleven different glycosyl residues including Apif, AcefA, 2-O-Me Fucp, 2-O-Me Xylp, Dha and Kdo (unusual sugars) (Whitcombe *et al.*, 1995). Hence, RG-II is not structurally related to rhamnogalacturonans (RGs) that consist of a backbone made up of repeating disaccharide 4)-α-D-GalpA-(1,2)-α-L-Rhap (du Penhoat *et al.*, 1999).

1.4.3.3 Other primary wall components

1.4.3.3.1 Structural glycoproteins

These are O-glycosylated proteins, mainly hydroxyproline-rich glycoproteins (HRGPs) and are often referred to as ‘extensins’, even though their role in plant growth remains unclear. Generally HRGPs are glycosylated with arabinose, arabinobiose, arabinotriose, arabino tetraose and with galactose (Kieliszewksi & Shpak, 2001).

1.4.3.3.2 Arabinogalactan proteins (AGPs)

Gaspar *et al.* (2001) observed that Arabinogalactan proteins are a family of structurally complex proteoglycans. Polysaccharide portions which are rich in galactose and arabinose are 90% of the structural components of the molecule of AGPs.

AGPs are linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) membrane anchor (Youl *et al.*, 1998). Those AGPs that are not bound to the plasma membrane are present in the apoplast. Anecdotal evidence suggests that the plasma membrane-associated AGPs may have a role in cell expansion and cell differentiation (O’Neill & York, 2003).

1.4.3.3.3 Enzymes

Primary cell walls consist of a number of enzymes including those involved in wall metabolism Fry (1995), namely endoglycanases and exoglycanases, methyl and acetyl esterases, and transglycosylases and enzymes that may generate cross-links between wall components (e.g. peroxidases). Proteins referred to as expansins that have been proposed to break hydrogen bonds between Xyloglucan (XG) and cellulose are also present in the walls and hence are believed to regulate wall expansion (Cosgrove, 1999).

1.4.3.3.4 Minerals

Conditions of plant growth and the methods used to prepare the walls are the two major factors influencing the mineral content of a primary wall. However, minerals such as Ca, K, Na, Fe, Mg, Si, Zn and B together can constitute for up to 5% of the dry weight of eudicotyledon walls (Epstein, 1999). Calcium is believed to have a major influence on the deformation and flow of different components of

the primary wall. The increase of Ca^{2+} cross-linking leads to an increase in wall strength as cells mature.

1.4.4 Secondary cell wall

The secondary cell wall in higher plants is made up mainly of cellulose, lignin, and xylan. One of the cell types in higher plants that develops a secondary cell wall are the xylem elements. Xylem elements are involved in transporting water from the root system to the aerial tissues of the plant. The movement of water through the xylem elements is supported by negative pressure created by the aerial tissues of the plant during transpiration. In order to withstand these forces, the xylem cells develop a secondary cell wall matrix. In the secondary xylem, produced by the vascular cambium, the development of the secondary wall takes place when the cells have reached their final size (Turner *et al.*, 2001). The lignified secondary wall of xylem elements and associated fibres within the secondary xylem provides the major mechanical support for the transport of water and the growth of woody plants (Persson *et al.*, 2007).

As observed by transmission electron microscopy, the secondary cell wall in the xylem can be identified in three distinctive layers, namely S1, S2 and S3, as depicted in Fig 1.1 (A). The formation of three such layers is due to changes in the orientation of cellulose microfibrils during their deposition. Of the three layers, S1 and S3 are typically thin and consist of cellulose microfibrils oriented in a flat helix relative to the elongation axis of the cell (Fig 1.1 (B)). The S2 layer is a thick deposition of cellulose microfibrils oriented in a steep helix relative to the elongation axis. The mechanical strength of the fibres in wood is determined by the S2 layer (Zhong & Ye, 2009).

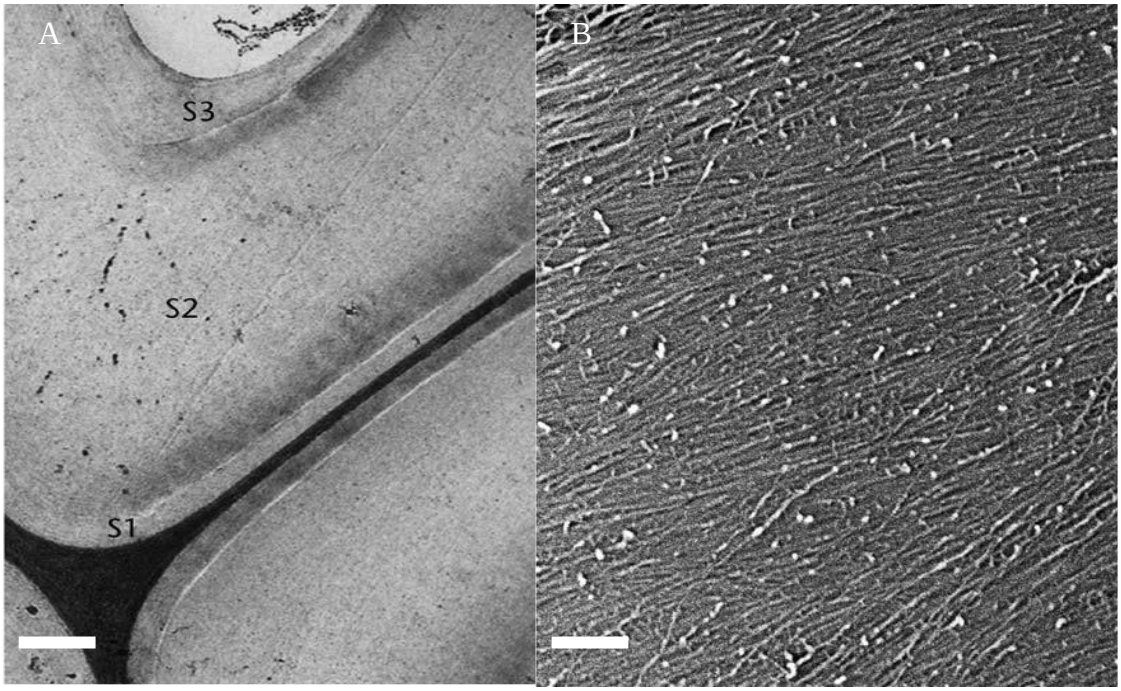

Figure 1-2 Descriptive view of the secondary cell wall under a transmission electron microscope. (A) Three distinct layers (S1, S2 and S3) of secondary cell walls in fibre cells of an *Arabidopsis* stem. (B) Cellulose microfibrils in the innermost layer of a mature fibre cell from an *Arabidopsis* stem are arranged in a flat helix. The vertical direction of the image corresponds to the elongation axis of the cell. Bar = 10µm

Source : Zhong and Ye (2009) ; Secondary cell walls. *eLS*.

Lignin is a complex phenylpropanoid polymer with hydrophobic characteristics. It is associated with the cellulose and hemicellulose network and gives a waterproof quality to the secondary cell walls. The waterproof quality enables tracheary elements to function as conduits for water transport. The lignin also forms an extensive crosslinked network providing a greater strength and rigidity to the secondary walls. Furthermore, lignin is a relatively inert polymer thus providing a stable and protective barrier to shield secondary cell walls from chemical, physical and biological attacks (Boudet, 2003).

Lignins have as a basis three hydroxycinnamyl alcohols, namely the monolignols, which subsequently become phenoxy radicals that then polymerize. Higher plants contain two types of lignin, guaiacyl lignin which is polymerized from coniferyl alcohol and syringyl lignin which is polymerized from synapyl alcohol. The secondary cell walls of vessels of dicots are rich in guaiacyl lignin and secondary cell walls in fibres contain both guaiacyl and syringyl lignin (Boudet, 2003).

Hydroxycinnamic acid-mediated cross-linking may occur between cell wall constituents after cell growth has ceased and the secondary wall has been formed. Subsequently, lower molecular weight components, such as phenolic acids, flavonoids, tannins, stilbenes and lignans are deposited which are thought to be important during the last stages of wall differentiation (Boudet, 2003).

Mechanical resistance of the walls are increased due to all of the above processes and therefore increase the resistance of the plant to abiotic and biotic stress factors (Nicholson & Hammerschmidt, 1992). All these different types of cell walls, especially the lignified walls, account for the larger part of the plant biomass and a pool of carbon in the form of lignocelluloses.

Typically lignification starts in the cell corner in the middle lamella and proceeds towards the lumen. During the process, lignin fills up pores in the already deposited polysaccharide network. The lignification continues until the protoplast disintegrates and the cell dies (Christiernin, 2006).

Stems of kiwifruit consist of a normal cambial structure but kiwifruit wood is diverse in structure. A common feature of kiwifruit wood is that it has fibre-tracheids. The wood is semi ring-porous; perforation plates may be simple, scalariform, reticulate or combinations of all (Condon, 1991). Water moves from one vessel to another or to adjacent parenchyma cells through numerous small openings called “pits” in the secondary walls. Pits between vessels consist of overarching walls that form a bowl-shaped chamber, called “bordered pits.” At the centre of each bordered pit is the pit “membrane”. The pit membrane is formed from the middle lamella and the original primary walls made up of cellulose micro fibrils that are hydrophilic. No lignification is found in the pit membranes (Zwieniecki & Holbrook, 2000).

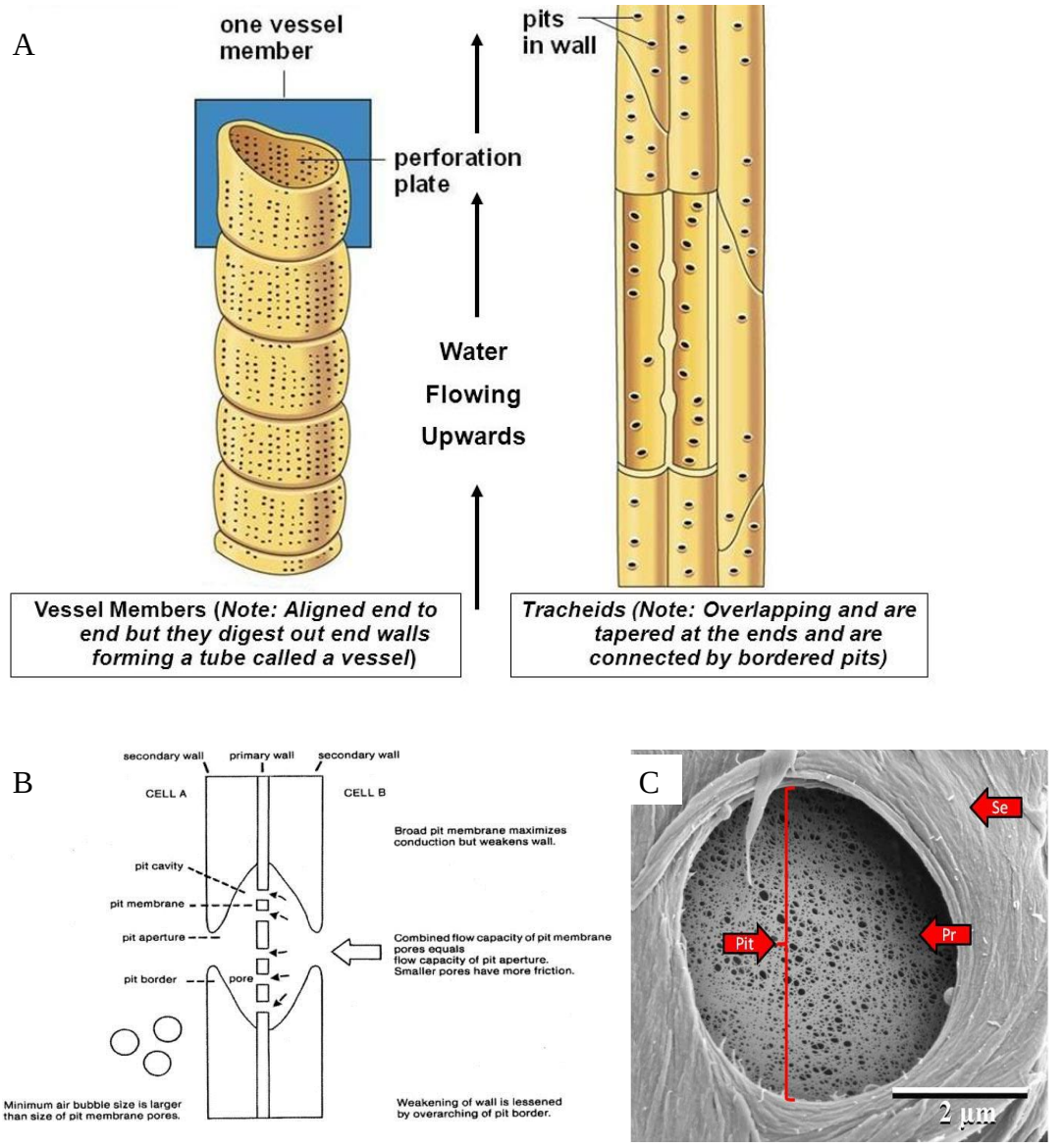

Figure 1-3 Xylem vessel structure. (A) Arrangement of vessels and tracheids. (B)Schematic diagram of a longitudinal section across a pit. (C) Scanning Electron Microscopy (SEM) view of a bordered pit

Secondary cell walls are evenly deposited on the inside of the primary walls except in the pit areas in fibre cells, where secondary walls are absent. Solute transportation and communication between two adjacent cells occurs through pits. (Zhong & Ye, 2009). Therefore if there was movement of the *Psa* pathogen through pit membranes it would not require any lignin degradation (Fig 1.2 (A),(B),(C)).

1.4.5 Composition of kiwifruit cell walls

There is an absence of research into the structure and composition of primary or secondary cell walls of the vascular system of kiwifruit stem and leaves.

However, extensive research has been conducted by Redgwell and co-workers on the composition of the primary walls of cells in the kiwifruit berry in relation to the ripening and post-harvest storage of the fruit (Redgwell & Fischer, 2001; Redgwell & Fry, 1993; Redgwell *et al.*, 1997; Redgwell *et al.*, 1988, 1991, 1992; Ross *et al.*, 1993). In their analysis of the cell wall polysaccharides of the kiwifruit berry at harvest, they observed that 40-50% of cell wall materials are made up of a heterogeneous mixture of pectic galactans. Furthermore, between 15-25% of the cell wall materials are hemicelluloses, the bulk of which were xyloglucans. They further observed that each tissue zone of the fruit contained similar types of polysaccharides. It was also observed that polysaccharides of the rhamnogalacturonan II type are associated with the pectic polymers of kiwifruit.

1.4.6 Cell wall degrading enzymes (CWDE)

Bacteria produce a range of CWDE, which contribute to their ability to invade the host plant. Research has been conducted to elucidate the mechanism adapted by microbes to disintegrate the different components of the host plant cell wall (Bateman & Basham, 1976). A thorough understanding of various CWDE produced by other pathogens, and their cell wall degrading mechanisms, would lead to a better understanding of putative CWDE activity of *Psa*. Therefore the well-known CWDE literature has been reviewed.

1.4.6.1 Pectinases

Pectinases are responsible for pectin solubilization and depolymerisation. Enzymatic and probably also non-enzymatic processes result in the solubilization of pectins into different fractions (Rose *et al.*, 2003).

1.4.6.1.1 Polyuroronide hydrolysis and polygalacturonase (PG)

The enzyme PG hydrolyses the α-1,4-D-galacturonan backbone of pectic polysaccharides (Gross & Wallner, 1979; Hobson, 1962; Huber, 1983; Redgwell & Fischer, 2001). As with many cell wall modifying enzymes, PG occurs as a family of genes and the corresponding proteins can act as either endo-, or exo-hydrolases (Hadfield & Bennett, 1998).

1.4.6.1.2 Pectin de-esterification: pectin methylesterase and pectin acetylesterase

It is believed that pectins are synthesized and deposited into the wall in a highly methylesterified form during cell wall synthesis, and then it undergoes enzyme-mediated demethylation (Kauss & Hassid, 1967; Lau *et al.*, 1985). Demethylation of the pectin galacturonosyl residues results in the generation of carboxylate groups. The carboxylate groups can then bind cations such as calcium and homogalacturonan chains non -covalently at specific junction zones. This results in the formation of a pectate gel (Pérez *et al.*, 2000).

1.4.6.1.3 Pectin depolymerization and pectate lyases

This is another group of plant pectinases. These enzymes catalyse the cleavage of unesterified α-1→4-galacturonosyl linkages by a β-elimination reaction, in contrast with the hydrolytic mechanism of PGs (Rose *et al.*, 2003).

1.4.6.1.4 Pectin side chain modification: galactanases/β-galactosidases and arabinosidases

Galactanases/β-galactosidases and arabinosidases are believed to be responsible for the loss of arabinan and galactan side chains from RG-I (Gross & Sams, 1984).

1.4.6.1.5 Rhamnogalacturonase

Rhamnogalacturonase A (RGase A), is a hydrolase that cleaves galacturonosyl-1,2-rhamnosyl glycosidic bonds (Schols *et al.*, 1990). This is regarded as another enzyme that may act together with PG and other pectinases to degrade the pectin network (Redgwell & Fischer, 2001).

1.4.6.2 Cellulose-interacting

Cellulase is a general term which refers to three cellulolytic enzymatic groups. The three enzyme groups are endoglucanase (Cx), exoglucanase (C1) and cellobiase (CB), which act synergistically to degrade cellulose (Zaldivar *et al.*, 2001).

Endo-1, 4-β-D-glucanases (EGases) compose a class of enzymes that can hydrolyse the 1,4-β-D linkages of cellulose. Potential substrates in plant cell walls

include cellulose, xyloglucan and glucomannan. Exo-1, 4-β-glucanases split off either cellobiose or glucose from the non-reducing end of the cellulose. 1,4-β-Glucosidase hydrolyses cellobiose and other water soluble cellodextrines to glucose (Levy *et al.*, 2002).

Additionally, oxidative enzymes are required to fully degrade the cellulose polymers, which include cellobiose:quinone oxidoreductase (cellobiose dehydrogenase), which reduces quinones and phenoxy radicals in the presence of cellobiose, that are then oxidised to cellobiono-δ-lactone (Ander *et al.*, 1990).

1.4.6.3 Expansins

Expansins are proteins that aggravate wall loosening but lack hydrolytic activity (Cosgrove, 2000a; Cosgrove, 2000b). However, most of the evidence suggests that they act in vivo at the cellulose-hemicellulose interface by weakening glucan-glucan interactions resulting in breaking of the hydrogen bonds (Cosgrove, 2000a; Cosgrove, 2000b; McQueen-Mason & Cosgrove, 1994; McQueen-Mason & Cosgrove, 1995).

1.4.6.4 Hemicellulases

The heterogeneity of hemicellulose molecules makes their degradation difficult, requiring a variety of enzymes, generally referred to as hemicellulases. These include xylanases, β-xylosidase, endoglucanases, endomannanases, β-mannosidases, β-mannanases, arabinofuranosidases, α-L-arabinanases and α –galactosidases (Karboune *et al.*, 2009; Saha, 2003).

1.4.6.4.1 xyloglucanases

Xyloglucan cross links are susceptible to cleavage by this enzyme (Pauly *et al.*, 1999).

1.4.6.4.2 Xyloglucan endotransglucosylase-hydrolase (XTH)

XTH catalyse the endo-cleavage and assumption of xyloglucans in a transglycosylation reaction. These transglycosylases were earlier named either xyloglucan endotransglycosylases (XETs) (Smith & Fry, 1991) or endoxyloglucan transferases (EXTs) (Nishitani & Tominaga, 1992). A ‘xyloglucanase’, or ‘xyloglucan-specific endo-β-1, 4-glucanase’, also exhibits

endotransglycosylase activity under certain conditions in vitro (Fanutti *et al.*, 1993; Farkaš *et al.*, 1992).

1.4.6.4.3 Mannanases

These enzymes depolymerise or solubilise galactoglucomannans and/or glucomannans, which are also hydrogen bonded to cellulose (Fischer & Bennett, 1991; Tong & Gross, 1988). They are known from the cell wall in ripening berries of kiwifruit (Redgwell *et al.*, 1991).

1.4.6.4.4 Xylanases

Xylanases are glycosidases (*O*-glycoside hydrolases, EC 3.2.1.x) that catalyze the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan. Being a widespread group of enzymes, they are involved in the production of xylose, a primary carbon source for cell metabolism. In addition to above, xylose are produced in plant cell infection by plant pathogens, and by a plethora of organisms including bacteria, algae, fungi, protozoa, gastropods and anthropods. When it was first reported in 1955, they were originally termed pentosanases. Then they were recognized by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1961 and were assigned the enzyme code EC 3.2.1.8. Their official name is endo-1,4-β-xylanase, but commonly used synonymous terms include xylanase, endoxylanase, 1,4-β-D-xylan-xylanohydrolase, endo-1,4-β-D-xylanase, β-1,4-xylanase and β-xylanase (Collins *et al.*, 2005).

Despite a lack of evidence of degradation activity by xylanases, endoxylanase activity has been detected in a wide range of fruits (Ronen *et al.*, 1991).

As described above, xylans are typically an important structural component of the secondary cell walls of eudicotyledons such as kiwifruit. Given the ability of *Psa* to colonize and form cankers on the woody secondary tissues of its host, a core objective of this research was to investigate xylanase activity as a possible strategy adopted by *Psa* to degrade kiwifruit cell wall material.

1.4.6.5 Lignin degrading enzymes

The enzymes that are responsible for the degradation of lignin are referred to as lignin modifying enzymes (LMEs) and are oxidative in their enzymatic

mechanisms, not hydrolytic. There are two general groups of LMEs with the first including the peroxidases, such as lignin peroxidase, manganese peroxidase and versatile peroxidase, and many phenoloxidases of the laccase type (Martinez *et al.*, 2005).

1.5 Vascular structure of the Eu-dicotyledon plants and vascular pathogens

1.5.1 Vascular structure

The plant vascular system performs two main functions. It is involved in transporting water and minerals from the roots to the aerial parts of the plant and transports sugars from the leaves to parts of the plant that are not involved in photosynthesis. The water and mineral transportation is carried out by the xylem while the sugar transportation is carried out by the phloem. The xylem structure is capable of transporting water to higher elevations through long tubes of connected dead plant cells. In contrast, the phloem consists of interconnected living plant cells (Vinatzer, 2012).

Xylem vessels are made up of elongated cells called vessel elements. At maturity the vessel elements consist of thick, lignified secondary cell walls. They are dead and hence lack all cytoplasmic content. The axial walls of vessel elements are perforated to allow relatively smooth water flow in the longitudinal direction, and a single vessel is comprised of many vessel elements arranged in series (Rudall, 2007).

Xylem is made up of nutrient-poor dead plant cells that are mainly transporting water and minerals. Sperry *et al.* (2006) observed that variation in the diameters of vessels, the tubes that transport water in most woody plants, is considered to be of central adaptive importance. Wider vessels conduct water more efficiently. However they are also likely to be more prone to breaking of the conductive stream and blockage by gas embolism (Olson & Rosell, 2013).

The hydraulic conductance of xylem is influenced by the geometry of the conductive pipeline. The diameter and the length of the pipeline play a major role in this conductance. This relationship is explained by the Hagen Poiseuille law. Water transportation in most woody angiosperms is dependent on a complex

network of vessels. Water movement can occur axially, tangentially and radially when vessels are in contact with adjacent vessels (Jansen *et al.*, 2011).

Inter-vessel pits play an important role within the xylem vascular structure. Since the axial length of a single vessel is limited, the inter-vessel pits facilitate passage of xylem sap from one vessel to another. When moving from roots to leaves, water passes through a large number of inter-vessel pit membranes and therefore connectivity of the vessel network plays an important role in water conductance of angiosperms (Jansen *et al.*, 2011). The small size of the pit membrane pores is a safety mechanism that limits the expansion of gas bubbles from one cavitated vessel to its neighbors and the movement of pathogens from one infected, water-filled vessel to its neighbors as water moves through the xylem system (Nakaho *et al.*, 2000; Sperry & Tyree, 1988; Tyree & Zimmermann, 2002b) unless those pathogens have a means of passing through the pit membrane (Newman *et al.*, 2003).

1.5.2 Vascular pathogens

The intracellular environment of xylem lacks nutrients required by many pathogens. Therefore, the pathogens adapted to survive in the xylem are often capable of surviving in nutrient rich areas outside the xylem tissue relatively well. As such, these pathogens are not considered as exclusive intracellular pathogens. In contrast, pathogens living in the phloem are considered intracellular pathogens since they have adapted to a nutrient rich, living environment (Vinatzer, 2012). It is important to know whether *Psa* is also a xylem adapted pathogen and therefore if it is not an exclusive intracellular pathogen.

Vinatzer (2012) in a commentary article stated that the economic loss caused by vascular pathogens in tree crops is much higher than that with herbaceous annual crops. Further he states that the recently emerged vascular plant pathogens are spread throughout many parts of the world. Phloem-limited *Candidatus* Liberibacter asiaticus, the pathogen thought to be responsible for citrus greening, poses a severe threat to the Florida orange industry. *Pseudomonas syringae* pv. *actinidiae* is affecting the kiwifruit industry in Europe and New Zealand and *P.syringae* pv. *aesculi* is affecting horse chestnut trees in the United Kingdom. Another species of vascular pathogens that poses a great risk to the wine industry

in California and the citrus industry in Brazil is the xylem-adapted *Xylella fastidiosa*.

Type II secretion (T2S) is one of five protein secretion systems in Gram-negative bacteria. T2S allow the export of proteins from within the bacterial cell to the extracellular milieu and/or into target host cells. T2S can uplift the virulence of human, animal and plant pathogens (Cellini *et al.,* 2014). *Ralstonia solanacearum* is another example of a vascular pathogen which uses a type II secretion system to secrete cell-wall-degrading enzymes when invading plant roots. Once *R. solanacearum* reaches the xylem, it lives within dead xylem cells, which obviously cannot be manipulated through its injected effectors.

The xylem sap is poor in nutrients and it is still unknown how bacteria are adapted to life in nutrient-poor xylem. Recent studies indicate that vascular pathogens *P. syringae* pv. *aesculi* and *P. syringae* pv. *actinidiae* contain genes that are capable of sucrose uptake and utilization (Green S, 2010; Marcelletti *et al.*, 2011). Genetic analysis show that sucrose is an important energy source for *R. solanacearum* in xylem (Vinatzer, 2012). It will be interesting to see if genes for sucrose uptake in *P. syringae* pathovars have a similarly important role.

Cucurbit yellow vine disease (CYVD), which can inflict heavy losses to watermelon, pumpkin, cantaloupe, and squash in U.S. is caused by a phloem-colonizing, squash bug –transmitted bacterium called *Serratia marcescens*. Symptoms are phloem discoloration, foliar yellowing, wilting, and plant decline (Bruton *et al.*, 2003).

1.6 Cell wall degrading activity of *Psa*

Marcelletti *et al.* (2011), observed that the *J-Psa* (a strain of *Psa* isolated in Japan, *Psa* biovar 1 in Chapmen et al.'s scheme), *I-Psa and I2-Psa* (the two strains known from Italy, *Psa* 1 and *Psa* 3 in Chapman et al.'s scheme) contain pectin lyase and polygalacturonase genes. These genes are completely similar to their orthologues in the genome of *Pto* T1 (the T1 strain of *P.syringae* pv. *tomato*). The soft–rot bacterium *P. marginalis* also contains the above enzymes. Additionally the *Psa* genome contains genes responsible for catabolism of plant derived aromatic compounds, such as lignin. These catabolism genes are also present in other *P. syringae* pathovars which infect woody hosts namely, *P. syringae* pv.

aesculi (pathogenic on chestnuts) and *Pseudomonas savastanoi* pv. *savastanoi* (pathogenic on olive).

In the above Marcelletti's study all three *Psa* strains were isolated from leaf spot symptoms. However they all show a set of genes involved in the degradation of lignin derivatives and other phenolics. Also all three *Psa* strains have genes supposed to catalyse the degradation of anthranilate to the catechol branch of the β-ketoadipate pathway and to the protocatechuate degradation via the protocathechuate 4,5-dioxygenase pathway. Utilisation of unsubstituted lignin-related compounds and other plant derived phenolic compounds such as mandalate and phenol is possible through these pathways. These lignin utilisation genes are also displayed in other *P. syringae* pathovars involved with woody hosts such as *P. syringae* pv. *aesculi* and *P. savastanoi* pv. *savastanoi* and *P. putida* (a soil-inhabiting species). Therefore it has been suggested that the most highlighted symptom of extensive degradation of the woody tissues of the main trunk and leaders on *Actinidia* plantations during winter is occurring due to the above lignin utilization ability of *Psa.*

1.6.1 Histological evidence of cell wall degrading activity of *Psa*

It has been found that the primary infection sites of the pathogen are the natural openings such as stomata and lenticels, and also lesions at the points of contact. In the leaves the pathogen invades the intercellular spaces of the spongy and palisade parenchyma first (Renzi *et al.*, 2012). *Psa* is considered a 'biotroph', hence, it invades the apoplasm first. Following the infection through the lenticels of stems, the pathogen has been observed in the intercellular and intracellular spaces (Hallett, 2012). It has been established that spongy and palisade parenchymas of leaves are infected by *Psa* and in the infected cells, the cytoplasmic organelles have disappeared and the cell wall has been ruptured in several locations, facilitating movement of the pathogen to neighbouring cells. In infected plants, the bacteria have been found in the dead phloem, suggesting that invasion and infection of the xylem is possible (Renzi *et al.*, 2012).

