Exploring the Disease Resistance Response (Chitinase, Lysozyme, Acid Phosphatase and Total Phenolic Content) of Kowhai (*Sophora microphylla* x *S. chathamica*) to Hormones (Salicylic Acid and Ethylene)

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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Abstract

The aim of this research is to find out if the disease resistance of kowhai (New Zealand native *Sophora* species) is induced by hormones. Kowhai is an indigenous legume belonging to the *Sophora* genus. Disease resistance in this case is represented by an increase in the level of plant defensive enzymes and total phenolic content for phenylpropanoid phytoalexins. The Kowhai species used in the study was identified using DNA amplified from the chloroplast *rbcL*, *matK* and *atpB-rbcL* genes. The nucleotide sequence results of the *rbcL* and *matK* genes were used to perform a DNA barcode search using a Basic Local Alignment Search Tool (BLAST) and the sequence result of the *atpb-rbcL* gene was used for a neighbour joining phylogenetic analysis. Combining the result of the DNA barcode, phylogenetic analysis and morphological analysis, the sample used in the study was a *S. microphylla* x *S. chathamica* hybrid, and hence, it is New Zealand native kowhai. The kowhai was treated with 20 μg g^{-1} of 2,6 dichloroisonicotinic acid (INA) and 1000ppm of ethephon. Chitinase, lysozyme and acid phosphatase activities and total phenolic content (phenylpropanoids) were tested by fluorometric and colormetric assays to determine if these hormone sources induced increases in enzyme and total phenolic content in kowhai. Total protein content was also measured by the Bradford method to minimize the experimental error. The result was analyzed by two sample t-test. Acid phosphatase activity and total phenolic content was significantly (p<0.05) induced by 1.77 times and 1.24 times by INA and chitinase and acid phosphatase activities were significantly (p<0.05) induced by 1.92 times and 1.76 times by ethephon. These results indicate that Kowhai has a response to ethylene of a similar order of magnitude to Eurasian legumes such as *Pisum sativum* (Pea). In contrast, the response to INA differed from other legumes and was very weak. This may be related to it being a woody perennial legume rather than a herbaceous annual legume on which most previous hormone research has been based.

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Abbreviation

18s: 18s ribosomal RNA

ANOVA: Analysis of variation

atpB-rbcL gene: ATP synthase beta subunit –rbcL intergenic region

CI: Confidence intervals

DNA: Deoxyribonucleic acid

GADPH: Glyceraldehyde-3-phosphate dehydrogenase

INA: 2,6-dichoropyridine-4-carboxylic acid or 2,6 dichloroisonicotinic acid

ITS: Internal transcribed spacer

matK gene: Maturase K gene

mins: Minutes

MIQE: Minimum information for publication of quantitative Real-time PCR experiments

NCBI-BLASt: National Centre for Biotechnology Information-Basic Local Alignment Search Tool

NTC: Non template control

PAL: Phenylalanine ammonia lyase

PCR: Polymerase chain reaction

rbcL gene: Ribulose 1,5-biphosphate carboxylase large subunit gene

RNA: Ribonucleic acid

SD: Standard deviation

TBE: Tris borate EDTA

sec: Seconds

Chapter 1

Introduction

1.1. Outline of the study

The ability of plants to resist pathogenic fungi and bacteria is linked to their ability to synthesize a wide variety of defensive proteins when challenged by pathogens (van Loon *et al.*, 2006). These proteins include hydrolytic enzymes such as chitinases and lysozymes which degrade fungal and bacterial cell walls and enzymes such as phenylalanine ammonia lyase (PAL) which controls the synthesis of phenolic compounds in plants via the phenylpropanoid pathway. Phenolic compounds may then act as phytoalexins which specifically target fungal and bacterial metabolism and also provide more general protection by forming physical barriers to colonisation e.g. cell wall lignin (MacDonald & D'Cunha, 2007; da Cunha, 1988). The synthesis of defensive enzymes such as chitinases, lysozymes and PAL is mediated by a variety of plant hormones, two important examples being ethylene and salicylic acid (van Loon *et al.,* 2006).

The quantitative induction of defensive enzymes and phenolic compounds by plant hormones such as ethylene and salicylic acid has been extensively studied in a wide variety of commercial food crop species, including legumes of the *Papilionoideae* subfamily which includes the pea, soybean and common bean (Boller *et al.,* 1983). While such legumes are of great importance as food sources they are typically herbaceous annual plants and only represent a tiny fraction of the *Papilionoideae* subfamily present in nature, many of which are woody, perennial shrubs and trees (Lewis et al., 1995).

Unlike the herbaceous legumes, the degree of induction of hydrolytic enzymes and phenolic compounds by plant hormones such as ethylene and salicylic acid has not been quantitatively studied in woody perennial members of the *Papilionoideae* subfamily. The degree of defensive enzyme induction observed in woody plants may differ significantly from that seen in herbaceous plants because of the different ecological lifestyles of herbaceous versus woody plants.

In addition commercial legumes such as peas, common beans and soybeans have been selectively cultivated by humans for several millennia and this selective

cultivation may have altered the quantitative defensive response of these plants by selection for certain characteristics. For example, it is known that in food legumes selection for plants bearing seeds with a reduced seed coat thickness and decreased content of toxic compounds has occurred. While such selection renders the legumes more edible it also makes them more vulnerable to pathogens and again selective breeding may have compensated for this by increased levels of defensive enzymes (Zohary & Hopf, 2001).

Thus, even if induction of defensive enzymes in response to hormones was originally similar in herbaceous and woody legumes, artificial selection by humans may have altered the responses in the food legumes. Since current knowledge of legume defensive responses to pathogens has been based almost exclusively on studies of the commercially important food crops (i.e. pea, beans) there is a gap in the understanding of these responses in non-commercial, non-herbaceous members of the *Papilionoideae* subfamily.

The object of this thesis was therefore to analyze the induction of defensive enzymes of a woody perennial legume of the *Papilionoideae* subfamily in response to treatment with hormones. The kowhai, a New Zealand native legume, was selected for this study because it is a woody perennial member of the *Papilionoideae* subfamily which has not been cultivated as a food plant due to the toxic nature of its seeds. The Kowhai displays both divaricating shrub and tree growth characteristics at different stages of its life cycle (Heenan et al., 2001).