Xylem invasion is further evidenced by a reduction in vessel size by approximately 50% after the first year of infection (Renzi *et al.*, 2012), although reduced vessel size could be caused indirectly by a reduction in growth following

infection. Furthermore, tyloses were observed in the outermost tree rings, which are important for water conductance. Formation of tyloses suggests activation of defence mechanisms to reduce spread of the pathogen within the xylem, resulting in a reduction in hydraulic conductance. Despite the suggestion that *Psa* invades the xylem, neither Renzi *et al.* (2012) nor Hallett (2012) have directly observed bacteria in the xylem during the initial infection period. However, from the New Zealand kiwifruit industry there have been many anecdotal reports of white bacterial ooze from the cut xylem of plants that are already severely affected, raising questions as to whether the bacterium spreads systemically within the xylem. Leaf inoculation experiments also suggest that the bacterium can spread from leaves into the stem, via the petiole (Ferrante *et al.*, 2012; Serizawa & Ichikawa, 1993 c), leading to speculation that it can spread systemically within the xylem. However, there have been no convincing reports of *Psa* movement within the xylem of stems or leaves (Spinelli *et al.*, 2011).

The entire invasion process from natural openings to possible invasion of xylem vessels strongly suggests the pathogen possesses the capability to either weaken or degrade cell walls of the host plant. However, whether the actual infection process is supported by cell wall degrading mechanisms is unknown (Renzi *et al.*, 2012).

1.6.2 Genomic evidence of cell wall degrading activity of *Psa*

Evidence of the capability of *Psa* to degrade cell w*all* material has been observed in a recent study of the draft genome of the pathogen (Marcelletti *et al.*, 2011).

In the genomic analysis of *Psa* by Plant and Food Research scientists in New Zealand, it has also been observed that there are annotations for well-known CWDE in the genome (McCann *et al.*, 2013). The analyses of the core genome *Psa*, combined with disease history, all support the hypothesis of an independent Chinese origin for both the Italian and the New Zealand outbreaks and suggest the Chilean strains also originate from China (Butler *et al.*, 2013).

1.6.3 Cell wall degrading activity by other pathogens

Despite the fact that cell wall degradation ability of *Psa* has not been previously investigated, the infection process of other xylem dwelling and canker forming

pathogens has been well established. It has been found that *Xylella fastidiosa* spreads through the xylem during the systemic infection of grapevine. In the healthy grapevine, the xylem pit membrane consists of pores with a diameter of 20 nm through which the pathogen of 250 nm x 1,000 nm size is unable to pass. However, the pathogen traverses the pit membrane and passes freely to the adjacent xylem vessel in infected plants. This observation has led to the investigation of a possible pit membrane degrading activity by *X. fastidiosa*. The walls of the vessel elements consist of a lignified secondary cell wall. However, the pit membrane is thought to consist only of middle lamella and parts of primary cell wall containing homogalacturonan pectins and xyloglucans. Sequence analysis of the *X. fastidiosa* genome has shown it to include genes possessing the potential to encode enzymes capable of degrading primary cell wall material. These genes include one polygalacturonase-encoding gene (pglA) and multiple genes encoding endo-1,4-β glucanase (engxcA). It has also been found that the alteration of pit membrane porosity occurs only when polygalacturonase and three endo-1,4-β-glucanases are present at the same time. When the bacterium secretes any one of the enzymes in isolation, it is unable to infect the plant (Perez-Donoso *et al.*, 2010; Roper *et al.*, 2007).

It has also been found that a Diffusible Signal Factor (DSF) produced within the pathogen tightly controls the expression of pglA and engxcA genes (Sun *et al.*, 2011). Mutants of DSF have been found to be hypervirulent on the host. Furthermore, the level of pectin and β-glucan in a culture media containing *X. fastidiosa* regulates the degree of expression of pglA and engxcA genes. This observation suggests that expression of pglA and engxcA genes may be controlled by endogenous factors present in the vessel system that are actively modified by the bacterium as it spreads through the host's xylem (Sun *et al.*, 2011).

These findings suggest that cell wall material degrading enzymes secreted by the pathogen are capable of modifying the porosity of pit membrane allowing the pathogen to pass through the xylem from the infection site to other parts of the plant, and that the degree of expression of cell wall degrading genes is a function of pectin and β-glucan content in the cell wall. If *Psa* is similarly capable of systemic spread through the xylem of kiwifruit plants, then the inter-vessel pit

membrane of kiwifruit also represents a barrier. Whether, and how, *Psa* can overcome the pit membranes of kiwifruit is unknown.

Xylem invasion has also been observed in the case of *Ralstonia solanacearum* during tomato root infection. It has been found that the pathogen initiates colonization on the surface of the root extremities and then progresses gradually towards the xylem. Electron microscope images of the root following colonization have shown the invasion of protoxylem by the pathogen. However, the xylem vessels with the lignified secondary walls have not been penetrated. *Ralstonia solanacearum* has been found to produce extracellular endoglucanase and exo-pectinase capable of degrading primary and secondary plant cell walls. Absence of invasion of lignified xylem vessels suggest that lignin and subarine coated secondary walls act as a physical barrier to the pathogen (Vasse *et al.*, 1995).

1.6.4 Evidence of pathogens altering the expression of host genes to facilitate invasion

It has been found that the chemical composition of the host cell wall material determines the degree of susceptibility to cell wall degrading enzymes of various pathogenic microbes (Lionetti *et al.*, 2007). Pectin is synthesised within the cell in a highly methylesterified form. Following its deposition on the cell wall, pectin molecules are demethylesterified by pectin methylesterase (PME) enzyme. Pectin, in its methylesterified form, is highly resistant to cell wall degrading enzymes, while the demethylesterified form is susceptible to degradation. In the host cells, expression of PME is inhibited by a pectin methylesterase inhibitor (PMEI), thus the conversion of pectin from methylesterified form to demethylesterified form is regulated. In *Arabidopsis*, PMEI is encoded by the expression of two genes, AtPMEI-1 or AtPMEI-2, and it has been found that constitutive expression of these two genes down regulates the expression of PME encoding gene AtPME3, thus reducing the plant's PME activity and rendering the cell wall more resistant to cell wall degrading enzymes of pathogens (Lionetti *et al.*, 2007).

Pectobacterium carotovorum and *Botrytis cinerea* have displayed a capability to utilize host derived molecules to facilitate pathogenicity process by forcing the host enzyme machinery to work in concert with their cell wall degrading enzymes to penetrate the cell wall barrier. The two microbes induce AtPME3 leading to the

expression of PME, forcing demethylesterification of pectin. Consequently, demethylesterified pectin becomes highly susceptible to cell wall degrading enzymes of the pathogens. In the plants infected by the pathogens, a relationship has also been observed between the activation of the jasmonic acid/ethylene (JA/ET) dependent defence pathway and up regulation of AtPME3 (Raiola *et al.*, 2011). Draft genome analysis of *Psa* reveals the presence of pectin lyase genes (Marcelletti *et al.*, 2011). Accordingly, it may be quite possible that *Psa* possesses a similar capability.

1.7 Aims and objectives of the thesis

Sequencing the entire genome of *Psa* by Plant and Food Research has resulted in the discovery of four genes displaying sequence similarities to the xylanase gene. Therefore, there is a significant likelihood that *Psa* possesses a putative xylanase activity. Therefore, the overall aim for this thesis was to investigate host cell wall degrading strategies, particularly the xylanase producing ability, of *Psa*. This investigation sought to test three hypotheses by pursuing the following objectives:

Chapter 2. Objectives: Analysis of the *Psa* genome for possible CWDE. Investigate whether *Psa* can grow in-vitro on a medium containing kiwifruit xylem material, and whether this growth is associated with the appearance of xylanase activity.

Hypothesis I: That the *Psa* genome includes genes encoding for plant cell wall degrading enzymes, particularly xylanase, and that xylanase is produced by the bacterium and results in cell wall degrading activity. Chapter 2 describes the experiments addressing Hypothesis I.

Chapter 3. Objectives: Investigate whether genes responsible for xylanase are expressed during in-planta infection. Identify the genes responsible for *Psa* produced xylanase during in-planta infection, and determine whether there is a reduction in stem strength associated with infection.

Hypothesis II: Bacterial genes encoding for xylanase are expressed during infection and the xylanases produced by the bacterium degrade components of the cell wall of the host plant, reducing the strength of the infected stem. Chapter 3 describes the experiments addressing Hypothesis II.

Chapter 4. Objectives: To ascertain whether xylem vessel architecture such as the length and the spatial distribution of vessels has a significant role in facilitating the movement of *Psa* within the plant. To investigate the movement of *Psa* after inoculation of the leaf.

Hypothesis III: That the kiwifruit vine contains long vessels that extend from the stem to the leaf, creating a possible pathway for Psa movement. That movement of the *Psa* bacterium from leaf to stem is correlated with the presence of open vessels between the leaf and stem. Chapter 4 describes the experiments addressing Hypothesis III.

CHAPTER 2

In vitro Psa Growth and Xylanase Enzyme Activity

2.1 Abstract

The host cell wall degrading activity and invasion strategies of a number of other *P. syringae* pathogens and also histological analysis of *Pseudomonas syringae* pv. *actinidiae (Psa)* infected host plants indicate a possible cell wall degrading mechanism of *Psa* pathogen. In this research the genome of *Psa* was examined to ascertain whether well-known cell wall degrading enzymes were annotated. The degree of similarity of the annotated genes was compared to the cell wall degrading genes of other *Pseudomonas* woody host pathogens. Nine genes with sequence homology to six types of well-known cell wall degrading enzymes (CWDE) were identified, with the degree of amino acid similarity varying between 58% and 98%. Patterns of *Psa* growth were examined in standard nutrient broth media, xylan and casein media and minimal media supplemented with kiwifruit cell wall material. *Psa* was able to grow in kiwifruit xylem media, but the pattern of growth differed from that in the other two media. In the standard nutrient broth media, a typical bacterial growth pattern was observed while in the kiwifruit xylem media the *Psa* count declined initially and thereafter recovered briefly, which is a pattern indicative of inductive bacterial growth. Based on the outcome of the bioinformatics analysis, *in vitro* enzyme assays were conducted for *Psa* in the aforementioned culture media to determine potential activity of xylanase enzyme(s). When *Psa* cultured in kiwifruit cell wall material were assayed with 3,5-Dinitrosalicylic acid (DNSA), a xylanase activity was observed. However, when *Psa* cultured in kiwifruit cell wall material was assayed with Remazol Brilliant Blue (RBB), no activity was detected.

2.2 Introduction

Pseudomonas syringae pathovars cause a number of diseases in woody dicot fruit trees worldwide, are increasingly difficult to control, and as a result are causing significant economic losses. The pathogen has the ability to systemically infect

and kill both young and older trees (Kennelly *et al.*, 2007a). New Zealand's kiwifruit industry had been a success story for a number of years until the *Psa* outbreak impacted the woody kiwifruit vine in 2010 (Vanneste, 2012). As *Psa* is a woody host pathogen, it is important to investigate the pathogen's potential to synthesise CWDE. Therefore in this thesis research, *in vitro* studies of *Psa*'s growth and propagation in various media, one of which was supplemented with kiwifruit woody xylem material, were conducted. An understanding of *Psa*'s survival and propagation on kiwifruit material under *in vitro* conditions provides important insights into the pathogen's capability to degrade and use woody kiwifruit tissue as an energy source under *in planta* conditions. Until now, there have been limited investigations of the *in vitro* growth response and enzyme activity of *Psa* to kiwifruit material (Nardozza *et al.*, 2015).

Genomic studies conducted into the *Psa* genome have revealed genes contributing to the pathogen's adaptation and virulence. The pathogen harbours genes encoding for well-known CWDE (Marcelletti *et al.*, 2011; McCann *et al.*, 2013). However, no research has been conducted to date to investigate the expression of these genes or the activity of the enzymes they encode for.

The importance of conducting *in vitro* experiments to detect expression of CWDE is further supported by the outcomes of a number of *in vitro* studies conducted on other Pseudomonads in low nutrient media. In *Pseudomonas fluorescens* 23F, the carbon-phosphorus bond cleavage activity (Phosphonoacetate hydrolase) to utilize phosphonoacetate as the sole carbon and phosphorus source without having to starve for phosphate was detected *in vitro*. The observation was vital in studying further behaviour of the pathogen within the host (McMullan & Quinn, 1994). In an *in vitro* study conducted into copper resistance in *Pseudomonas syringae* pv. s*yringae* recovered from blossoms of sweet and sour cherry, copper resistant isolates were detected by culturing bacteria on low nutrient glycerol medium amended with various concentrations of cupric sulfate. It was observed that 14 out of 17 copper resistant isolates contained a single plasmid of 46-73 kb. When the plasmids were transferred to copper-sensitive strains and infiltrated into bean leaves, populations of copper sensitive strains were reduced significantly (Sundin *et al.*, 1989). Important parallels can be drawn with *Psa* which also has genes coding for resistance to copper and antibiotics (Marcelletti *et al.*, 2011).

Composition of extracellular polysaccharides (EPS) produced by *Pseudomonas phaseolicola in vitro* were studied and it was found that the composition of EPS is dependent on the primary carbon source in the culture media (Gross & Rudolph, 1987). Therefore, there is a strong case to study the growth and propagation of *Psa in vitro*, especially on a medium containing material from woody parts of the kiwifruit vine, to determine whether *Psa* is able to utilize kiwifruit wood as an energy source by synthesising CWDE.

In this study the *in vitro* xylanase activity of *Psa* was studied using different enzyme assays, while also investigating the growth of *Psa* in different media. It was hypothesised that *Psa* produces xylanases *in vitro* when it is grown in energy-deficient media with a xylan-containing substrate. Xylanases are hydrolytic enzymes which randomly cleave the β1,4 backbone of the complex plant cell wall polysaccharide xylan. Different forms of these enzymes have been identified, with a variety of amino acid sequences, resulting in different structures, modes of action, substrate specificities (different xylanases have different activities against various xylan structures), hydrolytic activities and physicochemical characteristics (Collins *et al.*, 2005). Xylanases are classified as glycoside hydrolases, and a wide variety of xylanases have been identified as belonging to a number of different families within this more general class of enzyme. The most studied xylanases belong to glycoside hydrolase families 10 and 11 (Collins *et al.*, 2005; Li *et al.*, 2000). Since the assays for xylanase with *Psa* described in this thesis appeared to show different types of activity *in vitro* and *in planta*, it remains unclear which family they belong to. There were two objectives in this study. The first was to ascertain the pattern of *Psa* growth in a minimal culture medium supplemented with kiwifruit wood, compared to a standard rich medium and another minimal medium supplemented with xylan only. The second objective was to assess the potential for xylanase synthesis by *Psa* by examining the pathogen's genome for potential xylanase genes, and by testing for any xylanase activity produced during in-vitro culture.

2.3 Materials and methods

2.3.1 Bioinformatic analysis of *Psa* genome for putative genes encoding plant cell wall degrading enzymes.

A published genome (Genbank accession number NZ_MOMK01000370) was used in this research to identify putative genes encoding for cell wall degrading enzymes (CWDE) (McCann *et al.*, 2013). The bioinformatics analysis was carried out using Geneious version 7.1.9 (Biomatters, Switzerland) (Kearse *et al.*, 2012). Genes that were annotated in the *Psa* genome (referred to as *Psa*-V by McCann *et al.*, 2013) were full genes and not fragments or frameshifts.

The nucleotide (nt) sequence of each putative CWDE gene was translated to their respective protein amino acid (aa) sequence in all six open reading frames (ORFs) that were selected. The resulting six protein sequences were compared with protein sequences deposited in Genbank using BLAST (NCBI, 2016; Stephen *et al.*, 1997) to identify homologous proteins. The top 100 hits were obtained and investigated further. For each CWDE, the Enzyme Commission (EC) number was recorded using the ExPAsy Bioinformatics Resource portal (http://enzyme.expasy.org/).

2.3.2 Growth of *Psa* in nutrient broth

The *Pseudomonas syringae* pv. *Actinidiae* strain used in this experiment is referred to as "*Psa-V*" (McCann et al., 2013), which is the isolate responsible for disease outbreaks in Italy (in 2008) and in New Zealand (in 2010). For this research, a culture of *Psa-V* (Strain 10627) was obtained from Plant and Food Research, Ruakura, New Zealand. Bacteria were maintained at -80ºC in ampules containing nutrient broth and 5% glycerol. To culture in Nutrient Broth, 13 grams of standard Nutrient Broth 'E' (Thomas Scientific) was dissolved in 1 litre (L) of deionised water and 250 ml was dispensed into 1 L flasks. Three flasks were maintained including the negative control. The media were autoclaved for 45 minutes. Two flasks were inoculated each with 6.25 ml of 9.21 x 10^9 colony forming units (CFU) ml^{-1} *Psa* solution giving approximately 2.5% V/V bacterial suspension in the final solution. All the flasks including the negative controls were maintained in a shaking incubator with a shaking speed of 160 Rev min^{-1} at 25ºC. Serial dilutions, plating on nutrient agar and CFU counts were performed

from the first 8 hours post inoculation, every 8 hourly for the 1st day and every day for the following 10 consecutive days (Cappuccino & Sherman, 2008; Tortora, Funke, & Case, 2007).

2.3.3 Growth of *Psa* in Oat Spelt xylan, Casein and Ammonium Chloride NH_4Cl

Psa was grown on a medium designed to promote xylanase production in *Bacillus* sp. (Pham *et al.*, 1997). Two Erlenmeyer flasks containing the oat spelt xylan medium were inoculated each with 6.25 ml of 9.21 x 10^9 CFU ml^{-1} *Psa* solution giving approximately 2.5% V/V bacterial suspension in the final solution. The experiment was conducted in duplicate. Three flasks including the negative controls were maintained in a shaking incubator with a shaking speed of 160 Rev min^{-1} at 25ºC. Serial dilutions and plating were performed from the 1st day and every day for the following 10 consecutive days and CFU counts on Nutrient Agar plates were recorded.

2.3.4 Growth of *Psa* in minimal media supplemented with kiwifruit xylem

The minimal medium, a culture medium for microorganisms that contains the minimal mineral necessities for survival of the wild-type used in this experiment was prepared using a recipe published by Chakrabarty *et al.* (1973) and Demain (1958) with modifications to suit *Psa*.

1. PAS (phosphate ammonium salts) 7.75 ml/L
   0.1 M K_2HPO_4
   0.05 M KH_2PO_4
   0.160 M NH_4Cl
2. 100X Salts 10ml/L
   $MgSO_4$ (anhydrous) 19.5 gm/L
   $MnSO_4.H_2O$ 5gm/L
   $FeSO_4.7H_2O$ 5gm/L
   $CaCl_2.2H_2O$ 0.3gm/L
   Ascorbic acid 1.0gm/L

Pieces of stem obtained from mature *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' vines located at Plant and Food Research, Ruakura, New Zealand, were used to prepare the xylem powder. The bark of the stems was physically removed from the surface with a knife and the resulting xylem tissue was washed in hot water rubbing thoroughly to remove any sugars and starch associated with phloem and the bark. The tissue was cut into small pieces and oven dried at 60°C. The oven-dried pieces of kiwifruit were ground into xylem powder using a saw grinder (Pulverisette, Fritsch GmbH, Germany). The resulting powder is referred to as kiwifruit xylem in this thesis.

The medium used in this experiment was a minimal medium supplemented with 0.5% kiwifruit xylem (5g of kiwifruit xylem in 1L minimal media solution). The medium was autoclaved for 45 minutes twice to prevent any microbial contamination from the kiwifruit xylem. Duplicates of 0.5% kiwifruit medium were inoculated with 7.35 x 10^{11} CFU ml^{-1} *Psa* solution. Non-inoculated minimal media supplemented with kiwifruit xylem and nutrient broth were maintained as negative controls. The negative controls were also periodically checked for any bacterial growth by streaking on to Nutrient Agar plates. All the flasks including the negative controls were maintained in a shaking incubator with a shaking speed of 160 Rev min^{-1} at 25º C. Serial dilutions and plating were performed from the first day post inoculation, every day for 11 consecutive days and again on the 13^{th} day and the respective CFU counts on the plates were recorded (Cappuccino & Sherman, 2008; Tortora et al., 2007).

2.3.5 Enzyme assays

Two treatments were included in this experiment. The first was 0.5% xylem in minimal medium with *Psa* (henceforth referred to as treatment a), the second was 0.5% xylem in minimal medium without *Psa* (Blank Control, henceforth referred to as treatment b). *Psa* cultures, maintained in nutrient broth and glycerol at -80ºC, were used in this experiment to prepare the inoculum. One ampule of frozen *Psa* was added to 25 ml sterile nutrient broth in 100 ml Erlenmeyer flask. Flasks were kept in a shaking incubator at a shaking speed of 160 rev min^{-1} at 25ºC for

24 hours. A sterile loop of culture was spread on to a nutrient agar plate after 24 hours and incubated in order to ensure that only *Psa* was grown in the culture.

Minimal media (250 ml) supplemented with 0.5% kiwifruit xylem prepared earlier was added to two flasks and labelled as treatment (a) and treatment (b). 12.5 ml of *Psa* inoculum was added to flask (a) and 12.5 ml of sterile nutrient broth was added to flask (b). Both the flasks were kept in the shaking incubator, 160 rev min^{-1} at 25ºC. Following inoculation, 16 ml samples from the above cultures were taken on six days (i.e. Day 1, Day 3, Day 5, Day 7, Day 9, and Day 11) and assays performed.

Two different assays were used to test for xylanase in the resulting cultures. The DNSA assay is a general reducing sugar assay that detects sugars released primarily by exo-xylanase action on the reducing end of long-chain xylan molecules (Jeffries *et al.*, 1998). The RBB assay is an alternative assay that measures dye release from a conjugate of Remazol Brilliant Blue (RBB) and xylan, and is considered more suitable for quantifying the activity of endo-xylanases capable of hydrolyzing the internal β1-4 linkages of the xylan molecule (Bailey *et al.*, 1992).

Oat spelt xylan (0.2%) in 100mM hepes buffer at PH 7.0 was used as the substrate for the DNSA assay. DNSA reagent was prepared as follows.

Dinitrosalicylic Acid	1.36 g
Phenol	2.70 g
Sodium Sulphite	0.68 g
Sodium hydroxide	13.6 g
Potassium Sodium Tartarate	47.86 g

In water to 1000 ml total volume, and filtered through a 0.2 μm membrane filter.

The principle of the RBB assay is that RBB-Xylan is cleaved with endo-xylanase to low molecular weight dyed fragments which remain in solution on addition of ethanol to the reaction mixture. The high molecular weight fragments are removed

by centrifugation and the absorbance of the supernatant is measured at 595 nm. The endo-xylanase activity has a linear relationship to the absorbance of the supernatant measured. RBB xylan (0.1%, Sigma-Aldrich, M5019) in 50 mM citric acid at pH 5.5 was used as the substrate for the RBB assay.

While cultures were growing, a standard curve was constructed for DNSA using xylanase from *Thermomyces lanuginosus.* From the standard curve it was observed that 25 µl of 0.1 mg ml^{-1} of *Thermomyces* xylanase was required for the assay with 400 µl of 0.2% oat spelt xylan (starting substrate) to generate sufficient xylose product to create an absorbance within the linear portion of the standard curve.

Protocols were developed to concentrate and separate the cultures into bulk, extracellular and intracellular fractions. Using VIVASPIN ultrafiltration concentrators (membrane 10,000 MWCO PES), 16 ml of total culture were concentrated approximately 17 fold (5000 RPM for 60-90 minutes with Rotor F15 in BECKMAN Induction Drive Centrifuge). DNSA and RBB Assays for xylanase were performed as per the standard xylanase assay method with some modifications as recorded in Chapter 3 in triplicates (Bailey, 1988; Bailey *et al.*, 1992; Miller, 1959). The assay tubes were incubated for 30 minutes at pH 7.0 and 30ºC for DNSA, and 12 hours at pH 5.5 and 37ºC for RBB. Assays were conducted separately for culture retentate and the flow-through on day 1 and day 3. From day 5 and onwards, two 16 ml samples from inoculated culture (treatment a) were obtained. One of the 16 ml samples was concentrated 17 fold and the concentrate (retentate) and the flow-through assayed as done for day 1 and day 3 samples. A sample of 16 ml from the non-inoculated culture (treatment b) was also obtained from day 5, concentrated and assayed in the same way. The other 16 ml sample from treatment a, was centrifuged and the pellet and supernatant separated. The supernatant was concentrated using the same VIVASPIN concentrators and the retentate and flow-through assayed. The pellet (xylem particles and the *Psa* cells) were re-suspended in one ml of HEPES buffer (pH 7.0), distributed by briefly vortexing for 15 seconds and one portion of the suspension was assayed. The other portion was sonicated as per standard bacterial sonication and then assayed. As described above, xylanase from *Thermomyces lanuginosus* was used as positive control on day 5 for the DNSA assay. A cocktail

of M1, M3 and M6 xylanase (M1 – endo-1,4-β-Xylanase from *Trichoderma viridae*, M3 – endo-1,4-β-Xylanase from *Trichoderma longibrachiatum*, M6-endo-1,4-β-Xylanase from rumen microorganism, these enzymes obtained from Megazyme, Bray, Ireland) was used as the positive control for the RBB assay. The positive controls were used to confirm that the assay was working properly, as well as determine if there was any inhibition from the *Psa* culture (whether the organism or the medium) towards enzyme activity even if the enzyme was present. Therefore, assays were conducted with 100 µl xylanase from *T. lanuginosus*/M1, M3 & M6 and 50 µl xylanase from *Thermomyces lanuginosus*/M1, M3 & M6 and 50 µl of treatment b as positive controls in the activity. Concentrated whole culture samples from treatment a of each sampling day were kept at -20ºC and those samples were assayed after further concentration by Microcon Centrifugal Filter Units (MilliporeSigma, USA). In all the above assays, absorbance was measured spectrophotometrically at 575 nm for DNSA (UV-160, Shimadzu, Japan) and 595 nm for RBB (Microplate reader, BioTek, USA) against a blank without an enzyme.

t-test was performed on enzyme assay results to analyze whether the results are statistically significant at 0.05.

2.4 Results

2.4.1 Bioinformatic analysis of *Psa* genome

Nine sequences with homology to six types of known CWDE genes were identified as present in the *Psa* genome (Table 2.1). Among these sequences, four xylanases were identified as polysaccharide deacetylase, with transcript lengths ranging from 819 - 1162 nucleotides (Table 2.1). Amino acid translations of the annotated CWDE sequences from the *Psa* genome were compared with enzymes of other woody canker causing *Pseuodomonas syringae* species, and enzymes from non-*Pseudomonas* xylem dwelling pathogens that have been known to express CWDE (Table 2.2). Three of the four polysaccharide deacetylase sequences shared a high level of homology with known enzymes from other canker causing *Pseuodomonas syringae* pathovars and other *Pseudomonas* plant pathogens (Table 2.2).

Table 2.1 Selected annotated CWDE genes in the *Psa* genome. EC number, number of nucleotides (nt), number of amino acids (aa) and genome position are provided.

CWDE	EC Number	Gene (nt)	Protein (aa)	Genome Position
Polygalacturonase	3.2.1.15	1599	533	5,231,888 -> 5,233,486
Polysaccharide deacytyalse 1	3.2.1.8 3.2.1.32 3.2.1.136 3.2.1.156	1161	387	1,280,788 -> 1,281,948
Polysaccharide deacetylase 2	3.2.1.8	819	273	3,684,420 -> 3,685,238
Polysaccharide deacetylase 3	3.2.1.8	882	294	2,330,772 -> 2,331,653
Polysaccharide deacetylase 4 family protein	3.2.1.8	930	310	2,938,401 -> 2,937,472
Cellulase (Glucanase)	3.2.1.4	1212	404	1,925,122 -> 1,926,333
Pectin Lyase	4.2.2.2	1272	423	2,265,503 -> 2,266,774
Laccase multicopper polyphenol	1.10.3.2	729	243	5,385,657 -> 5,384,929
Catechol 1,2-dioxygenase	1.13.11.1	906	302	4,271,486 -> 4,270,581

Table 2.2 Comparison of *Psa* annotated CWDE enzymes with other *Pseudomonas syringae* organisms mainly infecting woody hosts and xylem pathogens. Grade % indicates the homology of annotated enzymes of *Psa* to CWDE proteins (enzymes) in the other pathogens.

Psa			**BLASTp Hits**			
Enzyme	Gene (nt)	Protein (aa)	Grade Score (%)	Bacterium	GenBank No. #	Protein (aa)
Polygalacturonase	1599	533	98.1	*Pseudomonas syringae* pv. *theae ICMP 3923*	WP_020325471	532
			97.7	*Pseudomonas syringae* pv. *tomato str. DC3000*	NP_793726	532
			96.4	*Pseudomonas amygdali*	WP_005740729	506
			93	*Pseudomonas savastanoi* pv. *savastanoi*	WP_002552584	532
			76.2	*Ralstonia solanacearum GMI1000*	NP_522441	531
			75.9	*Ralstonia solanacearum PSI07*	YP_003749800	530
			75	*Xanthomonas translucens* pv. *translucens DSM 18974*	WP_003475290	558
			75	*Xylella fastidiosa M23*	YP_001830252	544
			74.6	*Xylella fastidiosa Temecula1*	NP_779680	535
			74.6	*Xylella fastidiosa (PgIA)*	AFJ79979	544
Polysaccharide deacetylase 1	882	294	99.5	*Pseudomonas syringae* pv. *tomato str. DC3000*	NP_793964	293
			99.5	*Pseudomonas amygdali* pv. *morsprunorum*	WP_060402342	293
			99.3	*Pseudomonas syringae* pv. *pisi str. 1704B*	EGH41543	293
			99.3	*Pseudomonas syringae* pv. *viburni*	WP_044419948	293
			99.3	*Pseudomonas syringae* pv. *coryli*	WP_046234980	293

Table 2.2 (contd)

Psa			**BLASTp Hits**			
Enzyme	Gene (nt)	Protein (aa)	Grade Score (%)	Bacterium	GenBank No. #	Protein (aa)
Polysaccharide deacetylase 2	1161	387	63	*Pseudomonas syringae* pv. *morsprunorum str. M*	WP_005736640	403
			62.3	*Pseudomonas syringae* pv. *tomato T*	WP_007244172	403
			58.7	*Pseudomonas syringae* pv. *aesculi str. 0893_23*	WP_005733962	403
			58.6	*Pseudomonas savastanoi* pv. *Savastanoi*	WP_002556135	403
			58.1	*Pseudomonas syringae* pv. *avellanae str. ISPaVe013*	WP_003408510	403
			57.9	*Pseudomonas syringae* pv. *avellanae str. ISPaVe037*	WP_003414950	403
Polysaccharide deacetylase 3	819	273	98.7	*Pseudomonas amygdali*	WP_005737625	299
			98.3	*Pseudomonas syringae* pv. *Avii*	WP_057430319	299
			98.3	*Pseudomonas avellanae*	WP_024420634	299
			98	*Pseudomonas syringae* pv. *maculicola*	WP_054069241	299
			98	*Pseudomonas syringae* pv. *Persicae*	WP_046463531	299
Polysaccharide deacetylase 4 family protein	930	310	99.5	*Pseudomonas avellanae BPIC631*	WP_005619456	309
			99.2	*Pseudomonas syringae* pv. *delphinii*	WP_057435903	309
			99.2	*Pseudomonas syringae* pv. *viburni*	KPZ14289	339
			99	*Pseudomonas syringae* pv. *Tomato T1*	WP_007245254	309
			98.9	*Pseudomonas syringae]* pv. *tomato str. DC3000*	WP_005765316	309

Table 2.2 (contd)

Psa			**BLASTp Hits**			
Enzyme	Gene (nt)	Protein (aa)	Grade Score (%)	Bacterium	GenBank No. #	Protein (aa)
Cellulase (Glucanase)	1212	404	99	*Pseudomonas syringae* pv. *morsprunorum*	WP_005736640	403
			98.4	*Pseudomonas syringae* pv. *tomato str. DC3000*	NP_790865	403
			94.7	*Pseudomonas syringae* pv. *aesculi str. 0893_23*	WP_005733962	403
			94	*Pseudomonas syringae* pv. *avellanae str. ISPaVe013*	WP_003408510	403
			93.8	*Pseudomonas syringae* pv. *avellanae str. ISPaVe037*	WP_003414950	403
Pectin Lyase	1272	423	99	*Pseudomonas syringae* pv. *morsprunorum*	WP_005739467	421
			98.3	*Pseudomonas avellanae BPIC 631*	WP_005620151	421
			98.1	*Pseudomonas syringae group genomosp. 3*	WP_007246176	421
			91.8	*Pseudomonas syringae* pv. *avellanae str. ISPaVe037*	WP_003418628	421
			91.6	*Pseudomonas syringae pv. syringae B64*	WP_004416371	421
			91.2	*Pseudomonas savastanoi* pv. *savastanoi*	WP_002554938	415
Laccase multicopper polyphenol	729	243	98.8	*Pseudomonas syringae* pv. *morsprunorum*	WP_005740193	242
			98.8	*Pseudomonas syringae* pv. *tomato str.DC 3000*	NP_790674	242
			98.3	*Pseudomonas syringae* pv. *lachrymans str. M302278*	WP_005769280	242
			94.4	*Pseudomonas syringae* pv. *avellanae str. ISPaVe037*	WP_003414752	242
Catechol 1,2-dioxygenase	906	302	98.8	*Pseudomonas syringae* pv. *morsprunorum*	WP_005738670	301
			98	*Pseudomonas syringae pv. aesculi str. 0893-23*	WP_005733729	301
			98	*Pseudomonas savastanoi* pv. *savastanoi NCPPB 3335*	EFH98912	309

2.4.2 Growth of *Psa* in different media

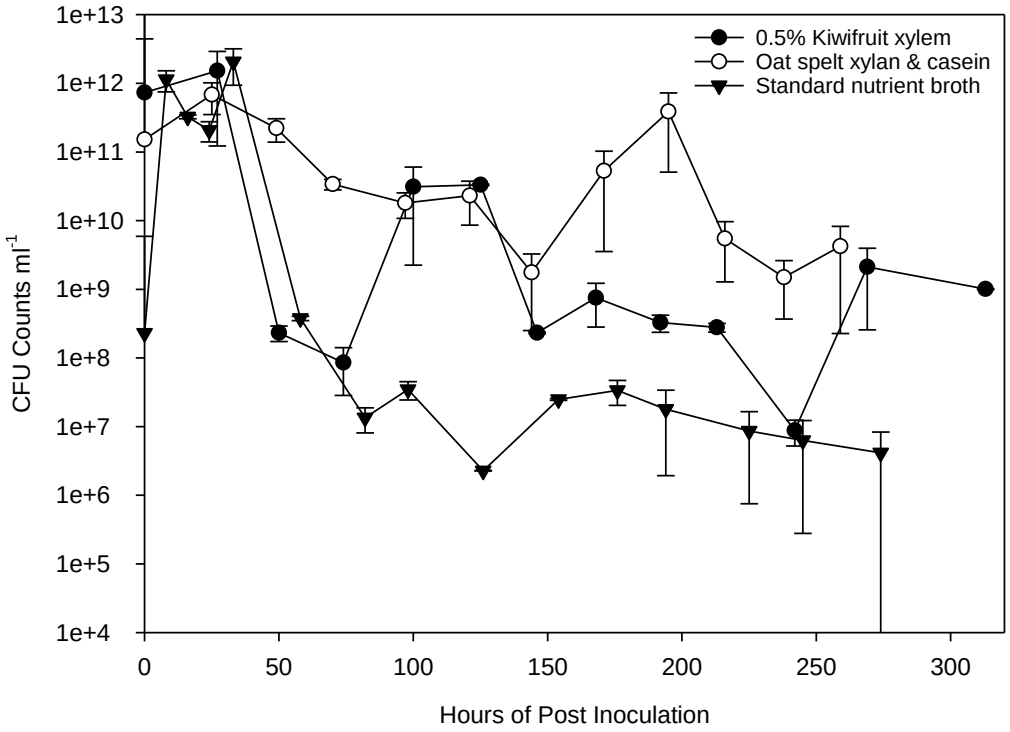

Figure 2-1 *Psa* colony density expressed as CFU in selected media. Liquid cultures held in conical flasks were incubated in a shaking incubator with a shaking speed of 160 Rev min-1 at 25ºC (n=5, means ± SE).

Psa in nutrient broth (NB) showed the highest growth rate in the first two days, then CFU declined during the 10 days thereafter. *Psa* in oat spelt xylan/casein and *Psa* in 0.5% kiwifruit xylem media showed increases and decreases in CFU counts reaching a peak at 195 hours post inoculation in oat spelt xylan medium and two peaks at 100 and 269 hours post inoculation in the kiwifruit medium (Fig 2.1).

2.4.3 *In vitro* xylanase activity of *Psa*

Xylanase activity was detected in *Psa* inoculated 0.5% kiwifruit xylem cultures using DNSA. *Psa* inoculated cultures showed an activity in the concentrated supernatant using the DNSA assay conducted post inoculation ($P > 0.05$; Fig 2.2 A). On day 9, *Psa* inoculated culture supernatant showed the highest activity which was 23.6 - fold higher than the non-inoculated cultures. The activity values were on average 17 to 20 – fold higher than that of non-inoculated cultures during the period

of the experiment. The level of activity was comparable to that measured with non-inoculated culture supplemented with a positive control. In contrast, there was no positive xylanase activity detected in *Psa* inoculated culture supernatant using RBB assays conducted post inoculation in minimal media supplemented with 0.5% kiwifruit xylem ($P > 0.05$; Fig. 2.2 B). However, the RBB assay indicated significant activity in the positive control.

Across all cultures, xylanase assay types and various levels of concentration, significant xylanase activity was only detected in the concentrated supernatant of centrifuged samples from xylanase-active cultures. No activity was detected in assays of re-suspended intact, sonicated (lysed) pellets or flowthrough, suggesting that the xylanase was present in an extracellular form.

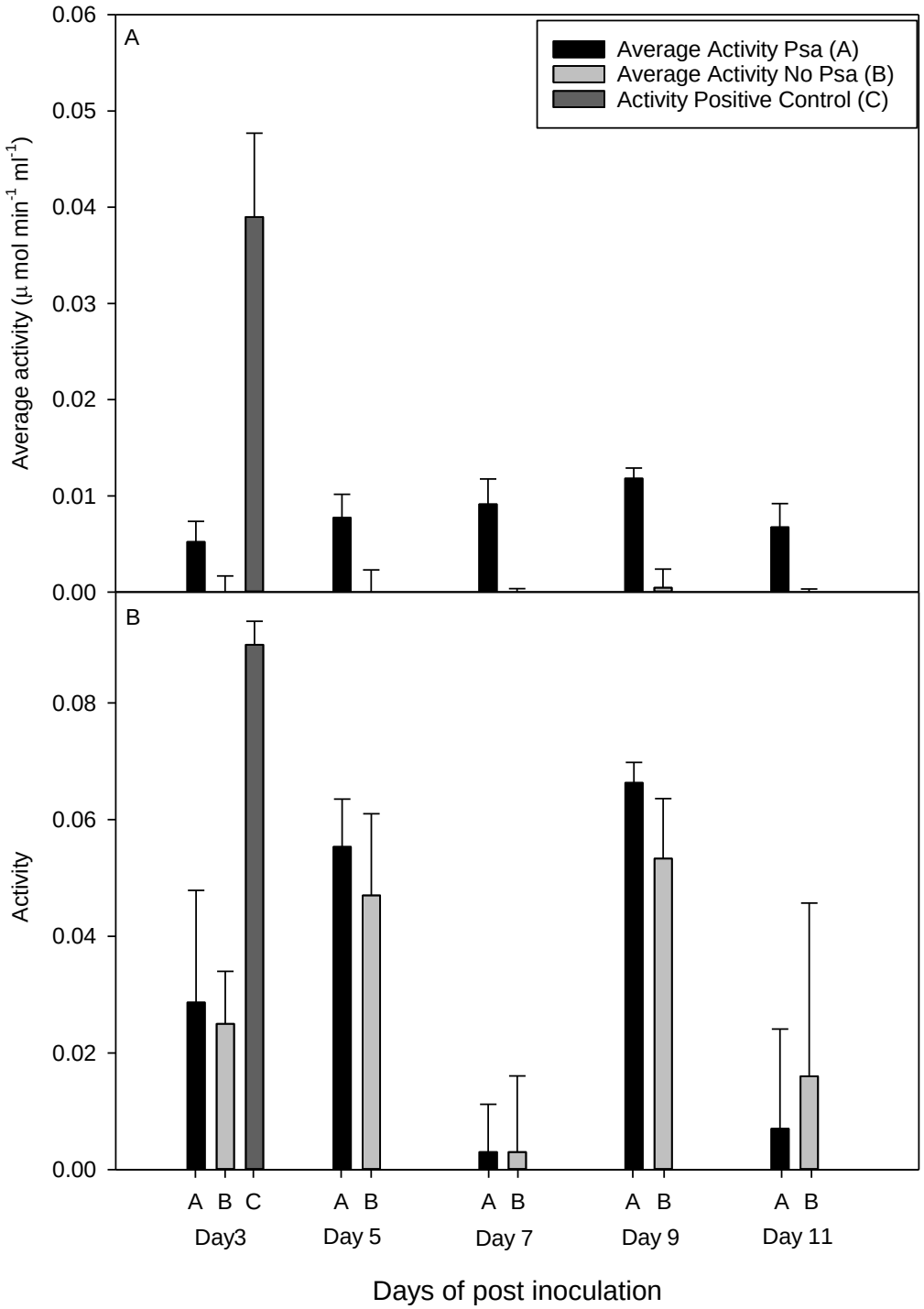

Figure 2-2 Xylanase activity in the concentrated supernatant of minimal media supplemented with 0.5% kiwifruit xylem. The positive control was only included in assays of the Day 3 culture. (A) DNSA assay; (B) RBB assay (n=5, means ± SE).

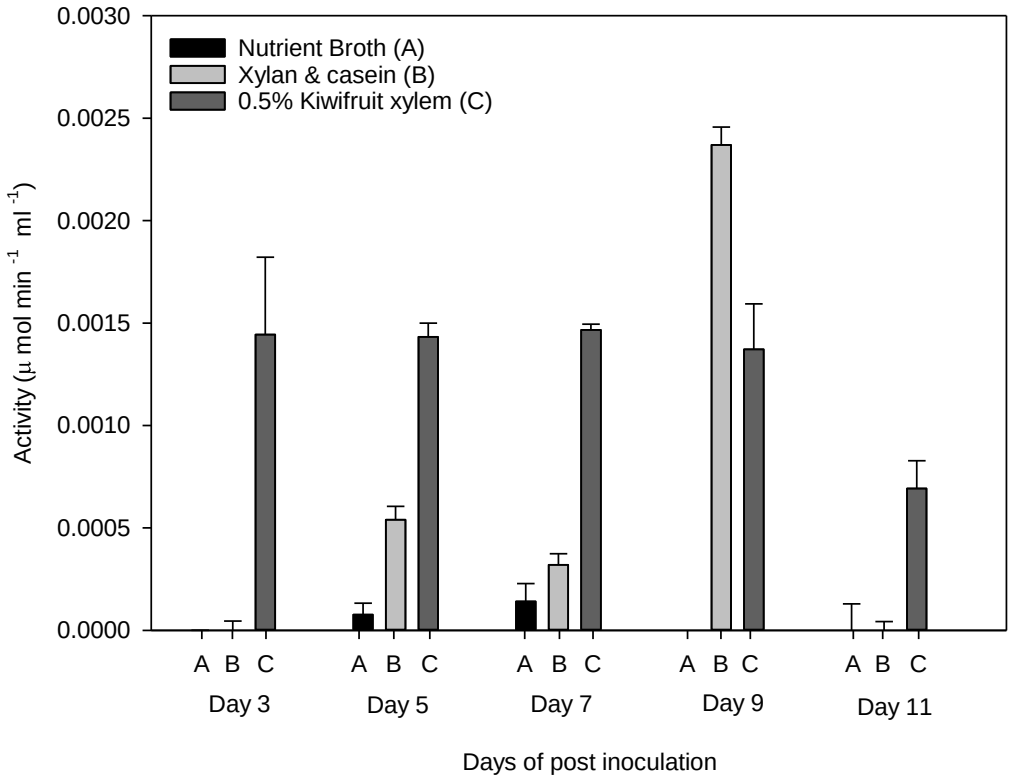

Figure 2-3 DNSA assay conducted in different media inoculated with *Psa* to evaluate xylanase activity. Assays were conducted on the concentrated supernatant of each inoculated media. (n =5, means ± SE).

The xylanase activity observed when *Psa* was cultured in minimal media supplemented with 0.5% kiwifruit xylem was on average 5-fold higher than activity detected in cultures grown in nutrient broth medium or xylan and casein medium on all days of post inoculation except on day 9 ($P < 0.05$; Fig 2.3). The first peak of *Psa* CFU counts in 0.5% kiwifruit xylem medium on the fourth day (100 hrs) post inoculation corresponded with xylanase activity detected on day 3 post inoculation in the same culture (Figs 2.1 & 2.3). In the xylan and casein medium, the first peak in *Psa* CFU counts was detected by the eighth day (195 hrs) of post inoculation and its xylanase activity was highest on day 9 post inoculation.

2.5 Discussion

The results were consistent with the hypothesis that *Psa* contains genes encoding for cell wall degradation (Table 2.1) and that a gene for xylanase is expressed and is active in in vitro cultures of *Psa*. Extracellular xylanase activity was detected in the culture of minimal media supplemented with 0.5% kiwifruit xylem using the DNSA assay (Figs. 2.2, 2.3).

2.5.1 Bioinformatics analysis of *Psa* genome for putative genes encoding plant cell wall degrading enzymes

Genes for six types of CWDE in total were found in the *Psa* genome. Polygalacturonase from the Psa genome has 74.6% homology to polygalacturonase present in *Xylella fastidiosa*, a well-known xylem dwelling pathogen (Table 2.2) whose polygalacturonase enzyme activity has been confirmed (Perez-Donoso *et al.*, 2010; Roper *et al.*, 2007). *Pseudomonas syringae pv. tomato str. DC3000 (Pto)* is a woody pathogen whose xylanase has 99.5% homology to *Psa* xylanase. Therefore one future research question concerns *Pto*'s enzyme activity. If *Pto*'s enzyme activity has not been established, future research could be envisaged in this direction. Furthermore, well known woody host pathogens *Pseudomonas savastanoi and Pseudomonas syringae pv. aesculi* proteins show 93.7% and 93.9% homology to *Psa* xylanase, respectively. Catechol 1,2-dioxygenase is involved in the lignin degradation pathway (Marcelletti *et al.*, 2011). *Psa* catechol 1,2-dioxygenase has 98.8%, 98% and 98% similarity to the proteins found in *Pseudomonas syringae* pv. *morsprunorum, Pseudomonas syringae* pv. *aesculi* str. *0893-23* and *Pseudomonas savastanoi* pv. *savastanoi NCPPB 3335*, respectively. Despite the evidence of genetic homology of *Psa* CWDE with above pathogen's CWDE, the actual cell wall degrading activity of these pathogens has not been investigated.

The *Psa* genome contains sequences with homology to laccase and catechol dioxygenase, enzymes typically associated with lignin degradation in wood degrading micro-organisms (Tables 2.1 and 2.2). Wood is highly resistant to decay because of the presence of lignin (Floudas *et al.*, 2012). Lignin is also a major pool of organic carbon, but white rot fungi in the class Agaricomycetes are the only organisms considered capable of substantial lignin decay. Surprisingly, Marcelletti *et al.* (2011) stated that extensive degradation of the woody tissues of the main trunk

and leaders in *Psa* infected kiwifruit during winter is due to utilisation of lignin by the pathogen. However, there is no direct evidence to support this claim. Potential mechanisms of lignin degradation by *Psa* should be an area for further research, starting from the enzyme sequences identified in this study.

The genes encoding polygalacturonase and pectin lyase enzymes display a significant degree of similarity to those found in *Pseudomonas savastanoi* and *Pseudomonas syringae pv. aesculi* (Table 2.2). This observation has already been made by Marcelletti *et al.* (2011). Furthermore, genes encoding for xylanase in *Pseudomonas syringae* pv. *morsprunorum* were 98.5% identical to the xylanase annotated in the *Psa*. Future experiments should be initiated to determine if cell cultures of these bacteria are expressing xylanase, leading to degradation of cell wall material.

2.5.2 Growth of *Psa* in different media

Psa in nutrient broth showed the characteristic bacterial growth pattern while *Psa*'s growth in xylan plus casein or the 0.5% kiwifruit xylem media deviated from a constitutive growth pattern. In the characteristic constitutive growth pattern of bacteria, upon inoculation there is an initial lag period followed by exponential-phase growth. During the first two to three days, bacterial density remains high and thereafter cells enter the death phase. After ~99% of the cells die, the survivors can be maintained under long-term stationary-phase culture conditions for months or years (Finkel, 2006). However, there can be instances where any one or several of these phases may be absent. The lag and the acceleration (exponential) phases may be suppressed under suitable conditions and the retardation (death) phase can be short and slight. Sometimes the stationary phase also becomes imperceptible. In addition more complex growth patterns are very common (Monod, 1949).

It is pertinent to draw parallels between the characteristic bacterial growth pattern and the growth pattern of *Psa*. *Psa* behaved in three different ways in three different media. *Psa* in nutrient broth followed a growth pattern typical of Gram negative bacteria; a log or exponential phase, followed by a stationary phase, and ending with a phase of prolonged decline. The rapid growth can be attributed to the rich medium in which the cells were initially cultured. *Psa* in the nutrient broth medium reached

its stationary phase within 24 hours and further reached the phase of decline after 48 hours (Figure 2.1).

An increase in *Psa* CFU counts was observed in the xylan and casein medium during the first 24 hours followed by a decline in CFU counts between 24 to 48 hours post inoculation with the decline continuing to day 6. From day 6 onwards, an increase in CFU count was observed until day 8. The second phase of growth was not significant compared to the initial growth phase. From day 8 onwards, a decline in CFU counts was observed.

Psa demonstrated a completely different growth pattern in minimal medium supplemented with 0.5% kiwifruit xylem with a deviation from the characteristic constitutive bacterial growth curve. The minimal medium does not contain a readily available source of carbon and energy for *Psa* to utilize for its survival or multiplication. Therefore, as observed in Figure 2.1, it is possible for *Psa* density to enter into the declining phase after the first 24 hours (Day 1). After the 3rd day, the bacteria multiply and showed a steady increase in CFU. The cell density reached a plateau by day 4 and maintained a constant density until day 5. From day 5 onwards, a decline in CFU occurred until day 10. After day 10, there was a rise but the 2nd rise was less significant compared to the 1st rise. This pattern of growth can be attributed to the ability of phytopathogens to produce a range of enzymes capable of degrading complex polysaccharides of the plant cell wall (Bateman & Basham, 1976). Therefore, the outcome of this experiment indicated the possibility of the bacteria surviving in the medium by producing enzymes that catalysed the degradation of cell wall components (mainly xylan and other cell wall polysaccharides of the xylem). The degradation products of xylan and xylem resulting from this process were likely consumed as a source of carbon and energy and metabolised by *Psa*, resulting in a delay while enzyme production was induced, followed by an increase in cell number.

A second peak in cell number during bacterial culture under low nutrient conditions can occur after cell death releases nutrients. Surviving *Psa* cells may then catabolise the organic debris resulting from cell death. Programmed cell death is a common phenomenon in cultures lacking abundant nutrients (Finkel, 2006; Jensen & Gerdes, 1995). A proportion of *Psa* cells under these conditions may have committed cellular "suicide", resulting in the survivors continuing to reproduce. The organic debris

released by cell death and known to be metabolisable by living cells mainly consist of amino acids from proteins, carbohydrates from the cell wall, lipids from cell-membranes, and even DNA (Finkel & Kolter, 2001). Therefore, the second peak in CFU during culture of Psa on xylem media may be attributed to this phenomenon.

Psa in nutrient broth (NB) showed the highest growth rate in the first 2 days and continued to decline over the next 10 days whereas *Psa* in xylan and casein medium or kiwifruit medium demonstrated increases and decreases in growth rate. Nutrient broth was a rich medium containing a readily available energy source, causing *Psa* to follow a characteristic pattern of bacterial growth. Overall, *Psa* in xylan and casien medium had the highest cell counts from day 3 onwards compared to NB and the kiwifruit xylem medium. Oat spelt xylan contains approximately 10% arabinose and 15% glucose residues, however these were presumably not initially available as an energy source. However, after a lag the surviving *Psa* cells should have been able to utilise these residues via the same type of xylanase activity as that detected in the xylem medium. Cell counts of *Psa* were possibly lower in the minimal medium supplemented with kiwifruit xylem compared to the xylan and casein medium because kiwifruit xylem was a less pure and energy rich source of polysaccharides than the xylan plus casein medium.

2.5.3 *In vitro* xylanase activity of *Psa*

As discussed above, there should be a correlation between in vitro xylanase activity and the delayed first peak of CFU counts of *Psa* growing on minimal media supplemented with 0.5% kiwifruit xylem. This behaviour is consistent with a possible utilisation of cell wall polysaccharides by *Psa* as an energy source, following extracellular enzymatic degradation to metabolisable sugars. The DNSA assay results were consistent with this hypothesis (Figure 2.2A), while the RBB assay results were not (Figure 2.2B).

The results suggest that the xylanase is probably active as an extracellular enzyme secreted by *Psa* as the activity was detected only in concentrated supernatant. This is typical of xylanases and other CWDE produced by similar plant pathogens (Barras *et al.*, 1994; Bateman & Basham, 1976; Kosugi *et al.*, 2001).

The rate of xylan hydrolysis is influenced by the chain length and the degree of substitution. For example, some gylocoside hydrolase family-11 xylanases can rapidly hydrolyse xylans that have a chain length greater than 8 xylose residues, and their hydrolytic rates are not sensitive to substituents on the xylan backbone (Li *et al.,* 2000). Others are more effective on xylans that have a long chain (greater than 19 xylose residues), but vary widely in their sensitivity to substituent groups (Li *et al.*, 2000). In this study oat spelt xylan $(C_{10}H_{11}O_8)n$ was used as the substrate for the DNSA assay (*Xylan Oat spelt*). It is possible that the xylanases produced in-vitro by *Psa* were active towards the oat spelt xylan substrate used in the DNSA assay, but inactive towards the RBB assay substrate. RBB xylan is generally considered a better substrate and assay method for endo-acting xylanases that can cleave at sites within longer chain xylans (Bailey *et al.*, 1992; Bhat & Hazlewood, 2001).

In the *in planta* study (Chapter 3), contrasting results were obtained with the two types of xylanase assays. The RBB assay conducted with *Psa* inoculated plant extracts indicated a positive activity, whereas the DNSA assay did not. Contradictory assay results between in-vitro and in-planta conditions are not unique to *Psa.* For example, when protein production by the pathogen *Fusarium graminearum* (*Gibberella zeae*) was compared between in-vitro culture and during infection of wheat heads, 49 in-planta produced proteins were not found during growth on any of 13 different culture media. Several proteins found only during *in planta* growth may be important in the interaction between *F. graminearum* and its host plants (Paper *et al.*, 2007).

Another example of contradictory activities between in vitro and *in planta* growth conditions has been observed with other *Pseudomonas syringae* pathovars. The amounts of ethylene produced by *Pseudomonas syringae* pv. *glycinea*, pv. *phaseolicola* and pv. *pisi* in synthetic medium were different to the amount of ethylene production *in planta* in bean. The bean strains of *P. syringae* pv. *phaseolicola* and strains of 17 other pathovars did not produce ethylene *in planta*. (Weingart & Volksch, 1997).

An association is evident between the xylanase activity and starvation of *Psa* in the minimal media supplemented with 0.5% kiwifruit xylem (Figs 2.1 & 2.3). In

contrast, xylanase activity was absent or very low in the nutrient broth and xylan and casein media. Only on day 9 did *Psa* produce a significant xylanase activity in the xylan and casein medium, a timing that corresponded with the 2nd peak in CFU count that *Psa* achieved on Day 9 in this medium (Figures 2.1, 2.3). It is a future area of research to investigate whether starvation or the reduction of CFU counts in the culture media triggers xylanase production by *Psa*.