The methodology employed in this study was to treat the leaves of kowhai with the plant hormones ethylene and 2,6-dichoropyridine-4-carboxylic acid (INA-an analogue of salicylic acid) to determine the level of induction of the enzymes chitinase, lysozyme and acid phosphatase. At the same time any changes in the level of phenolic compounds were also monitored.

This research is the first quantitative study of the defensive responses of a woody member of the *papilionoideae* subfamily to hormone treatment and the first such study of any member of the New Zealand flora.

1.2. Legumes and Kowhai

1.2.1. Legumes

The Leguminosae family (also called Fabaceae) is the third largest flowering plant family on the earth and is characterised by the presence of seeds in pods. There are 727 legume genera and 19325 species under this family and the family can be further divided into three subfamilies; the *Caesalpinoideae*, *Mimosoideae* and *Papilionoideae* (Lewis *et al.,* 2005). Most of them are capable of fixing atmospheric nitrogen through root nodules (Lewis *et al.,* 2005). Many legumes are known for their ability to disperse across oceanic barriers and to tolerate saline conditions (Hurr *et al.,* 1999).

1.2.1.1. Role in the eco-system

Most of the legumes including *Sophora* are important to the eco-system, because they are involved in nitrogen fixation. It is a critical process in the nitrogen cycle (Sharma, 2005). Atmospheric nitrogen (N_2, unavailable for biological use) is fixed to ammonium (NH_4^+, for biological usable). The nitrogen fixation process is catalysed by symbiotic rhizobium (also called root nodule bacteria) instead of the legumes themselves (Zhakhia *et al.,* 2004). The rhizobium forms the nodule structure at the root of the legume plant which is assimilated by the plant and supports growth, particularly in nutrient deficient soils. In return the rhizobium is supplied with nutrients, and is protected inside the nodule structure O'gara *et al.,* (1976). This nitrogen fixation symbiotic relationship between *Sophora* genus (and other legumes) and rhizobium are important to the eco-system in worldwide.

1.2.2. The Kowhai

New Zealand has around 2,000 indigenous vascular plant species (Brooker *et al.,* 1989), a high proportion of which are unique to New Zealand. Among flowering plants alone, 75% of species only grow in New Zealand (Salmon, 1991). New Zealand has 33 species of legumes that are native, comprising of four genera: *Sophora, Carmichaelia, Clianthus,* and *Montigena* (Salmon, 1991). Kowhai is the

Maori name for members of the New Zealand native *Sophora* genus which belongs to the legume subfamily Papillonidae under the *Soporeae* tribe of the Leguminosae family.

1.2.3. *Sophora* genus

The generic name, *Sophora*, is derived from *sophera*, an Arabic name for a pea-flowered tree which belongs to the *Papilionoideae* (a subfamily of legumes) and means yellow. 45 species of *Sophora* are widely distributed from south-east Europe across southern Asia, the Indian Ocean, the Pacific, South Atlantic and South Western America (Song *et al.,* 2008).

In New Zealand, the native *Sophora* plants are called kowhai, the Maori word for yellow, which refers to the yellow colour of the floral organ (shows in Figure 1). Maori use the wood of kowhai for beaters, mauls, paddles, weapons, spade blades, weeders, digging sticks and bird spears (Cooper *et al.,* 1991). The juice of the bark of kowhai was used by Maori for back pain treatment (Brooker & Cooper, 1959).

Figure 1. An image of kowhai flowers (Wikimedia, n.d.)

1.2.3.1. Geographic origins of the *Sophora* genus

The limited amount of fossil evidence found in New Zealand suggests that the native forms of the *Sophora* genus were not common in New Zealand until the Pleistocene when several of the other Papilionoid genera made their first appearance. *Sophora* pollen is a reticulate tricolporate type common among the dicotyledons, and might be misidentified or overlooked, which could affect the age estimates for the time *Sophora* has been present in New Zealand. While *Sophora* tends to be locally abundant, it was never a dominant part of the vegetation. Thus, it is possible that the fossil data underestimates the time of origin of the genus in New Zealand (Hurr *et al.,* 1999).

Molecular genetics analysis suggests that *Sophora* originally came from the North Western Pacific, from a Eurasian ancestor, and arrived in New Zealand about 2 to 5 million years ago (Heenan *et al.,* 2004).

Another study also supports this hypothesis by comparing the Internal Transcribed Spacer (ITS) sequence of *Sophora* genus from Section. *Edwardsia* (which grows across the Southern Pacific) to sect. *Pseudosophora* (which grows across Eurasia) and sect. *Wightia* (grows in China). Mitchell *et al.,* 2002 found that the sect. *Edwardsia* is likely to have an Asian or Eurasian ancestor or maybe the sect. *Edwardsia* is derived from a China lineage of *Sophora*.

1.2.3.2. *Sophora* species in New Zealand

New Zealand *Sophora* belongs to a subset known as "*Sophora* sect. *Edwardsia*" (Heenan *et al*., 2004). This is the largest group in the *Sophora* genus that includes 19 species whose distribution is centred on islands in the southern Pacific Ocean and there are 8 ecological species native to New Zealand. They are *S. chathamica*, *S. fulvida*, *S. godleyl*, *S. longicarinata*, *S. microphylla*, *S. molloyi*, *S. prostrate* and *S. tetraptera* (Heenan *et al.,* 2001). *S. microphylla* is the most common and widespread species in New Zealand (see Figure 2). In contrast, *S. chathamica* is the most common species found in Auckland region (see Figure 3) (Heenan, 1998).