2.6 Conclusion

The *Psa* genome comprises at least four sequences for xylanase, as well as sequences with homology to some other well-known cell wall degrading enzymes. *Psa* displays the behaviour of a characteristic Gram negative bacteria in a rich medium, however, it showed a different growth behaviour in energy deficient media. The results with one type of xylanase substrate indicated that *Psa* produced xylanase activity when grown on energy deficient media. The appearance of this activity corresponded with peaks of *Psa* CFU counts during culture on these media, leading to the conclusion that *Psa* is capable of degrading plant cell wall material, and of utilizing the resulting products as a substrate for growth.

CHAPTER 3

Putative *In Planta* Xylanase Activity of *Psa* in *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A'

3.1 Abstract

In order to better understand the invasion process of *Psa* within the host, studies of *in planta* activity of *Psa* xylanase were conducted. Stems of *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A'plants were inoculated with *Psa*, and when disease symptoms appeared in the inoculated plants, both inoculated and non-inoculated shoots were harvested. DNA from the infected shoots was extracted and amplified using a pair of duplex *Psa* specific primers to confirm that the symptoms were due to *Psa* infection. For detection of xylanase activity, ground stem pieces were subjected to xylanase extraction buffer. Remazol Brilliant Blue (RBB), 3,5-Dinitrosalicylic acid (DNSA) and Azurine-Crosslinked Polysaccharides (AZCL) assays were conducted on the stem extracts. Xylanase activity was detected using the RBB assay in extract from *Psa* inoculated stems, but not in extract from non-inoculated stems. No xylanase activity was detected using the DNSA and AZCL assays. The RBB assay detected xylanase activity exhibited typical enzyme kinetic responses to temperature and other assay conditions. A penetrometer test was used to determine whether *Psa* infection caused a reduction in stem strength. The average strength per mm thickness of xylem was less in infected stems, suggesting that infection and xylanase activity is associated with a loss of cell wall strength. Anatomical observations confirmed the presence of bacteria within the stem cortical tissue, and the movement of bacteria between fractured cell walls. cDNA was synthesised from RNA extracted from inoculated and non-inoculated stems and amplified using primer pairs specific to genes annotated in the *Psa* genome for xylanases (Polysaccharide deacetylases-PDs). Expression of a *Psa polysaccharide deacetylase* gene with a nucleotide length of 882 (PD 882) was observed only in the inoculated stem samples. Therefore at least one gene in the *Psa* genome for a putative xylanase

was expressed during *Psa* infection. This gene may be responsible for the observed xylanase enzyme activity and reduction in strength of the infected stems.

3.2 Introduction

Microorganisms colonising plants must first overcome the plant cell wall barrier to achieve infection. Enzymatic degradation of plant cell walls is one of the most striking invasion strategies of plant pathogenic microorganisms (Robinson, 1991). Cellulases, pectinases, proteases and xylanases are cell wall degrading enzymes often secreted by phytopathogenic bacteria and fungi for breaking down components of their host's cell walls (Choi *et al.*, 2013; Rajeshwari *et al.*, 2005). The plant cell wall is a mechanical support and a barrier; nonetheless the wall is a dynamic, metabolically active organelle (Robinson, 1991). Although the wall contains peroxidases, phenolics and other compounds which are toxic to living tissues, the wall is also a nutritional source for microorganisms and animals (Cooper & Jellis, 1984). Since the wall is comprised mainly of carbohydrate, it can be a rich source of glucose or other monomers (Showalter, 1993).

Once infected, all polymers of a plant cell wall can eventually be degraded by microorganisms. This suggests that for every type of chemical bond in the wall there must be an enzyme that can cleave it (Walton, 1994). Lignin is the cell wall component that is most resistant to microbial degradation, however even the ability to degrade lignin has been reported for some plant pathogenic fungi (Dashtban *et al.*, 2010)

In-planta expressed xylanase has a major role in virulence in many bacterial and fungal diseases. For example, *Xanthomonas oryzae* pv. *oryzae* is the causal agent of bacterial leaf blight, a devastating disease of rice (Rajeshwari & Sonti, 2000). Several proteins, including a xylanase, are secreted through type II secretion systems (T2S) and are important for virulence of *X. oryzae* pv. *oryzae*. The *xynB* gene has a xylanase catalytic domain present and encodes for the 32 kDa secreted xylanase in *Xanthomonas oryzae* pv. *oryzae*. The *xynA* and *xynB* genes are adjacent to each other. Mutations in *xynB* but not *xynA* affected secreted xylanase activity and caused a significant reduction in virulence (Rajeshwari *et al.*, 2005). There are about 30 to 40 genes potentially encoding for cell wall degrading enzymes in the genome sequences of *Xanthomonas campestris* pv. *campestris* and *X.axonopodis* pv. *citri.*

These genes are likely to be important in the plant pathogenesis of xanthomonads (Young J.M. *et al.*, 1996).

Botrytis cinerea is a fungal plant pathogen attacking a number of hosts, causing economic losses worldwide (Y. Elad *et al.*, 2004). *B. cinera* can degrade xylan by the coordinated action of a group of extracellular enzymes. Among these, endo- β-1,4-xylanases carry out the initial breakdown by cleaving internal bonds in the polymer backbone. When the gene, xyn11A, coding for an endo-β-1,4-xylanase belonging to family 11 of glycosyl hydrolases was disrupted, a moderate decrease (30%) in disease symptoms occurred. When the gene was deleted, an even more significant decrease in symptoms occurred (Brito *et al.*, 2006).

Certain Pseudomonads are known to derive their pathogenicity from production of pectinolytic enzymes, but not xylanase. *Pseudomonas marginalis* produces substantial amounts of pectate lyase (PL) to macerate tissues of herbaceous plants, causing bacterial soft rot (Membré & Burlot, 1994). Furthermore, an extracellular PL produced by *Pseudomonas viridiflava* is responsible for maceration of tissues of harvested vegetables, also causing soft rot (Liao *et al.*, 1988). *Pseudomonas syringae* has been demonstrated to be involved in a number of diseases of hosts ranging from herbaceous plants to various species of woody trees, resulting in the emergence of highly destructive diseases such as bacterial canker of kiwifruit and bleeding canker of horse chestnut (Nowell *et al.*, 2016). However, neither the cell wall degrading ability in general, nor the xylanase producing ability in particular, have been investigated in detail for *P. syringae* pathovars that infect woody hosts.

In recent studies of the *Psa* genome, it has been noted that the *Psa* genome includes open reading frame sequences with homology to genes from other pathogenic bacteria known to encode for polygalacturonase (PG), pectin lyase, and xylanase enzymes, as well as genes involved in the degradation of lignin derivatives and other phenolics (Marcelletti *et al.*, 2011). During the research reported in this thesis, a preliminary gel diffusion experiment was conducted to determine if PG activity was present in *Psa* infected stem extractions, however no PG activity was detected.

Investigation of the xylanase activity of the *Psa* pathogen cultivated in different growth media indicated a significant level of xylanase activity especially when the pathogen was cultured on minimal media supplemented with 0.5% ground kiwifruit secondary xylem (Chapter 2). Observation of the symptoms of infected *A. chinensis* plants also suggested that the stems are weakened and lose strength during invasion by the pathogen. These observations prompted the question of whether the organism produces xylanase *in-planta*, during its infection of host tissue. Therefore, in this chapter it was hypothesised that genes for xylanase that are present in the *Psa* genome are expressed during infection, that xylanase activity is detectable during infection, and that associated changes in the composition of host cell wall material causes a loss of strength in infected stems.

3.3 Materials and methods

3.3.1 Plant material

Twenty clonal *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' plants were grown from rooted cuttings obtained from the Plant and Food Research orchard, Ruakura. Once rooted the cuttings were grown in standard potting mix (KKW NZ Tree seedling Mix, Daltons, NZ) in 3.5 L volume pots under glasshouse conditions. Eight month old plants were pruned back to several axillary buds two months prior to the experiment, so that at the time of the experiment, each plant had at least 2 vigorous shoots with a minimum length of 60 cm. During the experiment the plants were kept in an automated containment glass house, irrigated once a day, kept under continuous high relative humidity (80 - 90%, maintained using fogging), and a daytime temperature of 20-24 ºC under natural summer-time day length and light. Ten plants were designated for inoculation with *Pseudomonas syringae* pv. *actinidiae (Psa)* and ten plants were maintained as non-inoculated, and randomly arranged in five rows within the glasshouse (Fig 3.1 A).

3.3.2 Inoculation

Psa maintained at -80°C in an ampule consisting of previously grown *Psa* culture in nutrient broth preserved in 5% glycerol was used in this experiment. One thawed ampule (containing one ml) of *Psa* was added to 25 ml of sterile nutrient broth and was kept shaking in the orbital incubator on 160 Rev min^{-1} at 25°C for 48 hours. From previous experiments, these conditions were expected to result in a culture

containing 10^{12} CFUs/ml, with CFU checked by measuring the optical density (OD) of the culture. The bacterial culture was diluted to approximately 10^9 CFU ml^{-1}, centrifuged, the pellet washed twice in sterile water and re-suspended in 10 ml of sterile water in order to obtain a final 10^9 CFU ml^{-1} suspension. Serial dilution and plating were used to confirm the actual strength of the inoculum.

Two shoots of each plant were inoculated, with either the prepared *Psa* culture or sterile water (controls). Stems were inoculated by first creating a horizontal wound in the bark, 5 mm in length, penetrating to the cambium, 20 cm above the base of the shoot. Ten microliters of the prepared inoculum suspension or water were placed immediately on the wound, and stem morphology monitored at the wound site to follow the progress of infection.

3.3.3 Harvest

After approximately 3 weeks there were clear visual signs of infection in inoculated shoots. This was observed as browning extending to approximately 400 mm above the inoculation point. One shoot from each plant was used for enzyme assays and histological analysis and the second shoot was used for the strength test. The shoots were measured (length, number of nodes) and harvested using sterilised secateurs approximately 50 mm from the base of the shoot.

Leaves of the shoots used for assays and histology were removed and discarded. Each shoot was cut into pieces of 50-60 mm in length from 200 mm above the inoculation point, sealed into polythene bags and transferred to the laboratory for further analysis. Approximately 10-12 pieces 1 mm in length were cut from the stems, snap frozen in liquid N_2 and stored at -80^0C until extraction and assaying for xylanase activity.

3.3.4 Plant extract

Hort16A kiwifruit stem pieces were ground in liquid N_2 using a pestle and mortar. Seven hundred and fifty µl of xylanase extraction buffer (Ahmed, 2004; European Molecular Biology Laboratory) was added to 250 mg of the ground kiwifruit tissue, 50 mg of Polyvinylpolypyrrolidone (PVPP) added, and the mixture thawed on ice. The sample was mixed by vortexing and incubated on ice for a further 30 minutes. The sample was centrifuged at maximum speed $16.1X10^3$ rcf in an Eppendorf®

5415 R centrifuge for 10 minutes at 4ºC, and the supernatant transferred to a new microcentrifuge tube. Five hundred µl of extraction buffer was again added to the ground kiwifruit tissue pellet, vortexed to re-suspend, incubated for 10 minutes on ice, centrifuged at maximum speed for 10 minutes at 4ºC, and the supernatant combined with that from the first extraction.

3.3.5 Enzyme assays

3.3.5.1 RBB assay

Four hundred µl of 0.1% RBB xylan (Sigma M5019) in 50mM citric acid (pH5.5), 10 µl of 1 mg ml^{-1} Bovine Serum Albumin (BSA) and 100 µl plant extract were mixed well in a 2 ml Eppendorf tube and incubated overnight at 37ºC. The next day 0.8 ml of 96% ethanol was added and left for 20 minutes at room temperature. The sample was then spun at maximum speed for 10 minutes and absorbance read at 595 nm against a background control without the plant extract. A positive control consisting of a cocktail of three known endo-1,4-β-xylanases (M1- *Trichoderma viridae*, M3- *Trichoderma longibrachiatum* and M6- rumen microorganism, Megazyme, Bray, Ireland) was added into an assay run with non-inoculated plant extract.

RBB assay conditions were varied to confirm the enzymic origin of the xylanase activity present in the inoculated plant extract. Assay series were conducted at 21ºC (ambient), 37ºC and 50ºC using water baths. Another series was conducted at 4ºC, 10ºC, 20ºC, 30ºC, 40ºC and 50ºC. RBB assays were also conducted with variable plant extract volumes (100 µl and 300 µl), variable substrate concentration (0.5% RBB xylan instead of 0.1% RBB xylan) and variable incubation times (2 and 12 hours at 10ºC, 20ºC, 30ºC, 40ºC).

3.3.5.2 DNSA assay

For the DNSA assay, oat spelt xylan (0.2%) in 100 mM hepes buffer at pH 7.0 was used as the substrate. Four hundred µl of substrate and 100 µl of the plant extract described above were dispensed into 1.5 ml microcentrifuge tubes. The assay tubes were mixed by inversion, then incubated in a water bath at 30ºC for 30 minutes, after which 1000 µl of DNSA reagent (Chapter 2) was added. The tubes were again mixed and placed in a boiling water bath for 6 minutes. They were then transferred to an ice

bucket to cool before reading absorbance at 575 nm in UV-Visible recording spectrophotometer (UV-160, Shimadzu, Japan).

Aliquots of substrate incubated without enzyme (plant extract) were used as blanks. After addition of DNSA, the plant extract was added, the tubes were immediately immersed in boiling water, before being cooled and the absorbance recorded. The blank absorbance was subtracted from the sample absorbance to calculate the increase in absorbance. Enzyme activity in μmoles $minute^{-1}$ ml^{-1} was calculated using the slope of the line of best fit over the linear region of the standard curve, prepared using known concentrations of product. Known xylanase from *Thermomyces lanuginosus* (Sigma-Aldrich, St. Louis, USA) was added into assays run with non-inoculated plant extract as a positive control.

3.3.5.3 AZCL assay

Fifty mg of AZCL xylan from birchwood (Megazyme, Bray, Ireland) was dissolved in 30 ml of 0.2M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at pH 5.0. In a 2 ml microcentrifuge tube, 100 μl of the above substrate was added, 50-100 μl of plant extract was then added, mixed well and kept overnight at 37ºC. After incubation 150 μl of 3% Tris were added and incubated for 20 minutes at room temperature. The sample was centrifuged at maximum speed for 10 minutes and absorbance of the supernatant read at 595 nm against back ground controls, which were assays without plant extract. Known enzyme (the cocktail of M1, M3 and M6 described above) was added to the assays conducted with extract from non-inoculated plants as a positive control.

t-test was performed on enzyme assay results to analyze, whether the results are statistically significant at 0.05.

3.3.6 Re-isolation of *Psa* from the infected shoots.

A piece of fresh stem (5 mm in length) was surface sterilised by dipping in 1% hypochlorite solution for 3 mins, triple rinsed with sterile water, ground in 100 μl of sterile water and centrifuged at low speed to remove the solids. The resulting supernatant was spread on a nutrient agar plate and incubated at room temperature. After 2 days the plates were checked for any creamy-coloured colonies that

resembled *Psa*. Fresh samples were then taken from representative colonies and Polymerase Chain Reactions (PCRs) conducted to confirm their identity as *Psa*.

Duplex PCRs were conducted using 2 pairs of primers (Gallelli *et al.*, 2011):

a. KN-F and KN-R (McCann *et al.*, 2013; Rees-George *et al.*, 2010)
b. AvrDdpx-F and AvrDdpx-R (King EO *et al.*, 1954).

Positively amplified products of the expected size were purified using a DNA clean and concentrator kit (Zymo Research, USA), eluted in a final volume of 20 µl and sequenced by the DNA Sequencing Facility, University of Waikato, Hamilton, New Zealand.

3.3.7 Strength test

Figure 3-1 Digital force gauge used to measure the strength of the inoculated and non-inoculated stems.

A digital force gauge (Imada DS-2, Northbrook, USA) was used to measure the maximum force required for the probe (2 mm diameter) to penetrate the stem. The

gauge was mounted onto a level test stand and the probe was manually advanced through the stem at an approximately constant speed until the probe penetrated the xylem and entered the pith. The penetration force was measured adjacent to the site of inoculation, and 200 mm distal to the site of inoculation. Thick transverse hand sections were cut with a razor blade adjacent to the site of each penetrometer measurement, placed on a light box and photographed with a camera equipped with a macro lens. The thickness of the xylem from cambium to the pith was measured across two radii using image analysis (Image J; Schneider et al 2012) and force required to penetrate the stem per mm of xylem thickness calculated.

t-test was performed on strength test results to analyze, whether the results are statistically significant at 0.05.

3.3.8 Histology

Minimal fixative was prepared using 0.1% glutaraldehyde, 2% formaldehyde in a 0.1M phosphate buffer solution. Final pH of the minimal fixative was 7.2. Approximately 1 mm thick transverse stem tissue sections from inoculated and non-inoculated stems were cut into sectors on a wax bottomed dish under minimal fixative, fixed for 24 hours, dehydrated through a graded alcohol series, before infiltration and embedding in acrylic resin (LR White, Sigma-Aldrich). Transverse 1.5 µm sections were obtained using a diamond knife fixed to an ultra-microtome (Ultracut, Reichert-Jung, Germany). Cross sections were mounted on poly-lysine coated slides, stained with 0.5% Toluidine Blue in Na_2CO_3 buffer pH 11.1 (O'brien *et al.*, 1964; Sakai, 1973) and photographed using a light microscope (Leica DMRE).

3.3.9 Primer designing for xylanase annotations

Four pairs of gene specific primers (Table 2.1) were designed using Primer3Plus for each polysaccharide deacetylase annotation identified in the *Psa* genome (PD; Accession Number: AKT30846.1, AKT32033.1, KCU98886.1, AKT29083.1).

Table 3.1 PCR primers used in this study for xylanase (Polysaccharide deacetylase – PD) annotations. Nucleotide number in a particular gene is represented by the numeral after PD.

Annotated Gene	Forward Primer	Reverse Primer	Product size(bp)
PD 819	ATTTGTTGGGTTTCCACTCG	GGTGTAGCGGTTCAGGTCAT	383
PD 930	GATCCAGATCCTCACCGAAA	CTCATGGCTTTTGGCGTATT	406
PD 882	GTTTGGTGTGGATGTCGATG	TCAGGCTGTGGTCGTACTTG	425
PD 1161	AGGTCTGTTCTGGCTTCAGG	ATCGATGTTCCACAACGACA	904
PD 882 (cover full gene)	ATGGCCAAAGAAATTCTGTGTGCG	TCAGCGGGTACGGGGGTTGCGACG	882
PD (882 (Internal)	GTCGGCGACAAGTGGACCAAGATC (Product size 402)	TTGGTCCACTTGTCGCCGACGCGA (Product size 500)	

3.3.10 RNA extraction and cDNA synthesis

A CTAB method used by Chang *et al.* (1993) for total RNA extraction was modified to suit kiwifruit stem pieces and overcome the problem of mucilage. RNA was extracted from 600 mg of stem tissues as described and subjected to DNase treatment (DNA-free^{TM} ;Ambion-Invitrogen). cDNA was synthesized using Tetro reverse transcriptase manufactured by Bioline, USA (Nardozza *et al.*, 2013). Negative cDNA controls were maintained to test for genomic DNA contamination.

Housekeeping genes

The gene rpoD, encoding sigma factor 70 (Petriccione *et al.*, 2014) and dnaA, for initiation of chromosomal DNA replication (Fujita *et al.*, 1990) were used as bacterial housekeeping genes. These are typical constitutive genes required for basic cell functions and act as a control for positive PCR presence.

Table 3.2 Primers for housekeeping genes used in this study

Housekeeping gene	Forward primer	Reverse primer	Product size (bp)
dnaA	CGCAGGAAGAGTTTTTCCAC	GATATCGCGACCCATGAAGT	326
rpoD	CTGATCCAGGAAGGCAACAT	TATTCATGTCGATGCCGAAA	496

3.3.11 PCR experiments

PCRs were performed on programmable thermal controller (MJ Research, PTC-100^{TM}) in a total volume 20 µl in a sterile 200 µl PCR tube containing 10 µl Intron 2X i-Taq master mix, 1µl of 10 µM of each Forward and Reverse primer (Table 2.2) and 2 µl containing 30-50 ng of c DNA. A thermal cycling program of 94ºC for 2 min for initial hot-start step, followed by 30 cycles for denaturing at 94ºC for 20 sec, annealing at 63º C for 10 sec and extension at 72º C for 50 sec and final extension of 5 min at 72º C was adopted. Amplified products (5 µl) were resolved on 1% TAE agarose mini gels containing 1.5 µl ethidium bromide and visualized under UV light using a gel visualizer (Omega Lum^{TM} G, Aplegen).

3.3.12 Gene amplification and sequencing

Positive amplified products of the expected size (Table 2.1), were purified using a DNA clean and concentrator kit (Zymo Research, USA) and eluted in a final volume of 20 µl. DNA sequencing was conducted at the DNA sequencing facility, University of Waikato, New Zealand with the corresponding primers in Table 2.1. The sequences were aligned using Geneious 7.1.8. (Geneious; Kearse *et al.*, 2012). The consensus nucleotide sequence was aligned to the annotated gene.

3.4 Results

3.4.1 Symptoms and confirmation of infection

The Hort16A kiwifruit stems inoculated with sterile water formed healthy callus at the inoculation site (Fig 3.1 B), while stems inoculated with *Psa*, tissue exhibited browning and white exudate and there was no callus formation (Fig 3.1 C). *Psa*-like

colonies were re-isolated from symptomatic tissue excised from all 10 *Psa*-inoculated stems. Isolates from 9 of the 10 stems (plant 1-7 and 10) were confirmed as *Psa* using duplex PCR (Fig. 3.1 D). Isolates from plant eight were also subsequently confirmed as *Psa* after a more concentrated bacterial suspension was used for DNA extraction.

Figure 3-2 Preparation of Hort16A kiwifruit plants for inoculation, disease occurrence and confirmation of *Psa* infection. (A) Randomly assigned Hort16A kiwifruit plants at the time of inoculation; (B) A kiwifruit stem three weeks after inoculation with sterile water; (C) A kiwifruit stem three weeks after inoculation with *Psa* (D) Agarose gel electrophoresis of products from duplex PCRs for bacterial colonies re-isolated from the 10 Psa inoculated plants. Lanes 1-10, isolates from plants 1 to 10; lane 11, *Psa* genomic DNA; lane 12, no template negative control. M: molecular markers (Gene Ruler^{TM} 100 bp DNA ladder). Expected product sizes of the PCR are 492 bp and 226 bp. Bar in B and C = 1mm.

3.4.2 Xylanase activity

Positive xylanase activity was detected using the RBB assay in extracts from *Psa* inoculated stems ($P < 0.05$; Fig. 3.2 A). The level of activity was comparable to that

measured in extract from non-inoculated stems supplemented with a fungal xylanase preparation (positive control; Fig. 3.2 A). The positive control with non- inoculated plant extract had only 1.4 fold (40%) higher activity than that of inoculated plant extract. No xylanase activity was detected using the RBB assay in extracts from non-inoculated stems (Fig. 3.2 A).

In contrast to the RBB assay results, no activity was detected when inoculated stem extract was tested using the DNSA ($P > 0.05$; Fig. 3.2 B) and AZCL ($P > 0.05$; Fig 3.2 C) assays, even though these assays indicated significant activity using the positive control enzymes ($P < 0.05$; Fig 3.2 B and C). The activity of the positive control with non-inoculated plant extract was 8.2 fold higher than that of inoculated plant extract for the DNSA assay ($P < 0.05$; Fig. 3.2 B) and 11.6 fold higher for the AZCL assay ($P < 0.005$; Fig 3.2 C). However, it was noticed that plant derived mucilage in the stem extracts appeared to cause fluctuating absorbance readings in the AZCL assay.

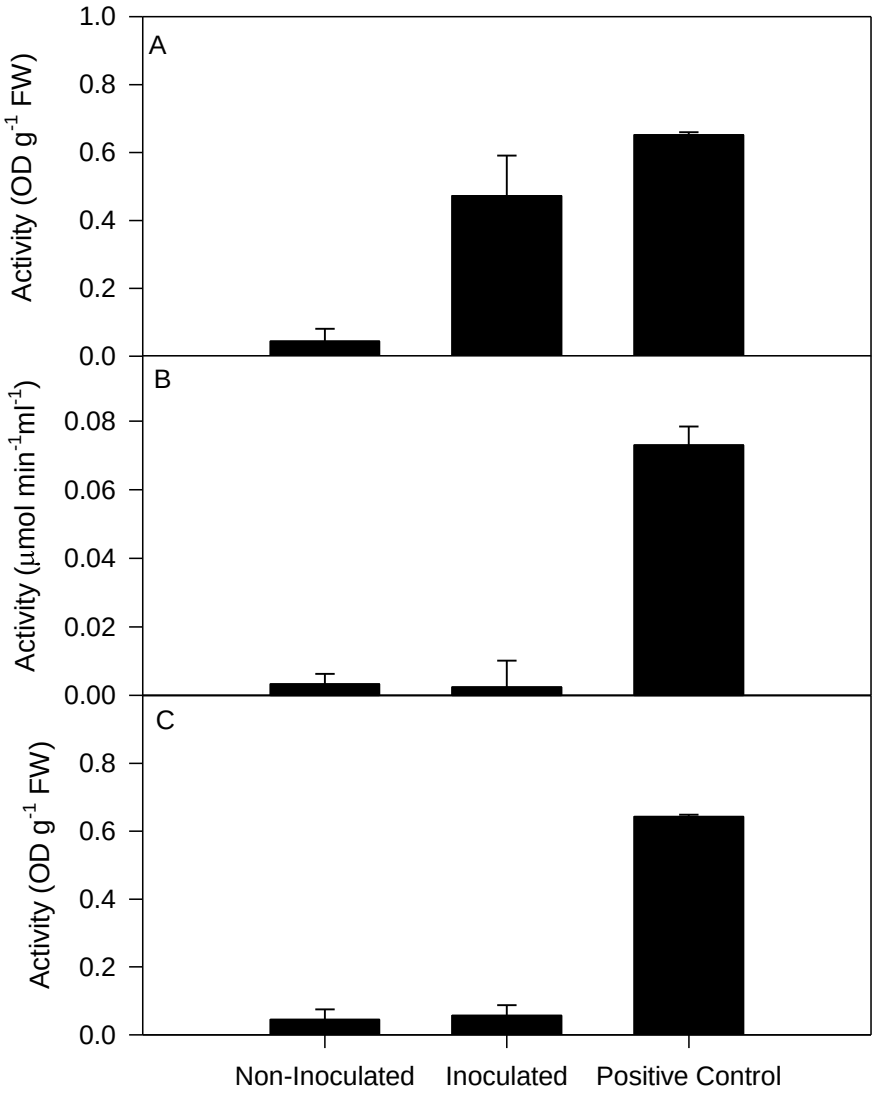

Figure 3-3 Assay results for xylanase activity in *Psa* inoculated and non-inoculated stem extracts of Actinidia chinensis Planch. var. chinensis 'Hort16A' (n=10, means ± SE). ; (A) RBB assay ; (B) DNSA Assay ; (C) AZCL assay

The RBB assay-detected xylanase activity of inoculated stem extract responded to temperature and appeared to have a low temperature optimum between 20^0C and 30^0C ($P < 0.05$; Fig. 3.3). The fungal xylanase used as a positive control had a higher temperature optimum under the same assay conditions ($P < 0.05$; Fig. 3.3).

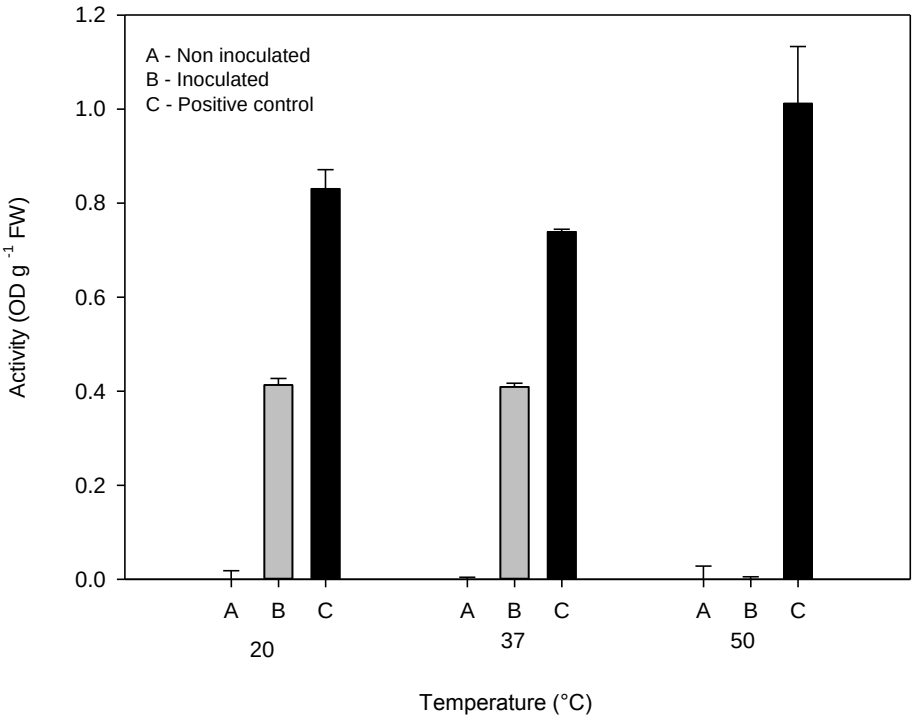

Figure 3-4 Response of RBB assay activity on *Psa* inoculated and non-inoculated stem extracts of *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' to temperature (n=5, means ± SE).

When the RBB assay with *Psa*-inoculated plant extract was repeated over a broader range of temperatures, activity increased between 4ºC and 30ºC, and decreased abruptly at 40ºC and 50ºC (Fig. 3.4).

Inoculated plant extract showed an activity in this RBB assay indicative of an enzyme, as activity varied with temperature and with a change of volume of inoculated plant extract in a fixed incubation time (Fig. 3.5). An assumption was made that there was no limitation of availability of RBB xylan substrate as 0.5% RBB xylan was used instead of 0.1% RBB xylan as the substrate.

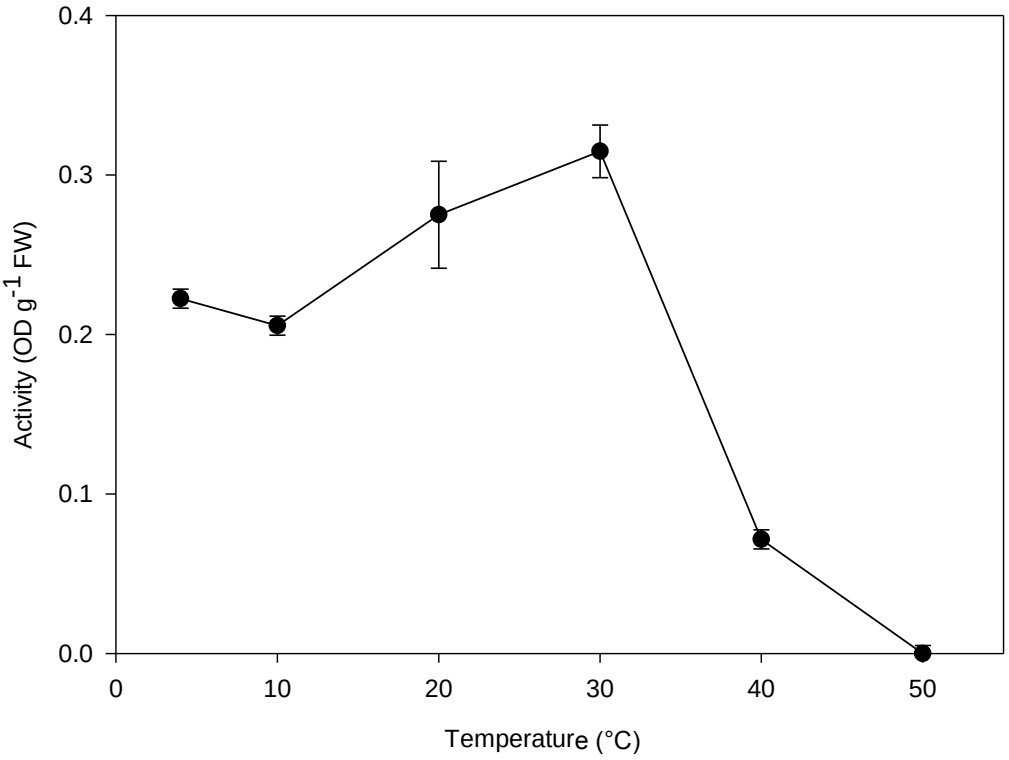

Figure 3-5 RBB xylanase assays conducted with *Psa* inoculated stem extracts of *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' over a range of temperatures (n=5, means ± SE).

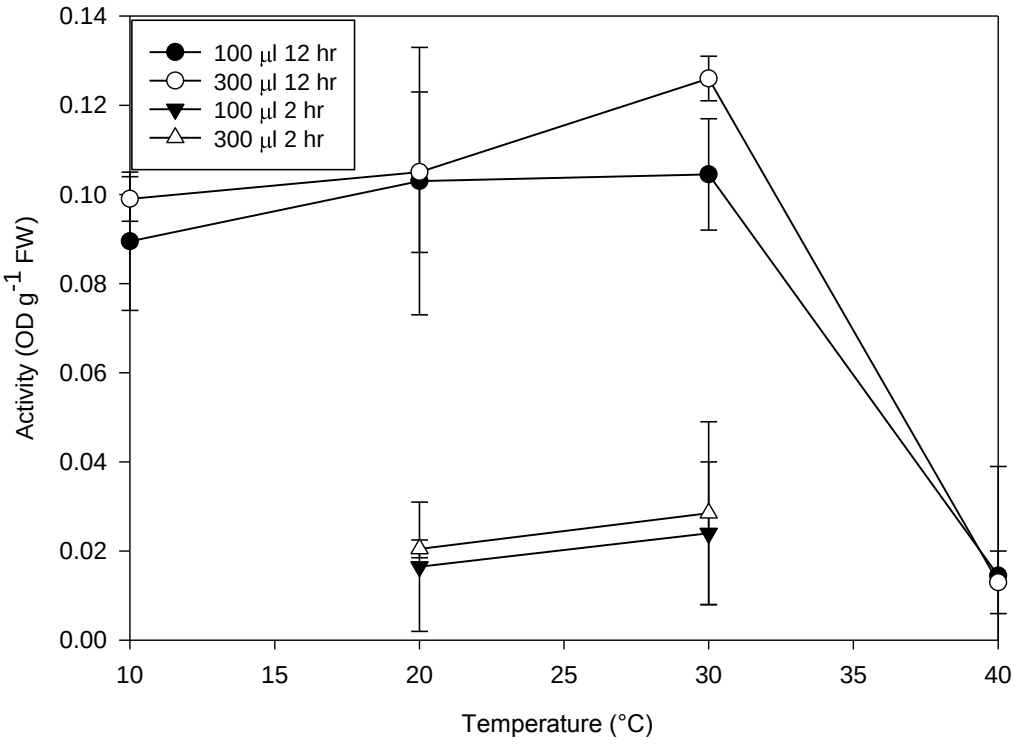

Figure 3-6 Effect of assay temperature, stem extract volume and incubation time on RBB xylanase activity. Two volumes of *Psa* inoculated *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' stem extracts were used with two incubation times (n=5, means ± SE).

3.4.3 Changes in stem strength and anatomy

The first observable external symptom of infection of Hort16A stems was slight browning of normally green stems. Three weeks after inoculation the bark of infected stems had shrunk and darkened at the point of inoculation, and tended to collapse more readily when cut (Fig. 3.6 C). At the same time, 200 mm above the inoculation point the bark was beginning to brown (Fig. 3.6 B).

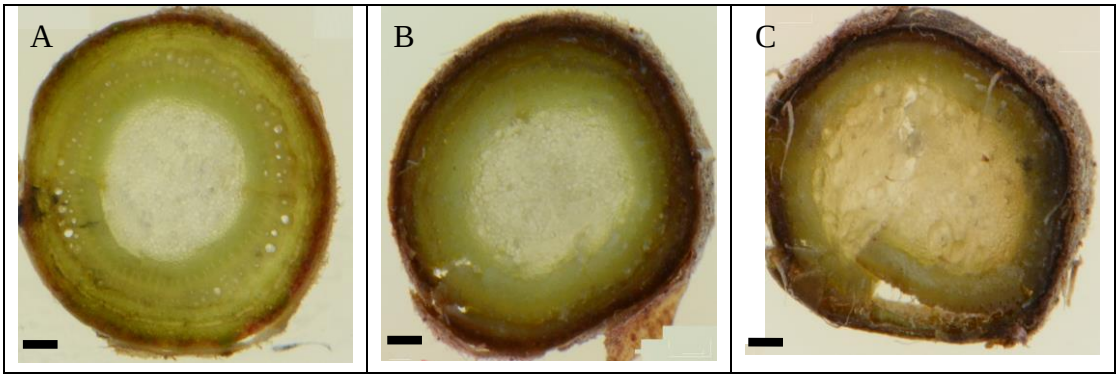

Figure 3-7 Transverse sections of *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' stems photographed under alight box. (A) Control (Non inoculated) stem above the point of inoculation; (B) Inoculated stem above the point of inoculation; (C) Inoculated stem at the point of inoculation. Sections are representative of n=10. Bar = 1 mm.

When measured with a penetrometer, the strength of the inoculated stems had decreased at the point of inoculation and 200 mm above the point of inoculation, compared to the strength of the non-inoculated stems at the same positions (t test, $P < 0.05$; Fig. 3.7).

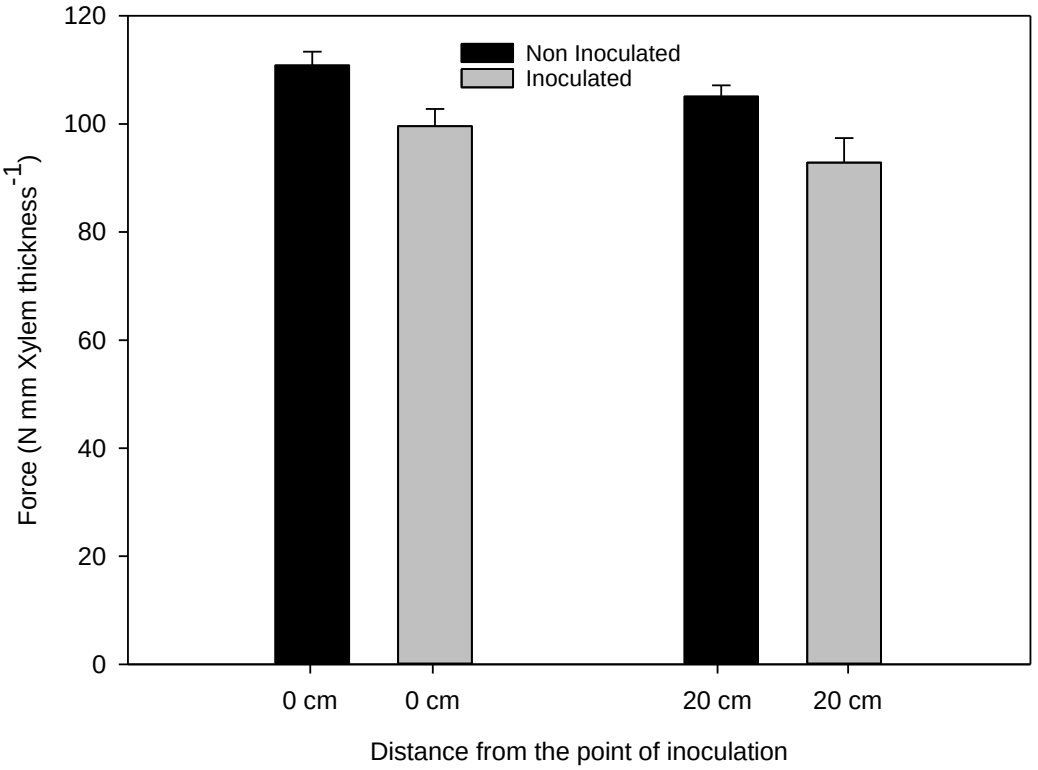

Figure 3-8 Strength of non-inoculated and *Psa* inoculated *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' stems, three weeks after inoculation, at the point of inoculation and 200 mm above the point of inoculation. (n=10, means ± SE).

Examination of the anatomy of infected Hort16A stems revealed more frequent fracturing and collapse of cortical tissues in inoculated stems compared to non-inoculated stems (Fig. 3.8). Pink staining bacterial masses were observed within the intercellular spaces and cell lumens in all of the inoculated stems examined. No bacterial masses were observed in non-inoculated stems. Bacteria were observed in both the inner and outer cortex, and observed to be crossing the fibre layer between the inner and outer cortex in all of the infected stems examined. Penetration of the fibre layer was associated with fractures in the cell walls of the fibre cells (Fig. 3.8).

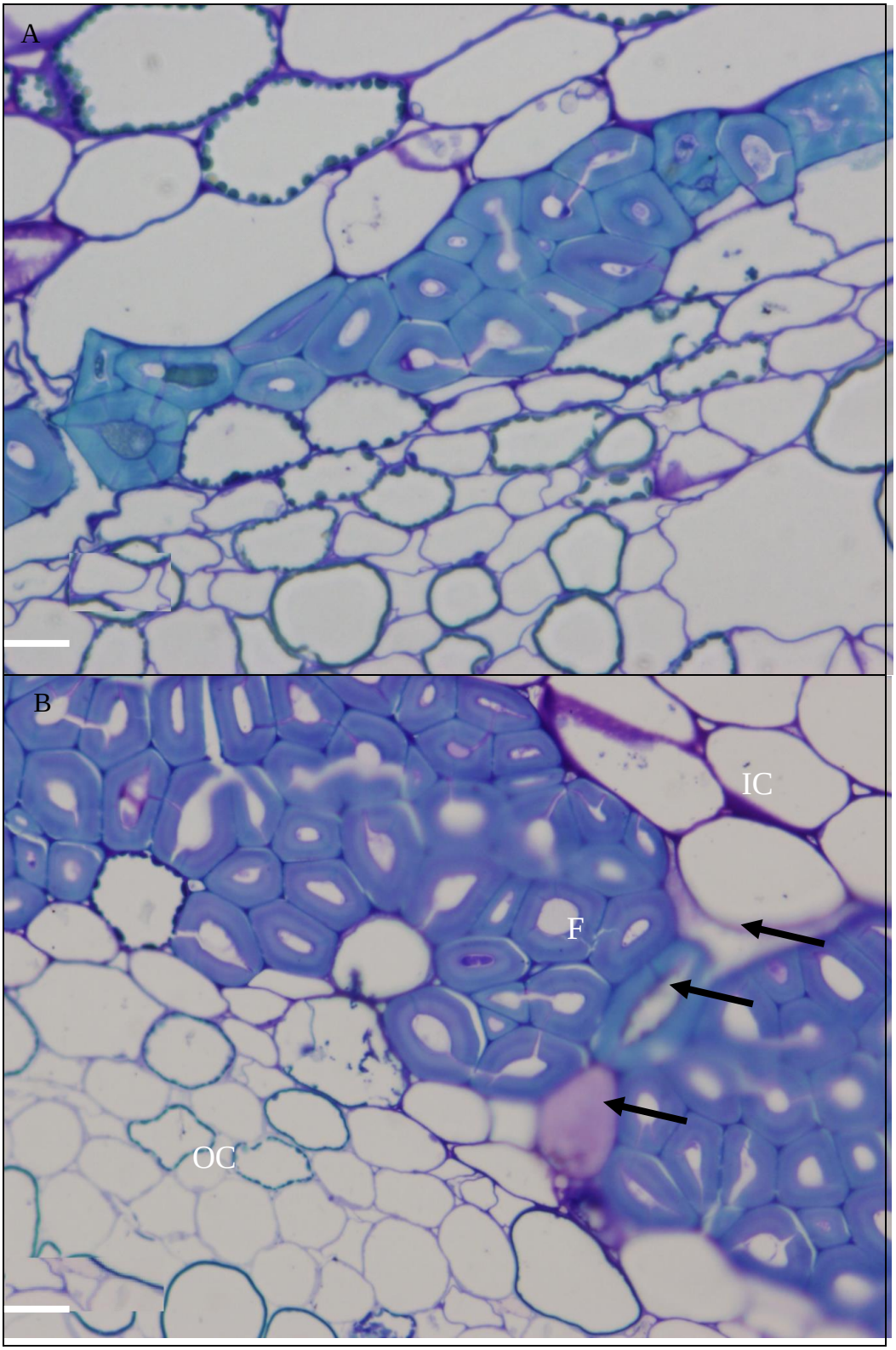

Figure 3-9 Transverse sections of *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' stems. (A) non inoculated; (b) inoculated. Bacterial passage through the fibre ring is evident (arrows). Bacteria are stained in pink colour with Toluidine blue stain. OC- Outer cortex, IC- Inner cortex, F- Fibre layer. Bar = 20 µm.

3.4.4 Bacterial gene expression

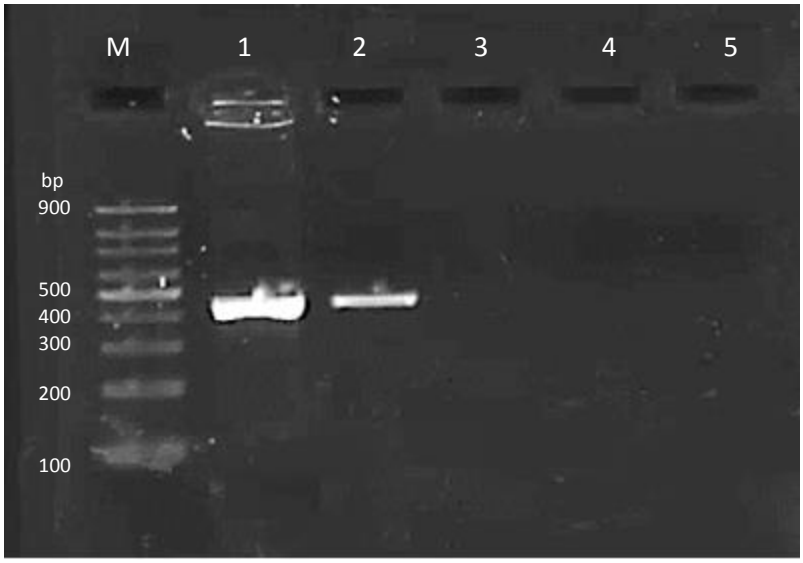

Figure 3-10 PCR conducted with primers specified for the annotated Polysaccharide Deacetylase 882 (PD 882) gene. PCRs were conducted using PD882 primers and cDNA synthesised from RNA extracted from inoculated and non-inoculated *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' stems. Lane 1, Positive control with *Psa* genomic DNA; Lane 2, cDNA from Psa inoculated stem; Lane 3, Negative control with no cDNA; Lane 4, cDNA from non-inoculated stem; Lane 5, Negative control with no template (Water). M: molecular markers (Gene Ruler^{TM} 100 bp DNA ladder).

One of the four Polysaccharide Deacetylase genes annotated in the Psa genome as potential xylanase enzymes was expressed during infection of the kiwifruit stems. cDNA synthesised from RNA extracted from inoculated Hort16A kiwifruit stems repeatedly produced the expected 425 bp product of gene PD 882 (Fig. 3.9). Primers for both housekeeping genes dnaA and ropD produced the expected amplicons in the presence of cDNA from inoculated stems, confirming amplification of expressed bacterial genes (Fig 3.10). None of the other three annotated Polysaccharide Deacetylase genes (PD 819, PD 930 and PD 1161) were detected as expressed in inoculated or non-inoculated *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' stems.

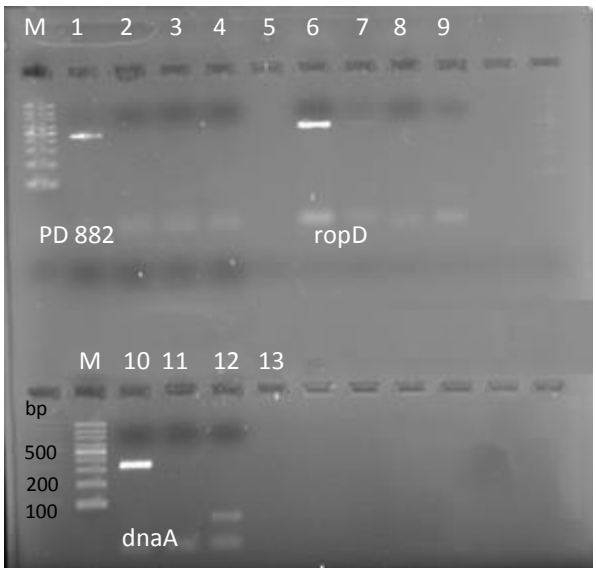

Figure 3-11 PCR conducted with primers for the annotated Polysaccharide Deacetylase 882 gene (PD 882) (Lanes 1-4) and bacterial housekeeping genes *ropD* (Lanes 6-9) and *dnaA* (Lanes 10-12), on cDNA synthesised from RNA extracted from inoculated and non-inoculated Hort16A kiwifruit stems. Lanes 1, 6, 10, cDNA from *Psa* inoculated stem; Lanes 2, 7, 11, Negative control with no cDNA; Lanes 3, 8, 12, cDNA from non-inoculated stem; Lane 4, 9 , 13, Negative control with no template (Water); Lane M, molecular markers (Gene Ruler^{TM} 100 bp DNA ladder).

The expressed PD 882 amplicon was purified and sequenced. Forward and Reverse nucleotide sequences were aligned and provided the expected consensus sequence comprised of 782 nucleotides with 100% identity to nucleotide positions 24 to 858 of the gene annotated as a potential xylanase in the *Psa* genome.

3.5 Discussion

Kiwifruit Hort16A stems were weakened by *Psa* infection, even during the early stages of colonisation, well before tissue necrosis and likely invasion by fungal saprophytes. Positive xylanase activity was detected with the RBB xylanase assay, consistent with the hypothesis that *Psa* produces xylanase during the colonisation of kiwifruit stems. Expression of a potential bacterial xylanase gene was also detected and may be responsible for the observed xylanase activity and stem weakening. Whilst xylanase activity was only detected with one of the three types of assays used, there are numerous types of xylanases (Honda *et al.*, 1985; Marui *et al.*, 1985; Okazaki *et al.*, 1984) and detection of their activity is known to be dependent on the assay (Bailey *et al.*, 1992).

3.5.1 Detecting xylanase activity

The results of the RBB assay were consistent with the hypothesis that *Psa* can produce xylanase. The contrasting levels of activity shown in the three different xylanase assays may have been caused by differences in the sensitivity of the assays the xylanase(s) produced by *Psa*. The three assays use different substrates and it is possible that the type of xylanase produced by *Psa* caused a higher level of hydrolysis of the substrate used in the RBB-xylan assay compared to the substrate used in the DNSA-xylan and AZCL-xylan assays (Bailey *et al.*, 1991; Bailey *et al.*, 1992; Puls & Poutanen, 1989), resulting in positive detection with the RBB-Xylan assay. The insolubility of the substrate used in the AZCL assay also posed a challenge due to its interaction with kiwifruit mucilage.

Xylanases have been isolated from a wide range of microorganisms (Kulkarni *et al.*, 1999). Xylanases are generally classified into glycoside hydrolase families (GH) 10 and 11 based on the primary sequence classification scheme of Henrissat and Bairoch (1993). Depending on the substrate binding site (cleft) of the xylanase, the protein can accommodate 4-7 xylose residues. The major difference between the GH10 and GH11 families is that GH11 enzymes hydrolyse unsubstituted regions of xylan, whereas GH10 enzymes are able to attack modified forms of polysaccharide (Pell *et al.*, 2004). GH10 and GH11 vary significantly in molecular mass, isoelectric points, substrate preferences, and the nature of oligoxylosides generated as products (Biely *et al.*, 1997). The family, the substrate binding cleft and mode of activity of the *Psa* xylanase detected in this study are unknown. The principle of the RBB assay is that the RBB-Xylan is cleaved by an endo-xylanase to low molecular weight dyed fragments which remain in solution on addition of ethanol to the reaction mixture. Higher molecular weight fragments are removed by centrifugation and the absorbance of the supernatant is measured at 590 nm (Aldrich). The AZCL assay measures the release of soluble dyed moieties from dyed AZCL xylan (Biely *et al.*, 1985) while the DNSA assay measures release of reducing sugars from partially soluble xylan substrates. Reducing sugars reduce the DNSA reagent, changing its colour from yellow. The absorbance measured is directly proportional to the amount of reducing sugar (Bailey, 1988; Tan *et al.*, 1985). Overall, the contrasting results from the three xylanase assays suggests that the xylanase produced by *Psa* during infection of kiwifruit stems is an endo-xylanase.

Microorganisms that are phytopathogenic tend to secrete a range of cell wall degrading enzymes to depolymerize the polysaccharides in the host plant cell wall. As a result, plants have evolved diverse defence mechanisms such as generating protein inhibitors to counter the cell wall degrading enzymes. In cereals endoxylanase inhibitors called novel pathogenesis-related proteins have been discovered (Juge, 2006). A new class of plant proteins that inhibit xyloglucan endoglucanase have also been discovered (Juge, 2006). Cell wall degrading enzymes themselves can also act as defence response elicitors. For example, cell wall degrading enzymes secreted by *Erwinia* soft rot bacteria cause both ethylene and jasmonic acid pathways together to regulate defence gene expression in *A. thaliana* plant (Norman-Setterblad *et al.*, 2000). Such discoveries point towards the potential for similar defense responses or inhibitor production by the kiwifruit plant against *Psa* secreted xylanases. Plant derived inhibition could provide a plausible explanation for the absence of positive xylanase activity with oat spelt xylan used in DNSA and AZCL xylan in AZCL assay. The lack of a plant defence response may also explain why DNSA but not RBB xylanase activity was detected when *Psa* was grown in vitro in the presence of dried and ground non-infected kiwifruit xylem (Chapter 2).

It is concluded that the product produced in the RBB assay was the result of xylanase enzyme activity. A number of experiments were carried out to determine if the activity followed a characteristic enzymatic behaviour. The activity did exhibit a characteristic enzymatic temperature response, with a gradual increase in activity to 30ºC, followed by an abrupt loss of activity at higher temperatures. Activity was also responsive to assay length, and to extract volume (Figures 3.3, 3.4, 3.5). The results are consistent with that expected of an enzyme catalysed reaction. These observations are also consistent with the literature published on temperature optima for other environmental *Pseudomonas* pathogens (Chang *et al.*, 2001; Gügi *et al.*, 1991; Membré & Burlot, 1994), which tend to have relatively low temperature optima. In contrast, the fungal xylanases, which were used as a positive control for xylanase activity, had a temperature optimum of at least 50ºC.

3.5.2 Weakening of inoculated stems

In this thesis it was hypothesised that there would be a loss of strength in infected stem and results were entirely consistent with the hypothesis. Stem strength was

significantly higher in non-inoculated stems compared to inoculated stems. Xylanases produced during the infection of susceptible kiwifruit stems, as indicated in the RBB assays, may have an important role in degrading hemicellulose in the plant primary cell walls of the stem. Histological analysis of infected stem cross sections further revealed tissue collapse including the cell walls (Fig 3.8). A study conducted in *Arabidopsis* during growth revealed that xyloglucan was essential to maintain mechanical strength of the primary cell walls (Peña *et al.*, 2004; Whitney *et al.*, 1999). Therefore, this is a potential area for further research to establish that the xylanase activity is the cause of loss of strength in the infected kiwifruit stems. Anatomical changes following *Psa* infection in the kiwifruit vine have been studied in Italy. They found that *Psa* can infect host plants by entering natural openings and lesions. In naturally infected kiwifruit plants, *Psa* was present in the lenticels as well as in the dead phloem tissue beneath the lenticels, surrounded by a lesion in the periderm indicating the importance of lenticels to kiwifruit infection. Biofilm formation was observed outside and inside plants. In cases of advanced stages of *Psa* infection, neuroses of the phloem occurred, which were followed by cambial dieback and most likely by infection of the xylem. Anatomical changes in wood such as reduced ring width, a drastic reduction in vessel size, and the presence of tyloses were observed within several infected sites. In the field, these changes occurred only a year after the first leaf symptoms were observed suggesting a significant time lapse between primary and secondary symptoms. *Psa* induced cambial dieback was studied by applying dendrochronology methods revealing that cambial dieback occurs only during the growing season (Renzi *et al.*, 2012)

Cell wall degrading enzymes are a known virulence factor for many plant pathogens, often facilitating the movement of the pathogen within the host. A number of studies have been published on the mechanism by which cell wall degrading enzymes enlarge the pore size of inter-vessel pit membranes in grapevines when infected with *Xylella fastidiosa,* enhancing a passive mechanism for a systemic spread of the bacterium (Chatelet *et al.*, 2006; Perez-Donoso *et al.*, 2010; Thorne *et al.*, 2006). The effect of pectinases have also been studied in a large number of pathogens and in greater detail than for any other wall depolymerase. *Pseudomonas viridiflava* has only one pectate lyase gene and mutation of this gene results in loss of the pathogen's ability to cause soft rotting in vegetables (Liao *et al.*, 1988). Mutation in

the single polygalacturonase (PG) encoding gene of *Agrobacterium tumefaciens* causes the loss of PG activity resulting in a substantial decrease in virulence (Rodriguez-Palenzuela *et al.*, 1991). A relationship between localized accumulations of H_2O_2 in plant cell walls with localized membrane damage adjacent to attached bacteria in the infection of lettuce leaves by *Pseudomonas syringae* pv *phaseolicola* has been established (Bestwick *et al.*, 1997). The current study suggests a similar relationship between cell wall degradation and the reduction of kiwifruit stem strength due to *Psa* infection, where the open wounds and fracture cell walls resulting from the reduction in stem strength is a strategy adopted by *Psa* to enter into and move within the plant.