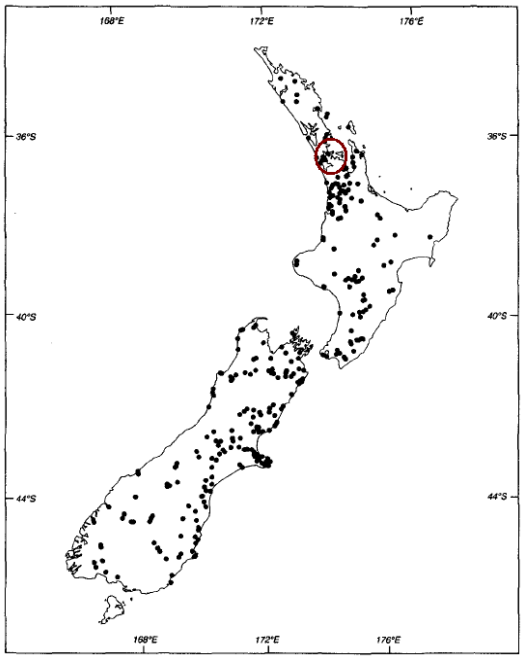


Figure 2. Distribution of *Sophora microphylla* in New Zealand. Red circle indicates the Auckland region (modified from Heenan et al., 2001).

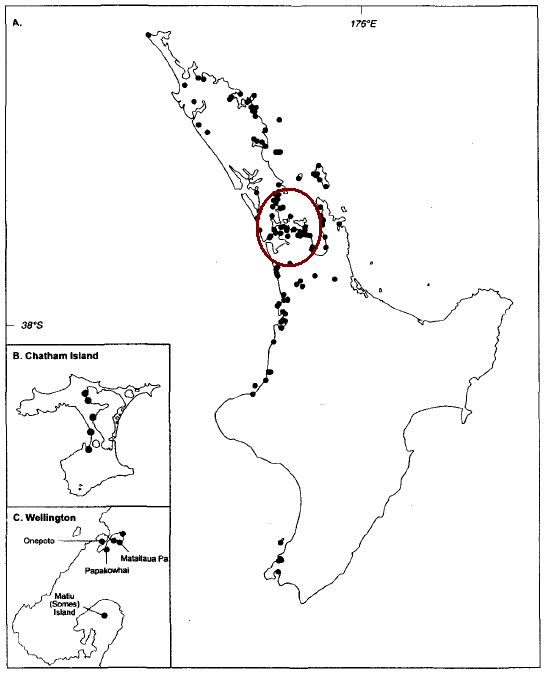


Figure 3. Distribution of *Sophora chathamica* in New Zealand. A: North Island, B: Chatham Island, C: Wellington. Red circle indicates the Auckland region (modified from Heenan et al., 2001).

1.3. Disease resistance of plants

Plants in nature are constantly threatened by pathogens which can impair their growth and reproduction (Jones & Dangl, 2006; Chatterjee & Gosh, 2008). Plant pathogens can be both necrotrophs (kill and consume the host by producing toxins) and biotrophs (live host is required to complete the pathogens life cycle) (Dangl & Jones, 2001). Although plants lack mobile defender cells and a somatic adaptive immune system found in animals, they have developed their own defence responses to infection (Jones & Dangl, 2006). The waxy skin layers on the plant surfaces are the first passive protection against pathogenic bacteria (Dangl & Jones, 2001). However, the insects chewing may overcome this barrier, and trigger the pathogenic bacterial infection via the wound sites. Plants have developed a wound response to confront this situation by producing anti-feedants such as protease inhibitors and alkaloid compounds to prevent the damage from insects (Dangl & Jones, 2001).

Moreover, the pathogenic bacteria can get access into plant intercellular spaces by entering through gas and water pores (Jones & Dangl, 2006). Also pathogenic fungi can infect the plant epidermal cells directly or extend their hyphae through the plant cell (Jones & Dangl, 2006). The passive protection, in these cases, cannot prevent the pathogen infection. Rather, inducible defence systems of plants will initiate after recognition of a pathogen attack. Unique signal molecules from pathogens (Pathogen Associated Molecular Patterns) are detected by receptor molecules of plants, which triggers a series of biochemical reactions to generate hormone signals which can activate the genes which are involved in the defence responses (Chatterjee & Gosh, 2008). These activated genes encode defensive enzymes which can attack pathogens directly (e.g. by glucosidases), or indirectly via producing antimicrobial phytoalexins (e.g. PAL). Defensive enzymes are also involved in other disease resistance processes such as programmed cell death known as the hypersensitive reaction (HR) (Jakobek *et al.,* 1993).

1.3.1. Plant defensive enzymes – The glucosidases

Glucosidases (EC 3.2.1) are glycoside hydrolase enzymes which are able to hydrolyse the 1,4-beta-linkages of polysaccharides. The typical defensive enzymes under this group are chitinase, chitosanase and lysozyme (Robertus *et al.,* 1998), which hydrolyse the polysaccharides present in the fungal (chitin) and bacterial (murein) cell wall. This research will mainly investigate the chitinase and lysozyme activities in kowhai.

1.3.2. Chitinase

Chitinases (EC 3.2.1.14), or poly (1,4-(*N*-acetyl-β-*D*-glucosamide))-glycanohydrolase (White & White, 1997), can be found in many organisms (Koga et al., 1999). They are essential in plants due to the fact that they are the defensive agents against fungal and insect attacks (Koga et al., 1999).

Chitinases have been classified by a single International Union of Biochemistry and Molecular Biology (IUB-MB) enzyme number (EC 3.2.1.14), but this classification cannot represent all the information on chitinases. Comparisons of their amino acid sequences have revealed that their catalytic domains can be grouped into two glucosyl hydrolase (GH) families, 18 and 19, based on differences in the amino acid sequence at the active sites. This classification also correlates with the structural and mechanism features of the enzymes (Henrissat, 1999). In addition, the family 19 chitinases are only found in higher plants (Kawase *et al.,* 2006).

Chitinases can also be divided into two chitinolytic enzyme systems, exochitinase and endochitinase. Exochitinase hydrolyzes chitin from the end of the chain while endochitinase hydrolyzes the chain internally at random positions (Sampson & Gooday, 1998).Plant chitinases are generally endochitinases.

Comparisons of chitinase sequences from various plant species showed that chitinases can also be separated into five classes. There is evidence that Class I, II and IV share a homologous main catalytic domain in addition to a signal peptide

(Hamel *et al.,* 1997; Bravo *et al.,* 2003; Kasprzewska, 2003). Figure 4 shows the difference between Class I to V chitinases. Class I chitinases are made up of two parts: a hevein domain (an antifungal peptide, also known as cystein-rich domain) located at the N-terminal and a glycoside hydrolase family 19 catalytic domain (labelled E E in Figure 4) (Hamel et al., 1997; Tair, 2010). Class II chitinase has a glycoside hydrolase family 19 domain homologous with Class I chitinase but lacks the hevein domain (Hamel et al., 1997; Tair, 2010). Class IV and Class I chitinases share homology but due to four deletions at the C-terminal, Class IV chitinase has a smaller size than Class I chitinase (Tair, 2010). Class III and V chitinases contain a glycoside hydrolase family 18 domain (labelled DXDXE in Figure 4). Apart from that, both Class III and V chitinases share a low homology since Class III has three disulfide bond linker regions while class V does not contain any. Also the molecular mass of Class III chitinase (30 kDa) is much smaller than Class V chitinase (40 kDa) since Class V chitinase contains a unique α/β domain (Tair, 2010). Moreover, Class I to IV chitinases includes bifunctional lysozyme/ chirinase enzymes (Hamel et al., 1997).

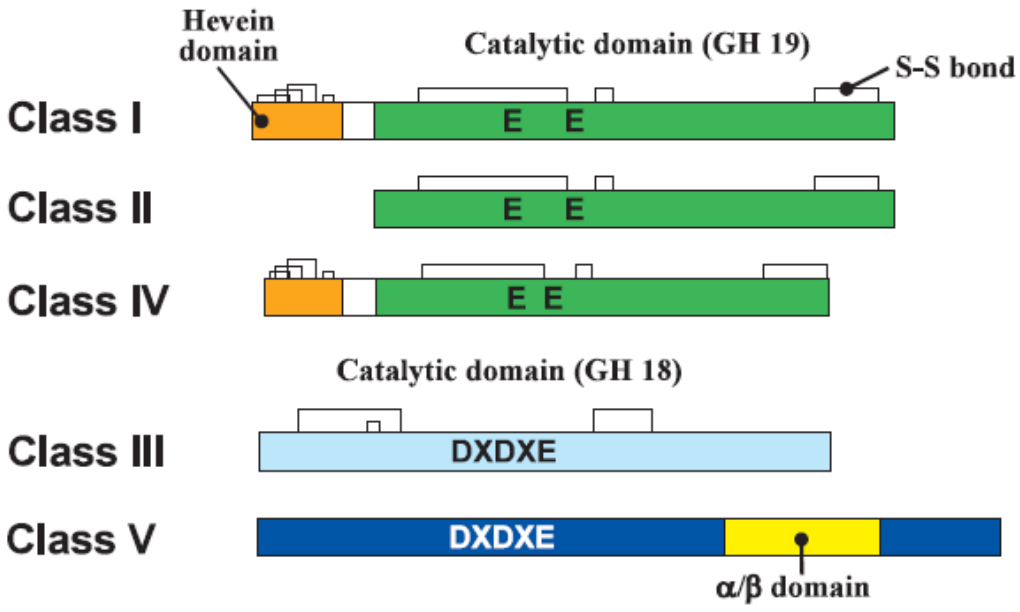


Figure 4. The classification of plant chitinase (modified from Taira, 2010), where the bar represents the protein from N terminus (left) to C terminus (right).

Based on their glucosyl hydrolase domain, the class I, II and IV chitinases belong to glucosyl hydrolase family 19 and the class III and V belong to glucosyl hydrolase family 18. The family 19 classes have a substrate-assisted catalytic mechanism (also called a double-displacement retaining mechanism) with a β anomeric configurated product. In contrast, the family 18 have an acidic catalytic mechanism (also called single-displacement inverting mechanism) with an α anomeric configurated product (Brameld & Goddard, 1998; Kasprzewska, 2003). Figure 5 shows two different hydrolysis mechanisms with the intermediate and products (Brameld & Goddard, 1998).

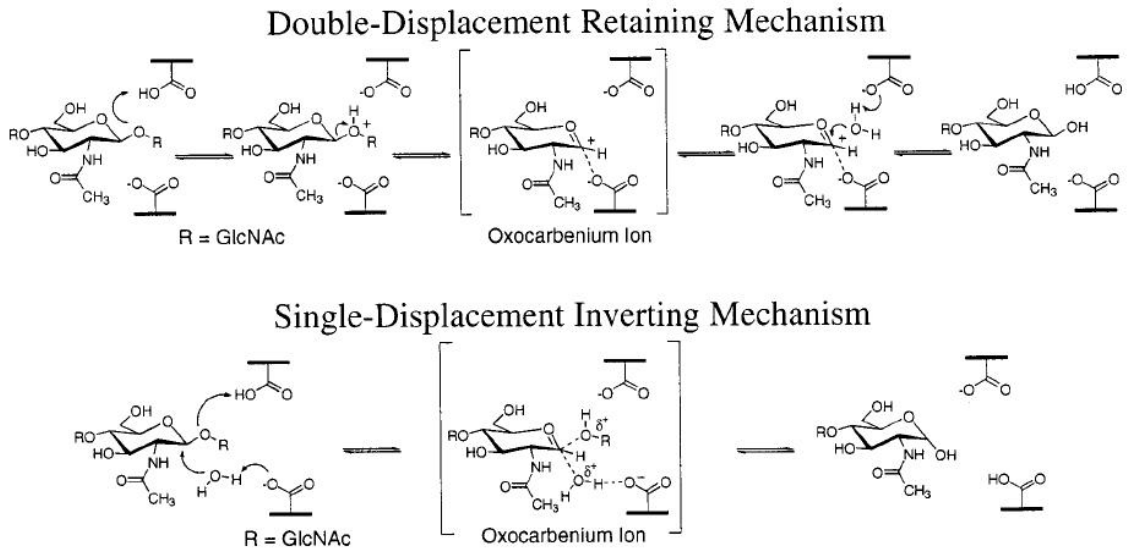


Figure 5. The difference between two chitin hydrolysis mechanisms representing families 19 and 18 respectively (Sampson & Gooday, 1998).