Whilst *Pseudomonas syringae* has not previously been shown to be reliant on cell wall degradation for virulence, the related bacterium *P. solanacearum* has been shown to cause cell wall degradation (Vasse *et al.*, 1995). The pathogenic interactions between tomato roots and *P. solanacearum* were investigated microscopically. The pathogenic strain invaded protoxylem vessels by degrading cell walls, and it was observed that partial wilting of the tomato plant occured if approximately 25% of xylem vessels in each vascular bundle were colonised. No studies have been conducted to determine the relationship between the wilting of the tomato plant and the strength of the infected root. The relevance of the above study is that it has significant parallels to the current study because the *Pseudomonas* pathogen invaded the xylem by degrading cell walls, resulting in partial wilting of the tomato plant. Wilting is also a symptom of *Psa* infection of kiwifruit plants.

3.5.3 RNA extraction and cDNA synthesis

There were four annotations for xylanase or polysaccharide deacetylase in the *Psa* genome as described in Chapter 2, Table 2.1. Of these four annotations, one polysaccharide deacetylase mRNA that was 882bp in length was expressed during the infection (Fig. 3.9). Expression of two bacterial housekeeping genes present in *Psa*, *dnaA* and *ropD* support the conclusion that bacterial gene expression was detected (Fig 3.10). There is no conclusive evidence that the gene expressed was encoding for xylanase or that this gene was responsible for the activity detected using the RBB assay. Next generation RNA sequencing could be used to investigate *Psa* gene expression patterns *in-planta* in more detail. A transgenic *Psa* with a PD882 knockout could be used to test whether the gene is responsible for the

observed xylanase activity, and contributes to virulence. Further experiments could be conducted involving recombinant expression of the protein *in vitro* by cloning the gene into a plasmid and transforming into the bacterium *Escherichia coli* for recombinant protein expression (Lee TH *et al.*, 2007; NCBI, 2016). Thus the recombinant protein could be examined by mass spectrometry and crystallography to provide insight into its structure and function. Furthermore, the recombinant protein could be applied and tested in the RBB assay.

3.6 Conclusion

In conclusion, *Psa* infection caused a loss of stem strength in kiwifruit, and was associated with xylanase activity in extracts from infected stem. The results indicate that the PD 882 gene, a putative xylanase gene, was expressed during the infection. The resulting enzyme produced by PD 882 may have contributed to the loss of stem strength and xylanase activity detected using the RBB assay. Further studies should attempt to characterise the detected xylanase protein in more detail, including investigating why there was variation in activity with alternative xylanase substrates, and the possibility that plant-pathogen interactions result in expression or inhibition of particular cell wall degrading enzymes. It would also be valuable to test the hypothesis that the PD 882 gene was responsible for the observed xylanase activity, and ultimately contributes to pathogenicity of *Psa* towards kiwifruit.

CHAPTER 4

The Role of Vascular Architecture in the Movement of *Psa* within the Kiwifruit Plant

4.1 Abstract

Studies conducted to explore the correlation between vascular architecture and pathogen movement in other plant species provide a strong rationale to investigate a possible correlation between the kiwifruit vascular architecture and the movement of *Psa*. Low pressure air injection was performed on *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' (Hort16A) seedlings to determine the longest vessel length of Hort16A. Low pressure air injection suggested that the maximum xylem vessel length of Hort16A kiwifruit seedlings was 345mm. Based on air injection, 71% of the leaves had at least one open vessel from stem to the leaf lamina and 79% of the leaves had at least one open vessel from stem to petiole. The average maximum vessel length and plant height showed a positive linear correlation. A negative correlation was observed between height of the plant and the number of nodes with vessels extending into leaves when air was injected into the stem from near the base of the plant. When air injection was performed from the stem below each node, a negative correlation was observed between plant height and the proportion of laminae and petioles that had an open vessel from the stem, suggesting that upper nodes were less likely to have an open vessel connection between the leaf and stem.

Cultivar and developmental differences in vessel length distribution were investigated by performing silicon injection on large shoots (canes) from mature plants of *Actinidia chinensis* Planch. var. *chinensis* 'G3' (G3) and *Actinidia deliciosa* (A.Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa* 'Hayward' (Hayward), and shoots from mature plants and seedlings of *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A'. The highest average vessel length was observed in mature shoots of G3, while the next highest average vessel length was observed in Hort16A mature shoots. Average vessel lengths in mature shoots were more variable between shoots of Hort16A than G3 and Hayward, but shoot to shoot variation was not as pronounced in Hort16A seedlings. Given that Hort16A is the cultivar considered

most susceptible to *Psa*, there was no relationship between susceptibility and vessel length.

Leaves of Hort16A were inoculated with *Psa* at the lamina tip and lamina base, adjacent to the lamina junction with the petiole, to investigate the potential for movement of the pathogen between the lamina and stem. Following tip inoculation, tissue samples were taken for attempted re-isolation of *Psa* from three sites; the lamina base, the midpoint of the petiole, and the stem immediately below the petiole. Following base inoculation, re-isolation was attempted from the lamina tip, the midpoint of the petiole, and the stem immediately below the petiole. *Psa* movement was observed in both the basipetal and acropetal directions following tip and base inoculations. After tip inoculation, there was an 88% probability of basipetal movement of *Psa* to the lamina base, an 85% probability of movement to the midpoint of the petiole, and a 77% probability of movement to the stem below the petiole. After base inoculations, there was a 96% probability of movement acropetally to the lamina tip, an 87% probability for movement basipetally to the midpoint of the petiole, and 87% for movement to the stem immediately below the petiole. The results suggest a high propensity for *Psa* to move well beyond the point of inoculation within the leaf lamina, to move between the leaf lamina and stem, and that movement between lamina and stem occurs more readily than the presence of open vessels between the stem and leaves suggests. It is concluded that if movement is occurring within the xylem of the kiwifruit plant, the vessel end wall is not a barrier for *Psa* movement.

4.2 Introduction

Psa is unusual amongst *Pseudomonas* canker diseases in that it appears to more readily invade into and spread via the vascular tissue (Froud *et al.*, 2015). In susceptible kiwifruit cultivars *Psa* enters the plant through natural openings such as stomata, lenticels or open wounds, and then systemically spreads, causing wilting and shoot death. *Psa* has been shown to move systemically from the leaves to young shoots of *A. chinensis* and *A. deliciosa* through the leaf veins and petioles (Ferrante *et al.*, 2012) possibly via the xylem (Balestra *et al.*, 2009; Ferrante *et al.*, 2012; Serizawa & Ichikawa, 1993a; Spinelli *et al.*, 2015; Spinelli *et al.*, 2011).

Kiwifruit is a liana, with wide and potentially very long vessels. With the increase in vessel length the integration and connectivity of vessel networks increase, particularly if vessels are both long and tortuous in their pathways (Jacobsen *et al.*, 2012; Loepfe *et al.*, 2007; Martínez-Cabrera *et al.*, 2009). Vessel length influences plant hydraulic function because longer vessels are less hydraulically limited by pit and end wall resistances, but long vessels may also have greater pit area that makes them more susceptible to hydraulic failure via cavitation (Hacke *et al.*, 2007; Sperry *et al.*, 2006; Wheeler *et al.*, 2005). Loepfe *et al.* (2007) has highlighted a potential risk of hydraulic failure due to increased length of the vessels. *Psa* clearly impacts kiwifruit xylem hydraulic functioning and may cause hydraulic failure, because wilting is a common early symptom of systemic infection. However, it is not known whether the architecture of the xylem of kiwifruit shoots influences pathogen movement or the development of wilting symptoms.

The architecture of the xylem, including the distribution of vessel lengths has been related to the movement of other xylem inhabiting pathogens (Chatelet *et al.*, 2006; Jacobsen *et al.*, 2012). The mechanisms of pathogenesis in diseases caused by *Xylella fastidiosa*, an obligate xylem pathogen, have been particularly well studied. Primary xylem vessels that are continuous between the stem and leaf of grape vines provide a pathway for passive movement of the bacterium (Chatelet *et al.*, 2006). The pathology of the disease also includes the ability to degrade pit membranes between neighbouring vessels, potentially overcoming movement limitations imposed by vessel length (Perez-Donoso *et al.*, 2010). Bacterial cells, tyloses, gums and the debris from cell wall degradation block xylem vessels of plants infected by *X. fastidiosa* (Hopkins, 1989; Sun *et al.*, 2013). *Psa* causes symptoms similar to *Xylella* such as leaf spots, leaf and young shoot wilting and dieback, suggesting similar behaviour of the pathogen in the vessels of kiwifruit plants.

The majority of research on vascular interconnectedness in the xylem has been conducted on the movement of water and not on the movement of pathogens (Choat *et al.*, 2005; Tyree & Zimmermann, 2002a; Zwieniecki *et al.*, 2002). Pit membranes present in the xylem may have a major role in restricting particle or microorganism movement via the transpiration stream. Investigations have been carried out into potential xylem pathways available to particles or bacteria between stems and leaves, however, often only an insignificant movement from stems to leaves or vice versa

was observed (Canny, 1997; Suhayda & Goodman, 1981; Wiebe *et al.*, 1984). In contrast to the leaves of grape vines, the majority of xylem vessels within leaves of other species end near junctions, specifically the petiole–lamina (Canny, 1997; Wiebe *et al.*, 1984) and the petiole–stem junction (Tyree & Zimmermann, 2002a; Wiebe *et al.*, 1984). If this pattern is the norm, then xylem-mobile microorganisms need to pass across pit membranes to leave the stem and move into a leaf blade or vice-versa (Thorne *et al.*, 2006). The degree of open-vessel connection between leaf and stem in kiwifruit is unknown.

Depending on the vessel architecture of kiwifruit, *Psa* bacteria that settle on the leaf surface, infect the lamina, and become xylem mobile may have to cross pit membranes to enter into the stem. Similarly, bacteria entering through lenticels and open wounds in the stem may also have to overcome the pit membrane barrier to enter into leaves. Chapters 2 and 3 of this thesis investigated whether *Psa* possesses cell wall degrading activity that might confer the ability to degrade pit membranes. This chapter describes the xylem architecture of kiwifruit, with the goal of determining whether vessel length and connectedness between stem and leaves are related to *Psa* movement. It was hypothesised that vessels are long in kiwifruit compared to other perennial deciduous fruit crops and that vessels extend from stem to leaf, enabling passive movement of the bacterium. Kiwifruit xylem vessel lengths were compared between cultivars that differ in susceptibility to *Psa* infection. Vessel connections from stem to leaf were also described in detail. Finally, bacterial movement from leaf to stem was investigated for comparison with leaf to stem vessel architecture.

4.3 Materials and methods

4.3.1 Plant material

Seedlings were grown from open-pollinated seed extracted from fruit of *Actinidia chinensis* Planch. var. *chinensis* ‘Hort16A’ (Hort16A). The average height of the plants was 821 mm and plants were approximately four months old.

Figure 4-1 *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' seedlings used for air injection

Actinidia chinensis Planch. var. *chinensis* 'G3' (G3) mature shoots, *Actinidia deliciosa* (A.Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa* 'Hayward' (Hayward) mature shoots and *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' mature shoots were harvested at dawn from nearby orchards, placed in water and bagged in humid bags to prevent further transpiration before the silicon injection. From the multiple cohorts of Hort16A seedlings grown, ten plants were used for silicon injection and 30 plants were used for air injection.

4.3.2 Air injection

Healthy Hort16A kiwifruit plants were taken into the laboratory. Age, plant height (mm), number of cotyledons, number of true leaves, number of fully expanded leaves (90% full size), cut height above soil (mm), nodal position of cut (node number below cut) were recorded for each plant.

Seedlings were cut approximately 137 mm above the soil and the deteached shoots were placed under water to avoid cavitation. Air was supplied to the cut base of the shoot at a maximum of 80 kPa, which was low enough to avoid inducing cavitation

in xylem vessels (Cohen *et al.*, 2003; Skene & Balodis, 1968). The shoots were cut back underwater, progressively from the tip to the base. Cuts were made at one to two mm intervals from the apex of the stem towards base. Cuts were also made in the leaf blades starting at the leaf margins from apex to base. With each incision, newly cut vein endings were examined for signs of bubbles emerging from the veins. The first appearance of a stream of bubbles was taken as evidence of an open xylem vessel, from the point of air injection in the stem to the location of the distal cut. This distance was recorded using a tape measure. When low-pressure air is forced through xylem vessels of fresh tissue, air will only pass through open vessels because wet pit membranes will block air flow (Cohen *et al.*, 2003; Skene & Balodis, 1968; Tyree & Zimmermann, 2002a). If vessels are already embolised, air will also be able to cross their pit membranes, which could compromise the accuracy of continuous vessel length measurements. To avoid that, all plant parts were well hydrated and stems were cut immediately prior to air injection, therefore embolisms in vessels were highly unlikely to contribute or detract from the lengths of open, continuous vessels (Thorne *et al.*, 2006).

In addition to recording maximum vessel length from a fixed position above the soil, air injection from an injection site two to three mm below each node was also used to record how often vessels extended from the node into a leaf, and the length they extended, for an additional ten seedlings, with five nodes measured on each seedling. Nodes were selected at a range of heights, then the average proportion of leaves with open vessels for each height class of node was calculated.

4.3.3 Silicone injection

4.3.3.1 Mature shoots

Shoots were harvested at dawn, enclosed in humidified plastic bags and their cut ends placed in water to prevent transpiration. The bark was removed from the cut end and the xylem washed and recut in the laboratory to give a mucilage free cut end for injection. Shoots were connected to pressurized degassed water supply using silicon tubing and flushed for 30 minutes at 75 kPa to remove air emboli from open vessels. A 1% w/w solution of fluorescent brightener (Uvitex, CIBA AG, Basel, Switzerland) in chloroform was prepared and mixed with silicone polymer plus hardener (Sylgard 184, Dow Corning Corporation, Midland, Michigan USA),

bubbles were removed and approximately five ml of the polymer was injected into the cut end of each stem via a three way stop-cock adjacent to the cut end. The shoots were left to set under a constant injection pressure of 30 kPa. Once set, the leaves were numbered and the transverse hand sections cut at five to six locations distal from the cut end (5, 10, 20, 50, 100, 200 mm). The sections were photographed using an epifluorescence microscope and the number of silicon filled and unfilled vessels was counted for each position using image analysis (ImageJ, (Schneider *et al.*, 2012)).

4.3.3.2 Seedlings

Plants were brought to the lab, kept in a dark stable environment, watered well and kept bagged in black plastic bags overnight to ensure full hydration. The plants were also kept bagged in dark bags as much as possible to prevent evaporation during injection. Stems were cut underwater, well below the point where the injection was to be made, and then cut again underwater at the point of injection. Bark was peeled and mucilage was washed off and the xylem recut with a clean razor blade.

The silicone polymer and brightener mixture was prepared as described above, transferred to five ml disposable syringes, and the syringes connected via short lengths of silicone tubing to the cut stem ends, taking care to remove any bubbles. Shoots were inverted with the cut end and syringe upwards, supported by a retort stand and a weight of about 600g loaded onto the syringe plunger to maintain a 30 kPa pressure at the stem end. The arrangement was left overnight for silicon to move into the vessels and set, before the stems were hand sectioned and imaged as described above.

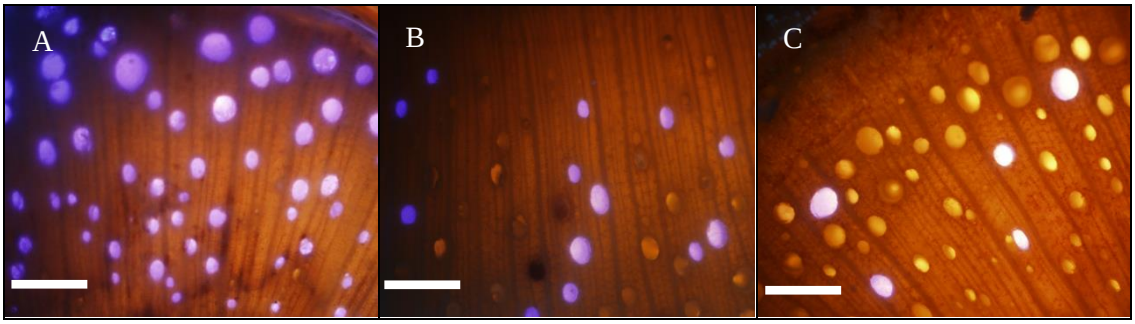

Figure 4-2 Vessels filled with silicone gel (appearing in purple) and non- filled (appearing in yellow) following silicon injection performed on *Actinidia chinensis* Planch. var. *chinensis* 'G3' mature shoots. Cross sections were imaged 24 hours after the injection; (A) Five mm away from the point of silicon injection; (B) 200 mm away from the point of silicon injection; (C) 400 mm away from the point of silicon injection. Bars=1 mm.

4.3.3.3 Determination of vessel lengths

Vessel length distributions were then estimated from the silicone injection results using curve fitting as described by Christman *et al.* (2009) and Sperry (n.d.)

4.3.4 Investigation of *Psa* movement from leaf inoculation site to petiole and stem

Hort16A kiwifruit seedlings were selected for leaf inoculation. Healthy Hort16A plants were randomly assigned to treatments (Fig 4.3 A). There were two treatments i.e. (a) lamina inoculation close (5-10mm) to the petiole ('base') (b) lamina inoculation near the distal tip of the lamina ('tip'). Recently fully-expanded leaves situated at least two nodes apart from each plant were subjected to leaf inoculation. Twenty plants were maintained as controls, with their leaves inoculated with sterile water. Petiole length, lamina length from petiole to tip and lamina width on the broadest part of the lamina of both experimental and control plants were measured and recorded before inoculation. For inoculation the leaf under surface was gently abraded at the midrib with 400 grit sand paper. One 10 µl drop of *Psa* inoculum with approximate strength of 10^9 CFU ml^{-1} (Source :Plant and Food Research, Ruakura) with 0.025% surfactant (Duwett, Nufarm Australia Limited) was added to the abraded surface patch. The plants were monitored for onset of symptoms (Fig 4.3 B & C). When the leaf spots were first observed, the plants were harvested. The duration from inoculation to harvest was two weeks on average. Samples were taken from three sites of each inoculation. For lamina base inoculations, samples were taken from the lamina tip, the midpoint of the petiole from the leaf blade to the stem,

and from the stem just below the node. In the plants where tip inoculation was performed, samples were taken from the lamina base, the midpoint of the petiole from the leaf blade to the stem, and from the stem just below the node. The distance between the inoculation site and the position of each sample was recorded. Each sample weighed approximately one g. The leaf and stem samples were surface sterilised and ground in 100 µl of sterile water using a sterilised plastic mortar and pestle for each sample.

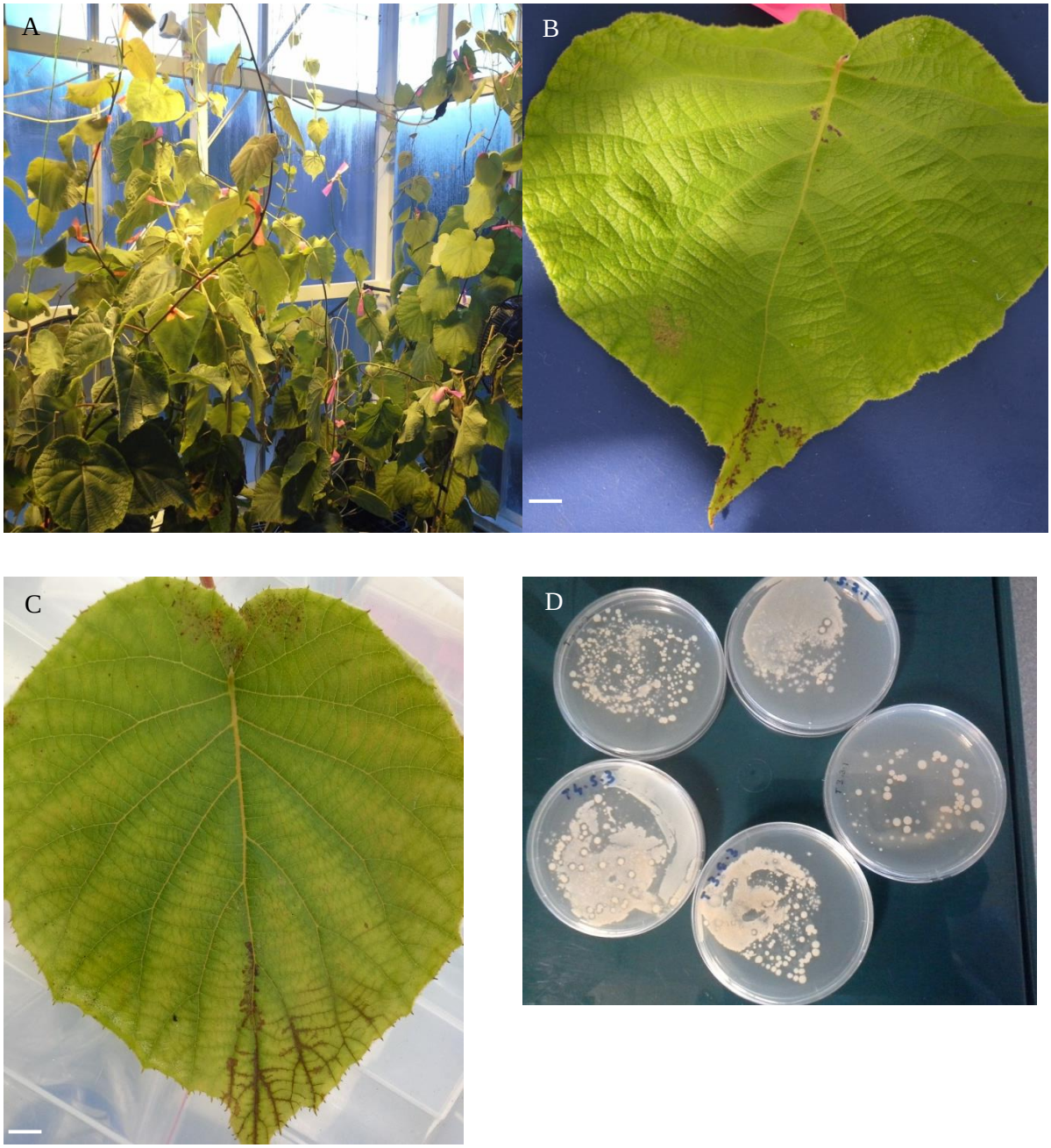

Figure 4-3 Preparation of five month old *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' plants for leaf inoculation, symptom appearance and confirmation of *Psa* movement; (A) Randomly assigned Hort16A kiwifruit plants at the time of inoculation; (B) A kiwifruit leaf inoculated close to the petiole with *Psa* after 16 days. Leaf spots are more frequent at the lamina tip despite the inoculation being performed close to the petiole. ; (C) A kiwifruit leaf inoculated with *Psa* at the tip after 16 days ; (D) *Psa* colonies formed on the nutrient agar plates following two days of culturing of the supernatant from ground tissue samples.
Bar = 1cm.

Re isolation of *Psa*

Extractions were spun and the supernatant was plated on to nutrient agar plates and allowed time for colony formation (Fig 4.3 D). Duplex PCR was conducted on selected colonies to confirm that they were *Psa* as described in Chapter 3 (Gallelli *et al.*, 2011).

For the two inoculation positions, the number of positive detections of *Psa* were collated for each sampling site. A Chi-square test of homogeneity was performed for each inoculation position to test whether the frequency of *Psa* detection differed between the three sampling points. 95% Confidence intervals for the probability of detection at each sampling site were estimated based on the normal approximation of the binomial confidence interval.

4.4 Results

4.4.1 Maximum vessel lengths and leaf to stem connections

The average longest vessel length of four month old kiwifruit Hort16A seedlings measured using air injection was 346 ± 19 mm (n=22). There was a positive correlation between the longest vessel length and the height of the plant ($P < 0.05$, $r^2=0.87$; Fig 4.4). In contrast, the number of nodes with vessels extending into leaves was negatively correlated with the height of the plant ($P < 0.001$, $r^2=0.72$; Fig 4.5).

The observation of decreasing stem to leaf vessel connection with height was also supported by air injection performed at the stem just below each node. The proportion of leaves with open vessels extending from the stem into the leaf blade and petiole also decreased with plant height ($P < 0.001$, Fig 4.6).

Overall 71% of leaves had an open vessel to the leaf lamina from the stem and 79% of leaves had an open vessel from the stem to the petiole. On average an open vessel from stem to leaf penetrated into the lamina up to a distance of 39.7 mm (Table 4.1).

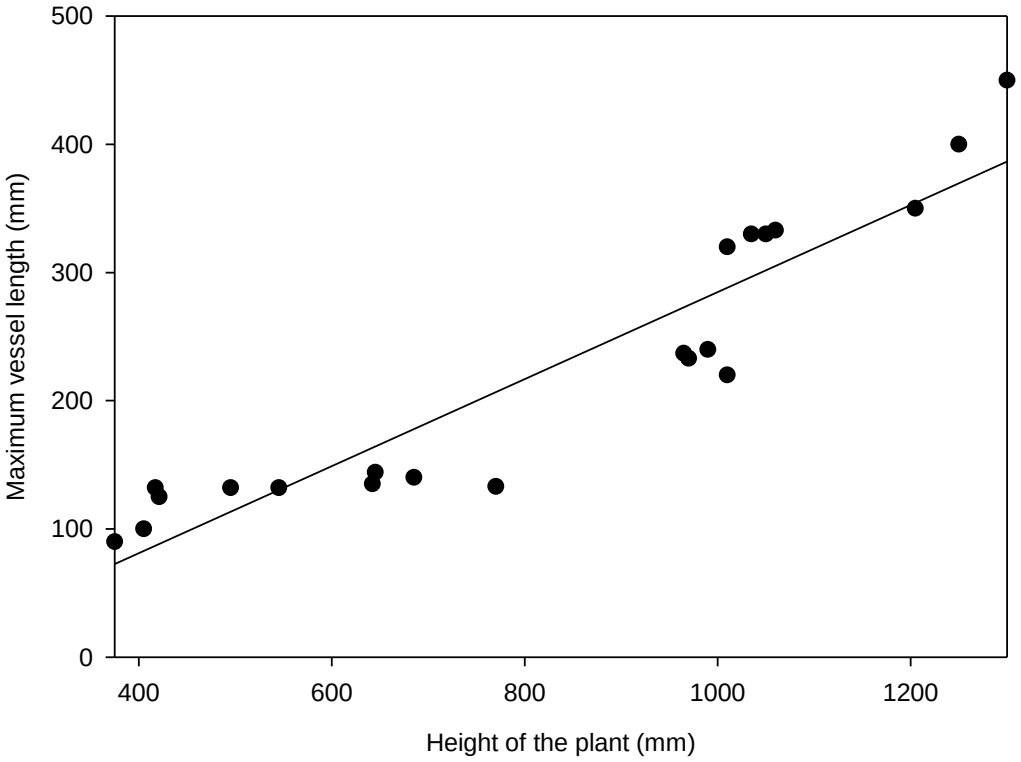

Figure 4-4 Variation in maximum vessel length with plant height when air injection was performed from the basal end of four month old *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A'seedlings (n=22).