Some chitinases were found to have the ability to cleave (hydrolyse) other chitin-like substrates, such as chitosan (Osswald *et al.,* 1992), N-acetylmuramic acid and N-acetyl-D-glucosamine residues found from the exoskeleton of crustaceans. Chitosan is the substrate of chitosanase (EC 3.2.1.132) (Robertus & Monzingo, 1999). On the other hand, the N-acetylmuramic acid and N-acetyl-D-glucosamine residues are the substrate of lysozyme (EC 3.2.17). The reason the chitinase has the chitosanase and lysozyme activities is because such enzymes have similar substrates, based on glucosyl polysaccharides with1,4-beta-linkages

derivatised wth N-acetyl-amine groups (Robertus & Monzingo, 1999; Lee & Yang, 2002; Huet *et al.,* 2008).

Besides the defence mechanism, plant chitinases have other functions or are involved in other processes. The class I chitinase are involved in interactions with root mycorrhiza, both class I and II chitinases take part in frost resistance, class III in nodulation, class IV in embryogenesis and programmed cell death (Kasprzewska, 2003). Furthermore, it has been suggested that some of the chitinases are inhibited by allosamidin, which is produced by certain fungi to inhibit the defensive chitinases produced by plants during infection (Sampson & Gooday, 1998).

Plant chitinases are considered to protect plants against fungal pathogens by degrading chitin, a major component of the cell walls of fungi. There are many reports of antifungal activity of plant GH-19 chitinases; however reports of antifungal activity of plant GH-18 chitinases are very limited (Taira, 2010). For example, Table 1 shows that most studies of antifungal activity have been associated with GH-19 chitinases (Class I, II & IV). Only class III chitinases from pineapple and gazyumaru show an outstanding antifungal activity. This table also highlights the emphasis placed on analysis of the chitinase activity of commercial crop plants. With the exception of *Leucaena leucocephala* all the species studied are of commercial importance. *Leucaena leucocephala* is a tree legume of the Mimosoideae subfamily.

Figure 6, also taken from Taira (2010), shows a study of the antifungal activity of various classes of chitinase against the fungi *Trichoderma viride*. The fungus is placed at the center of the plate and grows outwards towards wells loaded with chitinases. The chitinases in the wells comprise different classes and isoelectric points (pI) taken from various plants. The chitinases inhibit the growth of the fungi to varying degrees, indicated by the dark zone of inhibition around different wells. The results indicate that Class I enzyme (of glucosyl hydrolase Family 19) have the strongest antifungal activity.

Table 1. Antifungal activities of various chitinase using several fungi (modified from Taira, 2010).

Source	Class of chitinase	Test fungus
Bean	I	*Trichoderma viride*
Barley	II	*T. reesei*
Barley	II	*Alternaria alternaria*
Pea	I	*T. viride*
Pea	I	*F. solamo*
Pea	I	*Penicillium digitatum*
Barley	I	*T. viride*
Barley	II	*T. viride*
Arabidopsis thallaa	I	*T. ressei*
Barley	II	*T. ressei*
Barley	II	*F. sporotrichiodes*
Bean	I	*Rhizoctonia salani*
Maize	IV	*A. solani*
Maize	IV	*F. oxysporum*
Maize	IV	*T. viride*
Tobacco	I	*F. solani*
Tobacco	II	*F. solani*
Tobacco	I	*T. viride*
Tobacco	V	*T. viride*
Tobacco	V	*A. radicina*
Tobacco	V	*F. solani*
Tobacco	I	*T. longibrachiatum*
Tobacco	I	*F. oxysporum*
Grape	I	*Guignardia bidwellii*
Grape	I	*Botryis cinerea*
Rye	I	*F. oxysporum*
Rye	I	*R. solani*
Rye	I	*Trichoderma* sp.
Rye	II	*F. oxysporum*
Rye	II	*R. solani*
Rye	II	*Trichoderma* sp.
Leucaena leucocephala	I	*F. oxysporum*
Rice	I	*T. reesei*
Rice	II	*T. reesei*
Pineapple	III	*T. viride*
Pineapple	I	*T. viride*
Gazyumaru	I	*T. viride*
Gazyumaru	III	*T. viride*
Gazyumaru	I	*T. viride*

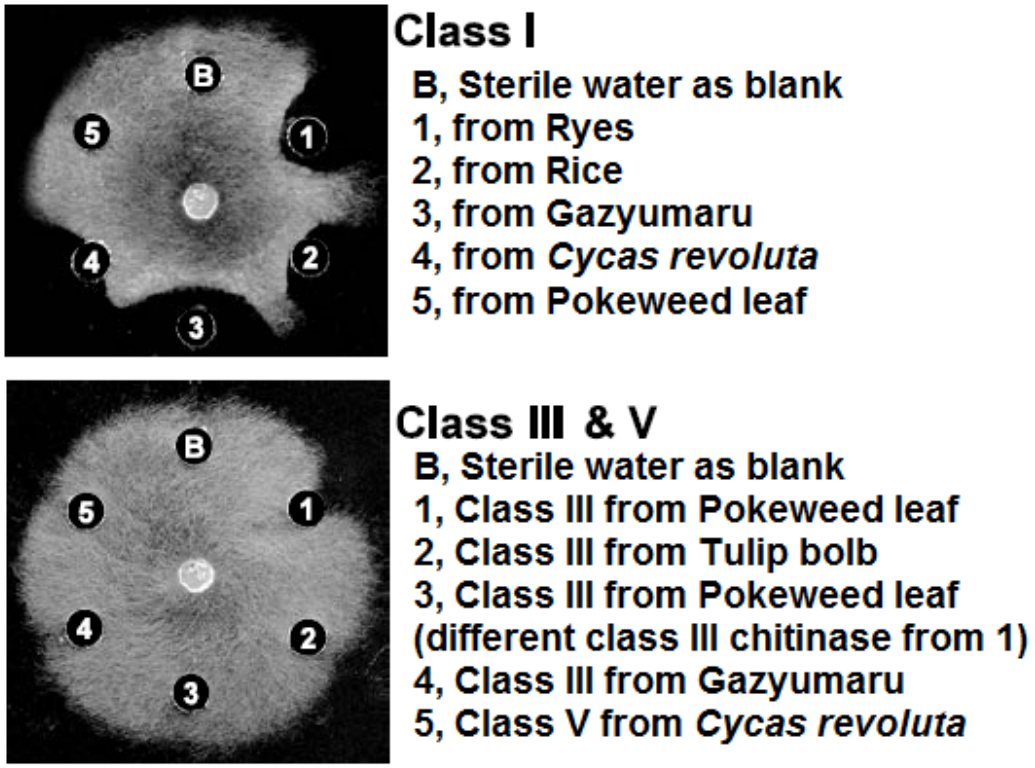


Figure 6. The antifungal activity of various classes of chitinase against the fungi *Trichoderma viride* (modified from Taira, 2010).