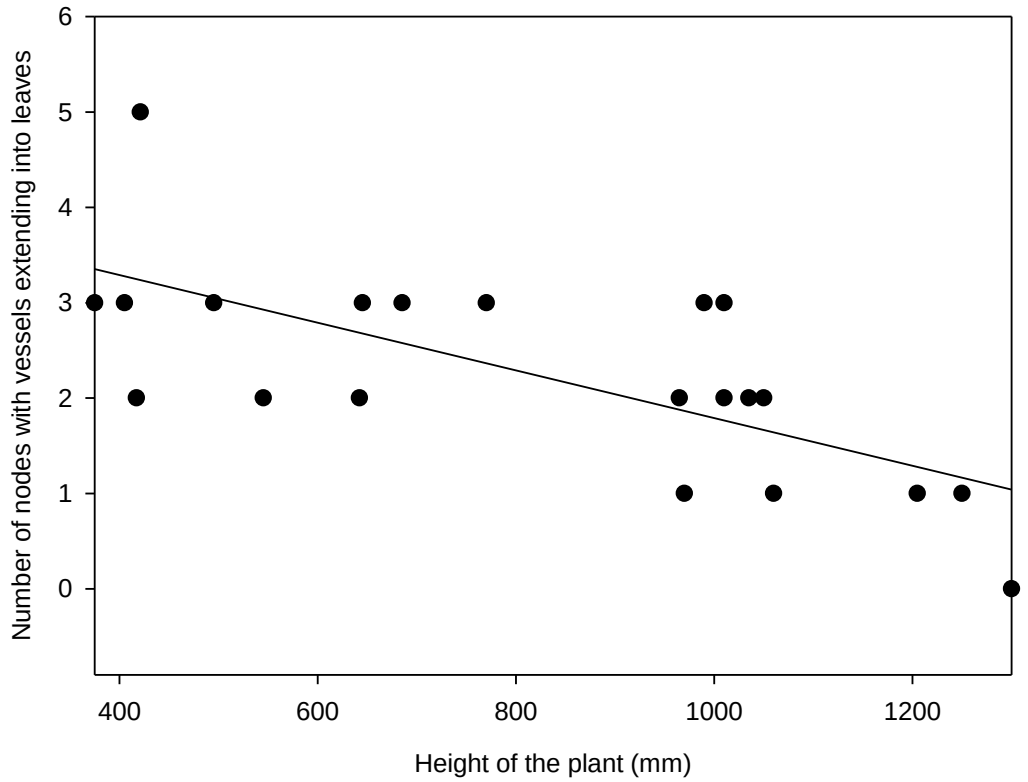

Figure 4-5 Variation in the number of nodes with open vessels extending from the stem into the petiole or lamina with plant height observed following air injection performed from a single point at the base of four month old *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A'seedlings(n=22).

Table 4.1 Proportion of leaf blades and petioles containing open vessels extending from the stem and following air injection performed on four month old *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' seedlings with air injected into the stem just below the node (n=10).

Distance vessel penetrated into lamina (mm)	Proportion of leaves that had an open vessel to the leaf blade	Proportion of leaves that had an open vessel to the petiole
39.7 ± 5.94	0.71 ± 0.093	0.79 ± 0.081

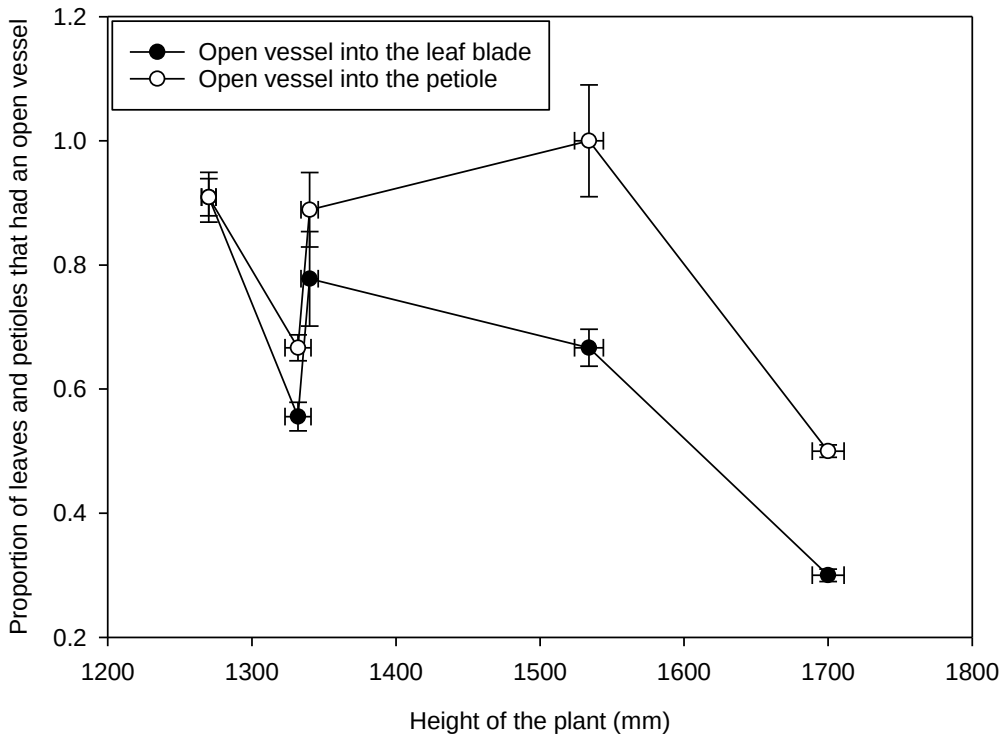

Figure 4-6 Variation in the number of leaf blades and petioles containing open vessels extending from the stem, following air injection performed on four month old *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' seedlings with air injected into the stem just below the node (n=10, means ± SE).

4.4.2 Vessel length distributions

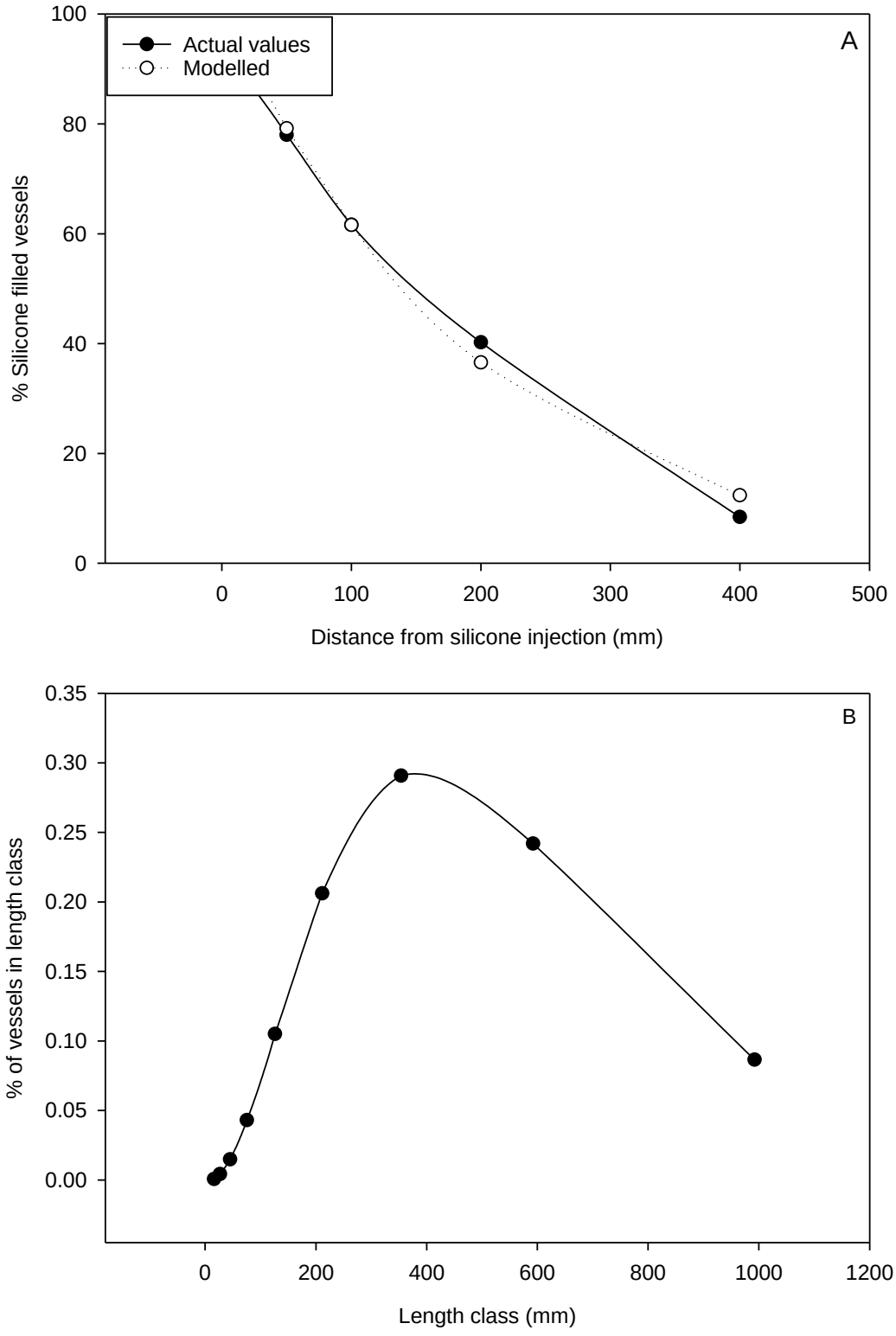

Figure 4-7 An example of vessel length distribution estimation for a mature shoot of *Actinidia chinensis* Planch. var. *chinensis* 'G3' using silicone injection. A Weibull function was fitted to the decrease in proportion of silicone filled vessels with distance from the injection site (A), and the fitted Weibull function was then used to predict the probability density of vessels in 9 length classes (B) (Christman *et al.*, 2009) .

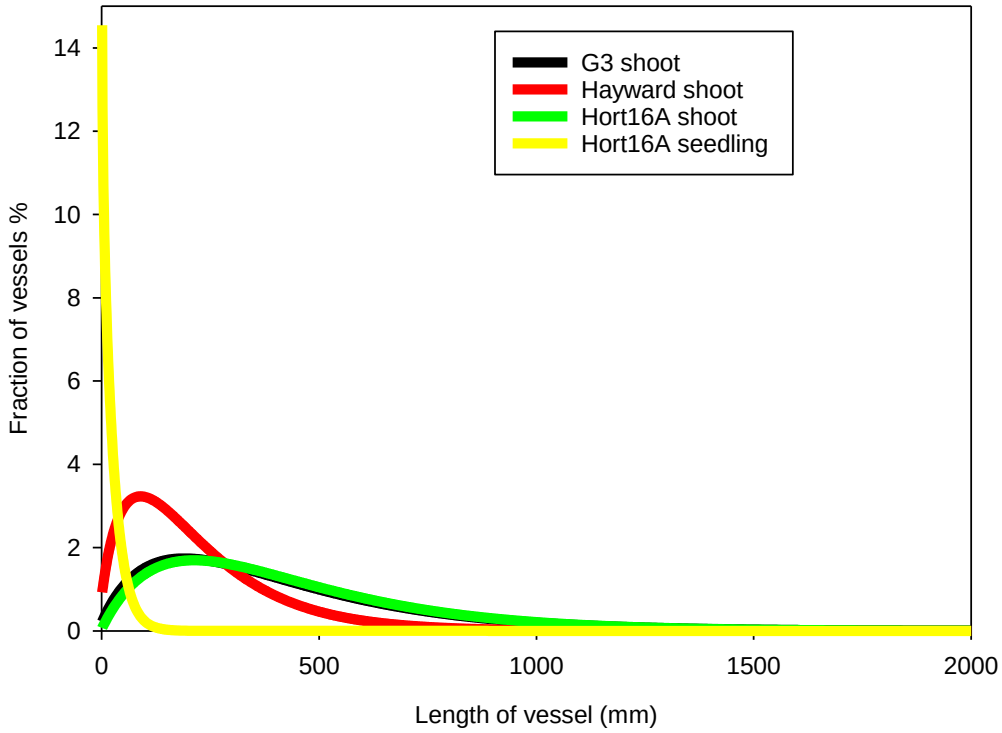

Figure 4-8 Vessel length distributions derived from mean best fit of each variety using the equations by Hacke et al., 2007 following silicone injection performed on mature shoots of each cultivar, and for seedlings of Hort16A (n=5).

Table 4.2 Average vessel lengths calculated following silicone injection and analysis according to Christman *et al.* (2009).

Kiwifruit Variety/Type	Vessel length (mm)			
	Mean	**Median**	**Mode**	**Maximum**
G3- mature shoots	1354 ± 102	840 ± 55	190 ± 10	9069 ± 78
Hayward - mature shoots	221 ± 10	179 ± 9	89 ± 6.8	927 ± 99
Hort16A - mature shoots	539 ± 130	452 ± 93	213 ± 40	2038 ± 886
Hort16A - seedlings	52 ± 9	87 ± 4	65 ± 5	350 ± 15

When cut stems were injected with silicone polymer the frequency of silicone filled vessels decreased with distance from the cut end (Fig. 4.7 A). A Weibull function fitted to these data was used to model the vessel length distribution for each injected stem (Fig. 4.7 B). When the results for all polymer injected stems were combined, there were pronounced differences between cultivars in their average vessel length distribution (Fig. 4.8, Table 4.2). G3 and Hort16A mature shoots had considerably longer average, median, and mode vessel lengths than Hayward (Fig. 4.8, Table 4.2). Average vessel lengths were consistent between shoots within the cultivars G3 and Hayward (Table 4.2), and more variable between mature shoots of Hort16A (Table 4.2). However, vessel lengths were shorter and less variable between seedlings of Hort16A (Table 4.2).

4.4.3 *Psa* movement from leaf lamina inoculation sites to the petiole and stem

Ninety per cent of the bacterial colonies grown from extractions from lamina, petiole and stem samples taken from inoculated leaves resembled *Psa*. Eighty Five per cent of these colonies tested positive for *Psa* using PCR.

Substantial movement of *Psa* occurred after both lamina tip and base inoculations, with Psa moving from the lamina to the stem from more than 70% of the inoculated leaf laminas. After tip inoculation there was a decrease in the frequency of Psa detection with the distance of the sampling site from the point of inoculation (Fig. 4.9 A, B). However, a test of homogeneity indicated no effect of sampling position on the probability of detection ($P>0.05$). With lamina base inoculation there was also

a high probably of movement to the stem or leaf tip (Fig. 4.10 A, B). A test of homogeneity indicated no effect of sampling position on the probability of detection ($P>0.05$). Across all sampling sites there was a 90% probability of recovering Psa, regardless of the distance between the point of inoculation and the sampling position.

When movement within the leaf blade was considered, the probability of *Psa* movement across the lamina was similar after lamina base or tip inoculation ($P >0.05$; Fig 4.11).

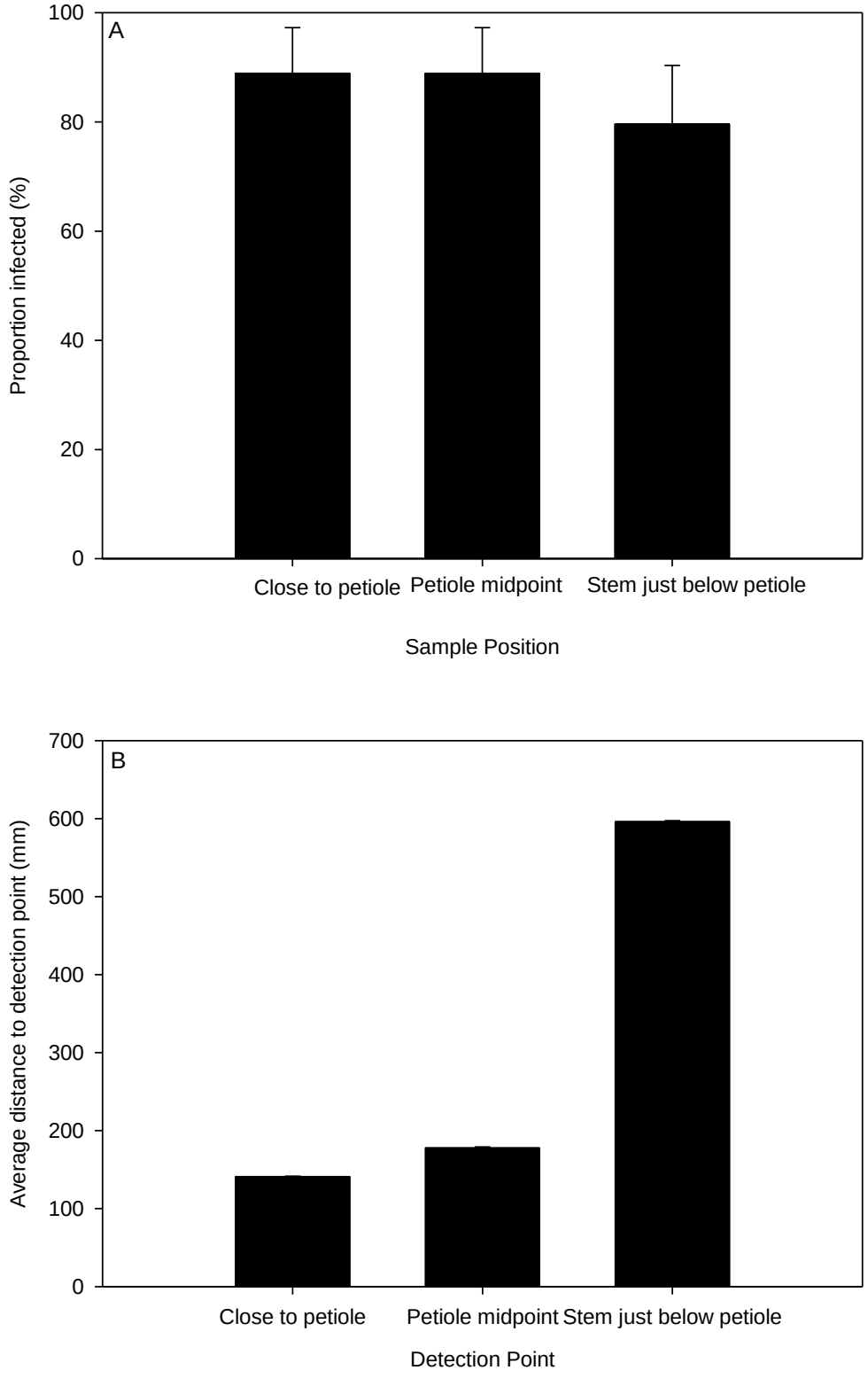

Figure 4-9 Movement of *Psa* within the leaf and petiole in five months old *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' seedlings following lamina tip inoculation.; (A) Probability of observing *Psa* at each detection site (error bars indicate the 95% confidence interval for the probability of detection). (B) Average distance to each detection site from the point of inoculation (n=54, means ± SE).

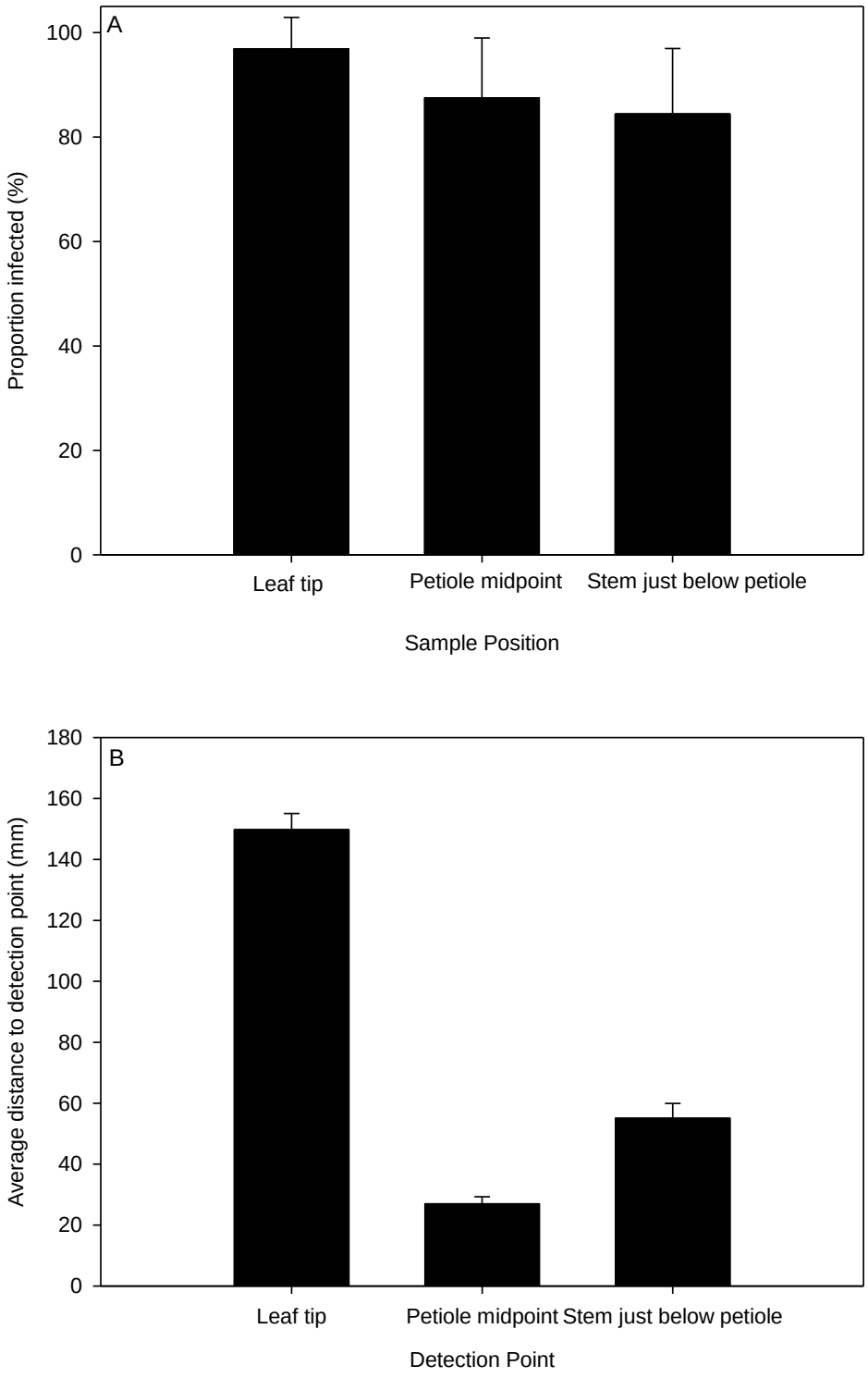

Figure 4-10 Movement of *Psa* within the leaf and petiole in five months old *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' seedlings following lamina base inoculation.; (A) Probability of observing *Psa* at each detection site (error bars indicate the 95% confidence interval for the probability of detection). (B) Average distance to each detection site from the point of inoculation (n=32, means ± SE).

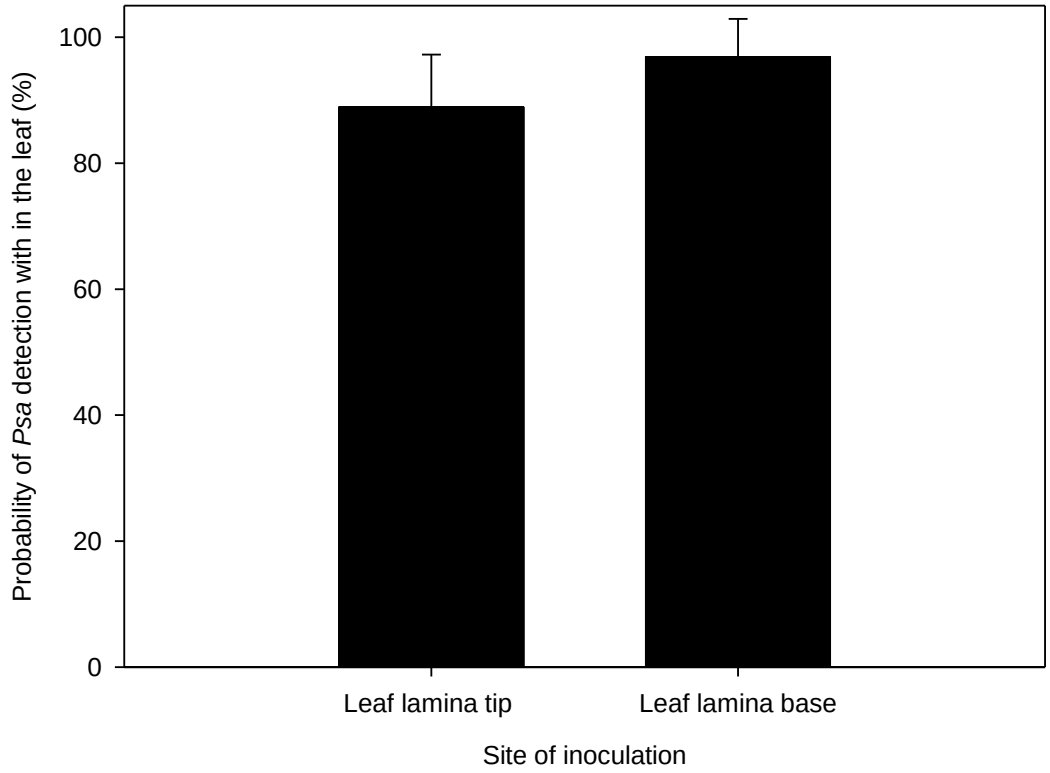

Figure 4-11 Comparison of *Psa* movement within the leaf blade towards the lamina base in tip inoculation and towards the tip in base inoculation in five month old *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A' seedlings. Error bars indicate the 95% confidence interval for the probability of detection. (n=54 and 32 respectively).

4.5 Discussion

The results clearly demonstrate that all three kiwifruit cultivars have very long vessels in their stems, with vessel length distributions that are as long, or longer, than other lianas that have been measured (Anna L. Jacobsen & Pratt, 2012; Ewers & Fisher, 1989; Ewers *et al.*, 1990). Long vessels may contribute to rapid movement of *Psa* if the pathogen enters the stem xylem. Despite long vessels within the stem, more detailed examination of stem to leaf vessel connections suggests that while open vessels between the stem and leaf do occur, these are less frequent and less extensive than in grape vine, the only other vine crop for which this trait has been examined (Chatelet *et al.*, 2006). However, *Psa* was able to move readily within the leaf blade and from the leaf to the stem, more often and further than the measured extent of open vessel connections. Assuming that *Psa* is present and mobile within

the vascular tissue after leaf inoculation, this observation suggests that vessel ends and the pit membrane are not significant barriers to *Psa* movement.

4.5.1 Vessel Anatomy

Kiwifruit seedlings have long vessels similar to other woody lianas like grapes; however, the kiwifruit vessels are longer compared to those of many other perennial deciduous crops (Ewers & Fisher, 1989; Zimmermann & Jeje, 1981). Vessel length of six species of tropical and subtropical lianas (woody vines) were examined (Ewers & Fisher, 1989). Stems tended to have vessels, with a maximum vessel length of 625 mm which is much shorter than kiwifruit. In an analysis of a xylem vessel lengths in woody plants, mean and maximum vessel length varied greatly within and among species (Jacobsen *et al.*, 2012) which is consistent with kiwifruit too.

The purpose of air injection experiments was to determine the maximum vessel lengths and to illustrate the xylem vessel interconnectedness between leaf, petiole and stem in Hort16A seedlings (Figs. 4.4, 4.5 & 4.6). Air injection results are consistent with those expected of a seedling since the vessel length increases with the growth of the plant in height (Fig. 4.4). The number of nodes with vessels extending into leaves decreases with the height of the plant due to the increase in distance from the point of air injection to the upper nodes, with the growth of the plant (Fig. 4.5).

However when air was injected into the stem immediately below each node, the proportion of leaves and petioles containing an open vessel decreases with height of the plant, suggesting that stem to leaf connections tend to decrease in the taller plants (Fig.4.6). The results of the air injection experiments also indicate the extent to which *Psa* is likely to move passively through the xylem vessels when the pathogen entered the plant from an infection site. In theory, from a point of entry into the xylem of a Hort16A seedling, *Psa* could move more than 300 mm by passive convection through the longest open xylem vessels before it would encounter a vessel end wall. Maximum vessel length calculated by air injection in Hort16A seedlings is 346 mm and maximum vessel length of Hort16A seedlings derived by curve fitting in silicon injection is 350 mm. Therefore, two results obtained from two methods for the same parameter are consistent. In this study 71% of the leaves had an open vessel from stem to the leaf blade and 79% of the leaves had an open vessel

from stem to the petiole (Table 4.1). The observation suggests that if *Psa* enters close to the petiole from the leaf or the stem end, there is a significant probability of the pathogen moving passively into the stem or leaf which is evident from the results of the movement experiments of *Psa* (Figs. 4.9 & 4.10). However in the few other investigations into potential xylem pathways available to particles or bacteria between stems and leaves, only an insignificant movement from one organ to another was observed (Canny, 1997; Suhayda & Goodman, 1981; Wiebe *et al.*, 1984). It was found that the average length of the leaf lamina in Hort16A seedlings was 198.6 mm, and open vessels from the stem, if they were present, penetrated into the lamina on average a distance of up to 39 mm. This is only a short distance beyond the end of the petiole. This observation is entirely consistent with the published literature where it is stated that within leaves, the majority of xylem vessels ended near junctions, specifically the petiole–lamina (Canny, 1997; Wiebe *et al.*, 1984) and the petiole–stem junction (Tyree & Zimmermann, 2002a; Wiebe *et al.*, 1984). Therefore, kiwifruit is similar to other plant species in vessel connections, while grape vine is different in this aspect. Furthermore, it was observed that vessels were not branched within the leaf lamina and air bubbles only appeared in the midrib; not in the other veins. However, in grapevine leaves, bacteria, beads and air moved through long and branched xylem vessels from the petiole into the secondary veins (Thorne *et al.*, 2006). Therefore in kiwifruit vessels, microorganisms have to cross pit membranes to leave the petiole and move a significant distance into a leaf blade. As stated in (Chatelet *et al.*, 2006), it is not clear if the observed movement of air in the xylem was restricted to a single xylem vessel or whether more than one vessel was seen connected to another through ruptured primary cell walls or ruptured pit membranes. However, *Psa* appears to move readily within the leaf and from the leaf through the petiole into the stem regardless of the position of open vessels, suggesting it is capable of crossing pit membrane barriers (Perez-Donoso *et al.*, 2010) or is moving outside of the vessels. Therefore, it can be ruled out that *Psa*'s movement in the plant is entirely a passive movement via xylem vessels similar to the movement of water in the xylem vessels (Jacobsen *et al.*, 2012). This is different to grapevine where bacteria, beads and air moved through branched xylem vessels from the petiole into the veins in leaves of two grapevine varieties. Kiwifruit has vessels extending from the stem into the leaf blade, but the frequency of these and the distance they extend appear to be less than in grape.

In another study of xylem structure of four grapevine varieties and 12 alternative hosts to the xylem-limited bacterium *Xylella fastidiosa (Xf),* no difference has been observed in stem or petiole vascular anatomy among the grape varieties examined. Furthermore, no difference in vascular anatomy was observed among the 12 alternative hosts. Among grape varieties, the more tolerant 'Sylvaner' had smaller stem vessel diameters and 20% more parenchyma rays than the other three varieties. Alternative hosts supporting *Xf* movement had slightly longer open xylem conduits within leaves, and more connection between stem and leaves, when compared with alternative hosts that limit *Xf* movement (Chatelet *et al.*, 2011). Therefore, it appears that *Psa* movement in kiwifruit and *Xf* movement in grapevine are different.

Liana vessels are commonly considered to be among the longest in the plant kingdom (Kramer & Kozlowski, 1979; Zimmermann & Sperry, 1983). Average vessel lengths are about 600 mm in the temperate liana, *Vitis labrusca* and over 1000 mm in the tropical liana, *Tetracera* (Ewers & Fisher, 1989). In a study conducted with woody, vessel bearing, flowering plants using liana, shrub, and tree species from different environments, it was revealed that maximum vessel lengths were typically 5.6 times longer than mean vessel lengths (Jacobsen *et al.*, 2012). This observation was consistent with kiwifruit cultivars in this research. In the above research, all species exhibited very similar vessel length distributions (i.e., the shape of the vessel length distribution curve), with the predominant difference between species occurring in shifts in the lengths associated with the peak and tail of the distribution (i.e., in the mean vessel length and maximum vessel length). In this study, kiwifruit cultivars G3 and Hort16A (mature shoots) displayed vessel length distribution curves similar in shape to each other except Hayward.

The average length of xylem vessels was determined in the Hort16A variety with highest susceptibility to *Psa* and the less susceptible G3 and Hayward varieties. The more *Psa* tolerant G3 variety had the longest average vessel length while more susceptible Hort16A had the second longest average vessel length. There are possible explanations in the literature describing the way the vessel lengths and vessel anatomy help microorganisms to invade the plants. The mechanism of water transport in xylem vessels only allows water and low molecular mass solutes to pass through pit membranes but not microorganisms and gas bubbles (Bové & Garnier, 2003; Choat *et al.*, 2005; Fosket, 2012; Tarbah & Goodman, 1987; Tyree &

Zimmermann, 2013; Zwieniecki *et al.*, 2002). However, bacteria might also be able to move through torn or remnant pit pore membranes, particularly if they possess a mechanism for enzymatically degrading the pit membrane (Schneider & Carlquist, 2004). If vessels are short and if numerous membranes have to be crossed, the bacterial movement could be impeded as the pathogen has to disintegrate a large number of pit membranes. Bacterial movement in the stem of *Vitis* is relatively unimpeded due to the presence of a large number of vessels that can be as long as 100cm (Chatelet *et al.*, 2006; Perez-Donoso *et al.*, 2007; Sperry *et al.*, 1987; Thorne *et al.*, 2006). Therefore, the observation in this study is consistent to some extent with the above literature as the most susceptible Hort16A variety had the second longest average vessel lengths. However, there is still a barrier for the bacterial passage into leaves if most of the vessels end at the stem—petiole and petiole—lamina junctions (Chatelet *et al.*, 2006). In addition to xylem vessel lengths, xylem network connections play an important role in water and nutrient transport in plants; however, they also facilitate the spread of air embolisms and xylem-dwelling pathogens. There are vessel relays found in grapevine xylem that form radial and tangential connections between spatially discrete vessels. Due to their spatial distribution within the *Vitis* xylem, vessel relays increase the connectivity between vessels that would otherwise remain isolated. Differences in vessel relays between *Vitis* species suggest these anatomical features could contribute to disease and embolism resistance in some species (Brodersen *et al.*, 2013). Future research can be envisaged investigating if vessel relays found in Hort16A make them more susceptible to *Psa* disease.

4.5.2 *Psa* movement from leaf inoculation sites to the petiole and stem

In this study, it is evident that systemic movement of *Psa* occurred in both directions in the leaf, from the lamina base to tip and from the tip to the lamina base and stem. The evidence suggests that *Psa* is moving actively from vessel to vessel, or is moving outside of the vessels, as movement occurs readily across the boundary where vessels between the leaf and the stem end. Movement also occurs from leaf to stem more often than the frequency of leaves that air injection results show have vessels extending from stem to leaf. It can be suggested that as described in Chapter 3 (Fig 3.2), *Psa* may have produced cell wall degrading enzymes like xylanases to penetrate the pit membranes and moved into the next vessel. The same observation

has been made by (Ferrante *et al.*, 2012). They have observed that *Psa* is capable of moving systemically from the leaves to young shoots of *A. chinensis* through the leaf veins and petioles. They recovered the pathogen from symptomless shoots one week after leaf inoculation. However, they claim that *Psa* probably moves from the leaf veins to the shoot through the xylem vessels as observed by Spinelli *et al.* (2011). In an experiment conducted using a GFPuv-labelled strain of *Psa,* light and transmission electron microscopic observations showed that bacterial cells colonize both the phloem parenchyma or sieve tubes, and xylem vessels (Gao *et al.*, 2016). Therefore as clearly shown in chapter 3 (Fig 3.8), *Psa* travelled not only in the xylem, but also in the phloem and parenchyma cells. Systemic movement of *Psa* was investigated in inoculated potted kiwifruit vines in a greenhouse and in naturally infected mature vines in the orchard in another study by Tyson *et al.* (2014). *Psa* moved basipetally and acropetally within the trunks, and through the scion and the rootstock, at similar rates. *Psa* moved systemically throughout mature 'Hort16A' and A. deliciosa 'Hayward' kiwifruit vines and was detected in symptomless tissues.

4.6 Conclusion

Susceptibility to *Psa* in different kiwifruit cultivars has no correlation to xylem vessel lengths of the cultivars since the least *Psa* susceptible variety G3 had the highest average vessel lengths while Hort16A being the most susceptible variety, had the second highest average vessel lengths for mature shoots. Movement of *Psa* between lamina and stem occurs more readily than the presence of open vessels between the stem and leaves suggests, and further than the measured extent of open vessel connections. If movement is occurring within the xylem of the kiwifruit plant, the vessel end walls and pit membranes are not a barrier for *Psa* movement.

CHAPTER 5

General Discussion

5.1 Novel Findings

This study advances our understanding of strategies adopted by *Psa* to infect and invade the host kiwifruit plant. The *Psa* genome includes six genes that have sequence similarity to known cell wall degrading enzymes. This is the first study to demonstrate that the pathogen's annotated xylanse gene is expressed during infection. Xylanase activity was detected by a reducing sugar-detecting assay when *Psa* was cultured *in vitro* in minimal media supplemented with ground kiwifruit wood. Interestingly, in the in-planta studies, the gene annotated for xylanase was expressed at the RNA level and xylanase activity was also detected. Secondly, this study is the first to describe kiwifruit vessel lengths in stems and leaves of several varieties of kiwifruit including the most *Psa* susceptible Hort16A cultivar.

Chapter two demonstrated that *Psa* is capable of surviving and multiplying in nutrient deficient media, especially in a minimal media supplemented with kiwifruit xylem. Further enzyme assay experiments revealed that xylanase activity was detected in *Psa* cultured in kiwifruit xylem media using the DNSA assay. However, with the RBB assay there was no xylanase activity detected in *Psa* cultured in kiwifruit xylem media. Bioinformatics analysis revealed that the *Psa* genome possesses six genes with sequence similarity to CWDE genes. In addition, some of CWDE proteins display a high homology to respective CWDE proteins of other well known woody host pathogens. The *Psa* genome contained four annotations for xylanase that differed in length, ranging from 819-1161 nucleotides, respectively.

Chapter three demonstrated that *Psa* synthesises a type of xylanase which can be detected by the RBB-Xylan assay in the extracts of *Psa*-infected Hort16A plants. However, the xylanase activity in *Psa* could not be detected using DNSA assay in the same infected Hort16A plants. This result is contradictory to the detection of *in vitro* xylanase activity. The strength of the stems of the infected plants was less than that of healthy plants, suggesting that xylanase activity detected using the RBB-

Xylan assay may have caused the reduction in stem strength by degrading hemicellulose. RNA extractions of infected kiwifruit stems demonstrated that one xylanase gene annotated in the genome (referred to as PD 882) was expressed during infection.

In Chapter four, kiwifruit vessel lengths and connections have been studied extensively. Low pressure air injection and silicone injection was performed on Hort16A seedlings to determine the longest vessel lengths and vessel length distributions, and leaf to stem vessel connectivity in these plants. Silicone injection was performed also on three different kiwifruit cultivars in order to ascertain their vessel length distributions. These were G3 mature shoots, Hayward mature shoots and Hort16A mature shoots. The G3 cultivar had the longest average vessel length, and Hayward the shortest. Hort16A average vessel length of mature shoots was intermediate compared to the two other cultivars and showed a considerable amount of variation among different shoots. Given that of the three cultivars considered, Hort16A is the most susceptible to *Psa*, these results suggest that vessel length on its own is not a major risk factor for *Psa* susceptibility. The average vessel length of Hort16A seedlings was much shorter than mature shoots (in keeping with the smaller overall length of the shoots) and was less variable.

Leaf inoculation experiments with Hort16A shoots revealed that there was movement of bacteria in both directions (towards stem and leaf tip) when *Psa* was inoculated near the tip and base of the leaf lamina. However, a significantly higher probability of movement was observed in the direction of leaf tip. *Psa* was able to move readily from the inoculated leaf lamina into the stem, moving past boundaries in open vessel connections between the stem and petiole or lamina, documented using air injection. This result reinforces the conclusion that if *Psa* is mobile in the xylem, as observed in other studies (Ferrante *et al.*, 2012; Renzi *et al.*, 2012; Scortichini *et al.*, 2012; Spinelli *et al.*, 2011), then pit membranes located at the vessel end-walls do not appear to be a barrier to movement. This finding agrees with the observations in Chapters 2 and 3 that the *Psa* genome includes a number of CWDE genes, that at least one of these genes is expressed, and that the pathogen produces active CWDE protein during infection.

5.2 The significance of the host cell wall degrading activity of *Psa*

Kiwifruit bacterial canker caused by *Psa* has become a widespread disease across kiwifruit growing regions globally. It has caused severe economic losses to the industry from 2008 onwards, and in a more aggressive manner when compared to pre-2008 outbreaks (Marcelletti & Scortichini, 2011). Following the first severe outbreak in Italy in 2008, many plant scientists and molecular biologists in the area of horticulture started researching this virulent pathogenic bacterium and its interactions with the host kiwifruit plant. One of the major areas of research includes genomic studies of the pathogen and phylogenetic comparisons and relationships between different strains of the organism (Chapman *et al.*, 2012; Marcelletti *et al.*, 2011; Mazzaglia *et al.*, 2012b; Scortichini *et al.*, 2012). More detailed genomic sequencing studies were published in 2013, including sequences for the plasmid that is unique to the most virulent strain of the pathogen (Butler *et al.*, 2013; McCann *et al.*, 2013).

In all of the above genome sequencing studies it was indicated that the *Psa* genome includes genes encoding for enzymes that may be responsible for degradation of plant cell wall material including the primary and secondary cell walls of the host plants. Some of these genes are present in the genomes of all sequenced strains of *Psa* while others may be unique to the most recently described virulent pathovar (Butler *et al.*, 2013; Marcelletti *et al.*, 2011; McCann *et al.*, 2013). Furthermore, investigation of host plant symptoms suggests a possible cell wall degrading activity of *Psa* during the process of infection of the host (Renzi *et al.*, 2012).

Therefore it is important to find out whether *Psa* is actually producing cell wall degrading enzymes in the process of invasion and propagation of the infection throughout the host plant and the nature of such enzymes. In this PhD study, in vitro experiments were conducted to describe bacterial metabolism and its ability to degrade model substrates. Furthermore, in-planta experiments were also conducted to determine the activity of *Psa* in inoculated kiwifruit plants with regard to cell wall degradation. This study is the first of its kind to demonstrate the activity of xylanase of *Psa* in vitro and in-planta.

Previous inoculation studies have shown that the bacterium can penetrate the plant through natural apertures (stomata, lenticels), fruit stalks, leaf scars and wounds (Ferrante *et al.*, 2012). Moreover, as described by Serizawa and Ichikawa (1993b), *Psa* is capable of moving systemically from leaves to young shoots of *A. chinensis* and green kiwifruit through the leaf veins and petioles (Ferrante *et al.*, 2012). Most probably, *Psa* moves from leaf veins to the shoot through xylem vessels as observed by Spinelli *et al.* (2011). In this study, experiments documented in Chapter four showed that vessel length has no impact on the pathogen's ability to move, since it has been observed that *Psa* moved quite quickly from the leaf and into the stem past the position in the leaf where stem to leaf vessels ended. Experiments documented in chapter two showed that *Psa* secreted a type of xylanase during infection and perhaps that xylanase contributed to the aforesaid rapid movement through the tissues, possibly in the xylem by breaking the pit membrane. Furthermore, it is also possible that *Psa* does not need to move specifically in the vessels and instead may only be exerting a thrust in and out of cell lumens and between cell walls. Xylanase may help to degrade primary cell walls along with either one or several other CWDE, such as pectinases and polygalacturonases (PGs).

It is relatively unusual for the *Pseudomonas syringae* species to invade woody plant tissues. However, contrary to the already established infection strategies of other *Pseudomonas syringae* pathovars, preliminary genomic and histological evidence suggests that the virulent strain of *Psa* possesses a cell wall degrading ability that may facilitate invasion of woody tissues. However, this possibility has not been investigated in detail and the actual cause of stem cankers and shoot wilting in host plants infected by *Psa* remain inconclusive. The outcome of this study leads to further studies aimed at producing resistant kiwifruit cultivars and identification of effective control mechanisms for the disease.

Observations of some of the infected plants show a pattern of localization of symptoms either in leaves or shoots. In certain kiwifruit plantations, the cankers appear in shoots with no symptoms in leaves while in other plantations only the leaf spots have been observed with no signs of canker (Vanneste *et al.*, 2011). Therefore, it is important to investigate the mechanism employed by the bacteria to move systemically from leaf to stem and from stem to leaf during the process of infection. Furthermore, in the absence of previous research with regard to bacterial movement

within the kiwifruit plant, it is important to investigate the above phenomenon while also considering the vascular architecture and anatomy of the kiwifruit vine.

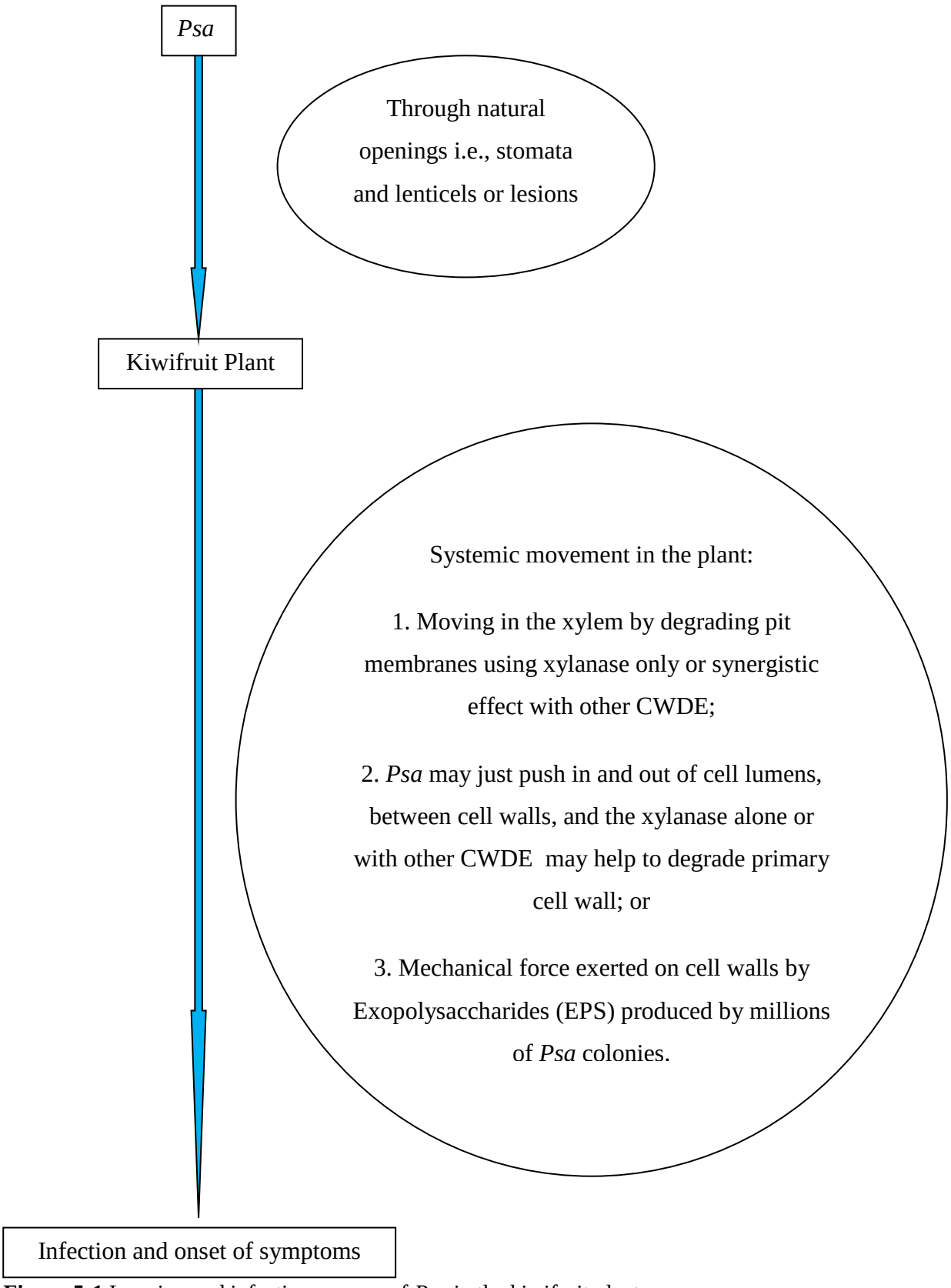

Figure 5-1 Invasion and infection process of *Psa* in the kiwifruit plant.

5.3 Future Prospects

The experiments conducted in this study have opened up many avenues to elucidate the activity of CWDE produced by *Psa,* especially in the process of understanding invasion strategies of the bacterium. For future studies, five research strategies are recommended. These are outlined in more detail below.

5.3.1 Investigate CWDE activity of annotated *Psa* genes in vitro and in-planta

As described in chapter one, six sequences were identified with homology to known CWDE genes in the *Psa* genome. An initial experiment was carried out to ascertain a possible PG activity in *Psa* infected stem extractions using PG acid gel method. However, no PG activity was detected using this method. Further experiments should be designed to conduct enzymatic assays for PG activity when *Psa* is cultured under a variety of conditions and/or in-planta. Investigations should also be conducted to detect pectin lyase and cellulase activity of *Psa*. Research on pectinases, PG and pectin methylesterase (PME) secreted by plant pathogens have been studied in more pathogens and in more detail than any other wall depolymerases (Walton, 1994). Pectinases are able to soften tissue, a distinguishing quality of soft-rot diseases, and induce many physiological effects in plants. Since a large degree of cellulose degradation occurs only late in infection, plant pathologists have generally concluded that cellulases are not particularly important in the development of disease (Cooper & Jellis, 1984). Therefore enzyme assays to detect cellulases could be conducted in very severe stages of the *Psa* infection. However there can be practical limitations of conducting assays for *Psa* cellulases in severe stages of infection as secondary organisms, particularly fungi, are interacting with assay results. Zymogram and SDS-PAGE analysis could also be carried out to confirm the activity and the length of the enzyme respectively.

The xylanase activity detected in *Psa* may be facilitating the rapid movement through the tissue that quickly goes beyond documented vessel lengths. Could xylanase alone facilitate this movement, or would other CWDE be needed to truly loosen or break the walls enough for the bacterium to move within and between cells? The type of xylanases and other types of CWDE needed for the aforesaid

movement can be investigated in detail. Kiwifruit stems could be infused with commercially available CWDE with properties similar to those produced by *Psa,* and measurements of hydraulic conductance used to determine the effect on pit membrane porosity, structure or strength of the xylem tissue. Similar types of experiments have been conducted in grapevine to determine the effect on xylem pit membranes of PG and endo-1,4-b-glucanase enzymes homologous to those produced by *Xylella fastidiosa* in grapevine (Perez-Donoso *et al.*, 2010).

5.3.2 Develop genetically modified (GM) *Psa* strains, knocking out the genes encoding for xylanse and carry out enzyme assays to confirm that activity is due to the xylanase gene observed in the genome

Genes encoding for xylanase can be deleted or knocked out from the *Psa* genome, and enzyme assays may be carried out both with wild type and knockout strains of *Psa* to detect the xylanase activity. A dramatic reduction of extracellular xylanase activity should be detected in cell cultures of genetically modified *Psa* when it was cultured in minimal media supplemented with 0.5% kiwifruit xylem as described in chapter 2, and in extracts from plants infected with knockout strains of *Psa* (Nélida Brito *et al.*, 2006). There are several examples from the published literature where xylanase or xylanase transcriptional activators of pathogenic microbes have been successfully knocked out, resulting in reduced xylanase activity in culture and during infection of the host (Fernando Calero-Nieto *et al.*, 2007; Nélida Brito *et al.*, 2006).

5.3.3 Use GM *Psa* inoculations to analyze the disease severity of the host plant and virulence of the bacteria.

The most susceptible variety Hort16A plants can be subjected to GM *Psa* (as described in 5.3.2) inoculations on the stem and the leaf and the resulting disease severity can be assessed against Hort16A plants that are inoculated with wild type *Psa.* It is expected to see yellow localized lesions in both plants inoculated with wild type *Psa* and GM *Psa*. However in GM *Psa* inoculated plants, spread of the infection and formation of white exudate should be less severe than in wild type *Psa* inoculated plants. Plant extracts of GM *Psa* inoculated plants should not produce xylanase and there cannot be any stem strength reduction in them. A number of instances (Nomura *et al.*, 2006; Woo *et al.*, 2006) can be quoted from the literature where researchers have tested disease severity with GM organisms. *Gibberella zeae*

infection on wheat using wild type (with toxins) and GM *G. zeae* (without toxins) is another example (Desjardins *et al.*, 1996). Successful knockout of xylanase activity from fungal plant pathogens has previously resulted in either no change in virulence (Fernando Calero-Nieto *et al.*, 2007) or substantial reductions in virulence (Nélida Brito *et al.*, 2006).

5.3.4 Use fluorescence to map out the pathways of movement of bacterium in the host plant and understand the rate of movement of *Psa* in the plant

The exact path of *Psa* movement within the plant remains inconclusive. There are several ways that *Psa* may move within the plant (Fig 5.1). It should be possible to transform *Psa* with a stable plasmid vector (pDSK-GFPuv) that strongly expresses the GFPuv (Green Fluorescence Protein) protein. GFP Psa should have 45-fold brighter green fluorescence than wild type bacterial cells, therefore allowing direct observation of individual bacterial cells or cell aggregations with under long-wavelength UV light (395 nm) (Gao *et al.*, 2016; Spinelli *et al.*, 2011). Moreover, (Chatelet *et al.*, 2006) has conducted experiments using GFP-*Xylella fastidiosa* (GFP-*Xf*) on grapevine to describe the movement of the pathogen within the xylem. A similar experiment is proposed for detection of *Psa* movement from stem to leaf lamina and vice versa using GFP-*Psa*, and to examine in more detail how the bacterium overcomes pit membrane and cell wall barriers in kiwifruit.

5.3.5 Host defense mechanisms deployed by the kiwifruit plant

The immune systems evolved by plants to defend against pathogenic microorganisms are quite powerful. Nevertheless, pathogens such as *P. syringae* have successfully developed mechanisms for injecting virulence protein into host plant cells to counteract and overcome the plant immune systems to cause diseases. The plant cell molecules targeted by bacterial virulence proteins that are vital for the development of plant disease have not been fully understood. It has been found that in *Arabidopsis thaliana*, an immunity-associated protein, *AtMIN7* is targeted by a virulence protein, *HopM1* produced by *P. syringae*. The outcome of this experiment illustrates the strategy adopted by bacterial pathogens to take advantage of host enzymes to undermine the immunity of the host plant and cause infection (Nomura *et al.*, 2006).

Further research into the effectiveness of host defence mechanisms deployed by kiwifruit plant in the face of *Psa* invasion could lead to breeding of resistant kiwifruit varieties. Successful *Psa* invasion and onset of symptoms ensue if the kiwifruit defence mechanisms are inappropriate, the kiwifruit plant fails to detect the pathogen or the defence responses triggered are ineffective (Hammond-Kosack & Jones, 1996). A comprehensive understanding of *Psa*-kiwifruit interaction and the kiwifruit's response would shed light on a possible weakness in the defence mechanism. It is already known that the G3 cultivar is more resistant to *Psa* than Hort16A. Future research may be envisaged on differences on defence mechanisms of Hort16A and G3 cultivars.

There are three common strategies that host plants mobilize for defence against invading pathogens; hypersensitive response, production of reactive oxygen species and cell wall fortification. For extracellular pathogens such as *P. syringae,* fortifying the cell wall could prevent leakage of cytoplasm into intercellular space thereby depriving the pathogen of nutrients. Pathogens produce a number of cell wall hydrolyzing enzymes and mechanical pressure exerted by the pathogen may facilitate pathogen entry. Although none of these enzymes or mechanical pressure is crucial for a particular mode of pathogenesis in isolation, some of the cell wall component fragments produced by the activities of these enzymes might trigger additional defence responses. For instance, in response to secretion of PG by some necrotrophic fungi to soften the plant cell walls, PG-inhibiting proteins are induced in bean that inhibit the activity of PG (Hammond-Kosack & Jones, 1996). Further research leading to characterization of defence mechanism related products such as PG-inhibiting proteins could pave the way to derive a better understanding of the pathogen invasion strategies and thereby developing resistance for specific invasion strategies. Therefore future investigations could be directed towards inhibitors produced by kiwifruit plant to CWDE secreted by *Psa* or whether the kiwifruit plant detects *Psa* CWDE as signs of infection.

Further investigations could also investigate whether there is a relationship between callose formation by the host plant and *Psa* CWDE activity. Callose is a structural barrier associated with wounding and infection, and is normally produced during the hypersensitive response in leaves (Vleeshouwers *et al.,* 2000). Callose formation is associated with higher levels of resistance by kiwifruit to *Psa* (Cellini *et al.,* 2014;

Southerland, 2013). Experiments could investigate whether callose formation is induced by CWDE activity, and whether callose formation acts to limit the bacterial movement facilitated by CWDE.

5.4 Conclusion

This study revealed the xylanase activity of *Psa* in vitro and in-planta. One gene annotated for xylanase in the *Psa* genome was expressed during infection. Moreover, this study revealed that there is a loss of strength in infected kiwifruit stems that may be associated with the observed xylanase activity. It is concluded that *Psa* probably does use at least one cell wall degrading enzyme to facilitate movement within the host, after entry via wounds or natural openings on the stem and leaves. Studies of vessel lengths in a number of kiwifruit varieties indicated that there is no relationship between disease susceptibility and vessel lengths or the location of vessel endings, even though others have found that the pathogen is mobile in the xylem tissue. This finding further reinforces the conclusion that *Psa*'s systemic movement in the plant is an active process that involves the activity of cell wall degrading enzyme(s) produced by the pathogen.

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