1.3.3. Lysozyme

Lysozymes (EC 3.2.1.17) also called peptidoglycan *N*-acetylmuramoylhydrolase (White & White, 1997) are a larger group of enzymes which can hydrolyse the glycosidic bonds of β-linked (1-4) copolymers of N-acetyl-D-glucosamine and N-acetyl-D-muramic acid. Since such polysaccharides are present in the bacterial cell wall, this cleavage step is believed to be the first step in breaking down the bacterial cells (Davies *et al.,* 1969; McKenzie & White, 1986). Some of the lysozymes are found to have the ability to also cleave chitin (GlcNAc-β1-4GlcNAc polymer) (Audy *et al.,* 1990; Lee & Yang, 2002).

Lysozyme was first discovered by Sir Alexander Fleming in 1921 from human nasal mucus (Jollès, 1996). Further studies found that lysozyme is present in chicken egg white, fish serum and insect saliva and contains both antibacterial and

antifungal factors (Lee & Yang, 2002). Not only animals have lysozyme, Fleming had also detected lysozyme activity in roots and flowers of higher plant (Audy *et al.,* 1990). Today, lysozymes can be divided into several types, they are chicken type lysozyme (c-type), goose-type lysozyme (g-type), plant lysozyme, bacterial lysozyme, T4 phage lysozyme (phage-type) and invertebrate lysozyme (i-type) (Wang *et al.,* 2005).

In plants, the term lysozyme is a definition rather than an individual enzyme present. It is defined as an enzyme which has a specific hydrolytic activity against the peptidoglycan found in bacterial cell walls (Beintema & van Scheltinga, 1996). The plant lysozymes are shown to have both chitinase and lysozyme activities but higher in chitinase and lower in lysozyme. Almost all of the enzymes with lysozyme function are classified as chitinases (Düring, 1993). Beintema and van Scheltinga (1996) had drawn a similar conclusion, “All plant lysozymes are chitinase, but only a limited number of plant chitinase are also lysozymes”. The plant lysozymes can be divided into three categories based on their structural similarity to chitinase, hevamine-type (h type or family 18 chitinase), barley-type (b-type or family 19 chitinase) and those without clear structural relationship to other enzymes (Beintema & van Scheltinga, 1996). Since the plant lysozymes are extremely similar to chitinase, this explains why they have chitinase activities. Moreover, the lysozymes are only found in angiosperms (Audy *et al.,* 1990).

1.3.4. Phenylpropanoid (phenolic) phytoalexins

Phenylpropanoids are phenolic compounds which have a wide range of functions in plants. They include flower pigments, UV protectants, insect repellents, signal molecules and phytoalexins (Hahlbrock & Scheel, 1989). The term phytoalexin is defined as low molecular weight antimicrobial compounds which are synthesised by plants and accumulate within the plant after exposure to microorganisms (Ebel, 1986). There are more than 150 phytoalexins known, which are structurally distributed among dihydrophenanthrenes, diterpenes, isoflavonoids, polyacetylenes, stilbenes etc (Ebel, 1986). The term phenylpropanoid phytoalexins are antimicrobial phenolic compounds.

L-Phenylalanine ammonia-lyase (EC 4.3.1.5) (White & White, 1997) are enzymes which can produce *trans*-cinnamic acid (cinnamate) and ammonia from L-phenylalanine by biotransformation with a cofactor, 3.5-dihydro-5-methyldiene-4H-imidazol-4-one (MacDonald & D'Cunha, 2007).

The above reactions and enzyme are important for plant defence against pathogens because they are the first step of the phenylpropanoid biosynthesis pathway that can lead to secondary metabolic pathways and phytoalexin production (MacDonald & D'Cunha, 2007; Cunha, 1988). Figure 7 summarizes the phenylpropanoid phytoalexin biosynthesis pathway in plants. Most of these phenylpropanoid phytoalexins can be found in legumes (Dixon, 1986). In addition, the signalling compound—salicylic acid can also be produced by PAL indirectly. Moreover, the phenylpropanoid products also have other functions, such as mechanical support and defense by lignins, antioxidant production to protect the plant against biotic and abiotic stress, signalling with flavonoid nodulation factors and pigments (MacDonald & D'Cunha, 2007).

PAL accumulates rapidly to reach high levels in the host plant during the resistance responses. PAL activity is induced by an increase in the supply of *L*-phenylalanine (da Cunha, 1987; da Cunha, 1988) and by fungal infection by chitin and chitosan found in fungal cell wall (Khan *et al.,* 2003).

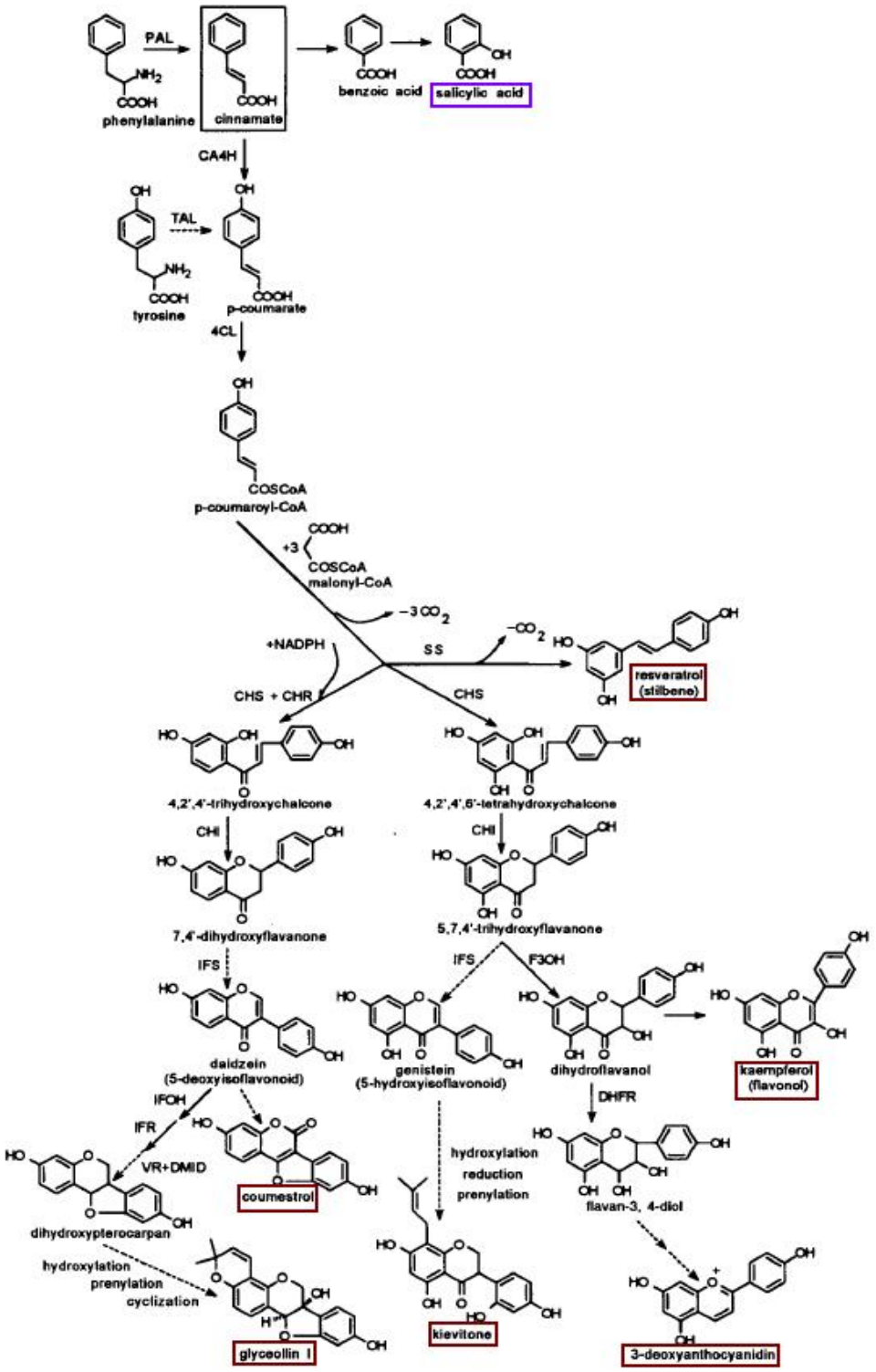


Figure 7. Phytoalexins biosynthesis from the phenylpropanoid (Red boxes indicate the phenylpropanoid phytoalexins and the purple box indicates the salicylic acid) (modified from Dixion, 1995).

1.4. Acid phosphatase

Acid phosphatases (EC 3.1.3.2), also known as orthophosphoric-monoester phosphohydrolase (acid optimum) (White & White, 1997), are one of the classes of phosphatases which are also called acid phosphomonoesterases, glycerophosphatases and phosphomonoesterases (Swiss institute of Bioinformatics, n.d.). Acid phosphatases have been reported in mammals, plants and microorganisms and all of them can hydrolyse phosphate monoesters to an alcohol and a phosphate group and acid phosphatases normally catalyze this reaction under pH 6.0 (dos Prazeres *et al.,* 2004). Figure 8 shows an *in vitro* assay system for the hydrolysis of a phosphate monoester (p-nitrophenyl phosphate) to produce an alcohol (p-nitrophenol) and inorganic phosphate (P_i) (Bioscience, n.d.).

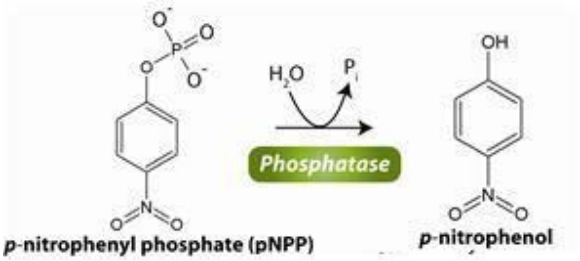


Figure 8. Phosphatase hydrolysis of a phosphate monoester to an alcohol (Bioscience, n.d.).

Phosphorus as phosphate plays an important role in energy transfer and metabolic regulation. For example, phosphorylation of ADP to ATP plays an important role in keeping organisms, including plants, alive (Nelson & Cox, 2004). It is also an important element to form the phospholipids (Hatch, 2007), proteins and nucleic acids (Proud, 2010). The development of plants similarity depends on these compounds and the Phosphorus is stored in plants as phytic acid. Many biochemical reactions and enzymes are involved in the release of phosphorus to form the above macromolecules, including acid phosphatase (Jakobek & Lindgren, 2002; dos Prazeres *et al.,* 2004).

In plants, acid phosphatases can be found in bulbs, roots, tubers, seeds and leaves (dos Prazeres *et al.,* 2004) and normally do not have high substrate specificity (Jakobek & Lindgren, 2002).

Importantly, from the point of view of the present research, some studies have reported that acid phosphatases are also involved in some plant defence mechanisms such as anti-insect (Liu *et al.,* 2005). Another example is during the hypersensitive defence response in *Phaseolus vulgaris* after bacterial infection (Jakobek & Lindgren, 1993).However, the mechanism of the defence response has not been elucidated.

1.4.1. Hypersensitive reaction

The hypersensitive reaction (HR) is a plant's response to pathogen attack which involves localized plant cell death, which rapidly restricts the pathogen growth (Heath, 2000). This is an active disease resistance and occurs after the plant 'recognizes' the pathogen infection (Pontier *et al.,* 1998). During the HR, there is morphological change of the plant tissue such as appearance of a brown colour and dead cells occur at the infection site (also known as localised necrosis) (Pontier et al., 1998; Heath, 2000).

Heath (2000) suggested that the HR can induce some defence responses against pathogens within the dying cells and their adjacent cells. She discovered this by a non-biotrophic pathogen infection which does not require the host cell to be alive, and found that the adjacent cells have a high defence response. So she concluded that the HR can drive the cell death and defence response induction, and also can occur without cell death (Heath, 2000). Goodman & Novacky (1998) also suggested a similar conclusion. Moreover, a study suggests that the phytoalexins are also involved in HR (Dixon, 1986).

1.5. Plant hormones

Plant hormones (also called phytohormones), are described as organic substances other than a nutrient, active in minute amounts, formed in certain parts of the plant and translocated from site to site (Moore, 1989). The physiology of the plant will change when the plant is in contact with hormones or elicitors. For example, increased grow rate and larger leaf size have been observed when plants are treated with hormones (such as ethylene) compared to the untreated control sample (Moore, 1989). Auxins, cytokinins, ethylene, and salicylic acid are typical plant hormones (Moore, 1989; Raskin, 1992). Only ethylene and salicylic acid will be investigated in this research, both hormones are known to induce plant defensive enzymes.

1.5.1. Ethylene

Ethylene is a simple gaseous hydrocarbon (C_2H_4) which acts as a plant hormone (Agrios, 2005). Ethylene is produced at a fast rate during growth and cell division. New growth and germinating seeds produce more ethylene than the amount which escapes from the plant, which leads to elevated concentrations of ethylene inhibiting leaf expansion (Moore, 1989).

Ethylene affects cell growth and cell shape. Ethylene production greatly increases when a growing shoot hits an obstacle while underground, preventing cell elongation and causing the stem to swell. This leads to a thicker stem that can exert more pressure against the object impeding its path to the surface (Wang et al., 2002). It also affects the stems natural geotropic response when the shoot does not reach the surface and the ethylene stimulus becomes prolonged, which makes the plant grow upright, allowing it to grow around an object (Wang *et al.,* 2002).

Ethylene has been shown to induce chitinase synthesis in a variety of plant species. For example, Table 2 shows that 24 hours of ethylene treatment on leaves from bean, pea and soybean caused large increases in the chitinase activity. Note that there is a wide variation in induction within the legumes treated

i.e. *P. vulgaris* (common bean), *Pisum sativa* (pea) and *Glycine max* (soybean), being 2.9-20 fold respectively (Boller et al., 1983). Cabello et al., (1994) suggested *Cicer arietinum* (chickpea) also had a similar induction of chitinase (for more details about the induction mechanism, refer to Section 3.1.).

Table 2. Effect of ethylene on chitinase activity. The listed plant species were treated with the hormone ethylene (modified from Boller et al., 2002).

Species	Factor of ethylene induced increase
Phaseolus vulgaris	20
Pisum sativum	2.9
Glycine max	5.2

In the present study ethylene will be generated *in vitro* from the chemical ethephon which spontaneosly decomposes to generate the gas when added to an appropriate buffer.

1.5.2. Salicylic acid

Salicylic acid was first recorded by a Greek physican around the 5th century BC as a bitter powder extracted from willow bark that could stop pain and reduce fevers (Raskin, 1992). As a plant hormone, salicylic acid influences flower development by induction as well as by inhibition (depending on different stages of development), photosynthesis, transpiration, ion uptake and transport (Raskin, 1992).

Salicylic acid is also involved in the Systemic Acquired Resistance (SAR) response to pathogen attack, a process which involves the induction of defensive pathogenesis related proteins such as the enzymes chitinase, lysozyme and PAL (Mauch-Mani & Métraux, 1998; van Huijsduijnen *et al.,* 1986). For example,salicylic acid treatment has been shown to induce chitinase activity in various plant species. Ward *et al* (1991) observed induction of both acidic Class III and basic chitinases in tobacco after treatment with either salicylic acid or a salicylic analogue methyl 2,6 dichloroisonicotinic (Me-INA). Van Kan *et al.,* (1995) observed induction of (total) chitinase mRNA in tomato after treatment with salicylic acid and its analogue 2,6

dichloroisonicotinic (INA). Derckel et al., (1996) observed chitinase activity induction in grape (*Vitis vinzfera* L.) treated with INA. Fan *et al* (2007) observed induction of a Class I chitinase with antifungal activity in plantain after treatment with salicylic acid. Metraux *et al* (1989) observed strong induction by salicylic acid of mRNA coding for a lysozyme/chitinase in cucumber. With specific regard to legumes, Esaka & Teramoto, (1998) observed induction of a Class III chitinase in winged bean *(Psophocarpus tetragonolobus)*, a herbaceous member of the *Papilionoideae* subfamily of legumes after treatment with salicylic acid. Likewise Regalado *et al.,* 2000 similarly observed induction of a Class III chitinase in lupin, another herbaceous member of the *Papilionoideae* subfamily of legumes.

Beβer *et al.,* (2000) showed that treatment of barley with the salicylic acid analogue INA induced defense response genes which resist the fungal disease powdery mildew. One of the genes induced was an acid phosphatase.

In the present study the salicylic acid analogue 2,6 dichoropyridine-4-carboxylic acid (also known as 2,6 dichloroisonicotinic acid -INA) was used instead of salicylic acid itself, since it was observed to give the strongest defence response in a study of salicylic acid and its analogues in a fungal infection model in *Phaseolus vulgarus i.e.*common bean (Kataria *et al* 1997).

1.6. Aims of the research

As discussed earlier in the outline of the study (refer back to Section 1.1.), the primary objective was to explore the plant defence system of kowhai in response to hormone treatment. The chitinase, lysozyme, and acid phosphatase (ACP) defensive enzymes and total phenolic content (representing phenylpropanoid phytoalexin content) were studied. Since there are no published studies concerning these defensive systems within the kowhai species (or other New Zealand flora), this research is significant to understanding the New Zealand biota and may also be more generally applicable to woody perennial legumes than previous studies with commercial legume species.

Since the plant disease is one of the major impacts of agricultural and horticultural production (over US$ 76 billion per year from global harvest of crop are lost due to plant disease) (Chakraborty & Pangga, 2004), futher development from this research may be applied to agriculture and horticulture to minimize the lose.

1.7. Limitation

Ideally, to measure biochemical responses in plants, they should be grown in a controlled environment. However, since kowhai is a slow growing species, there was insufficient time to grow plants in this manner for this project. Therefore, plants growing in a natural environment were studied. This meant that the plants were exposed to factors that could not be controlled such as, temperature, wounding, phytohormones and fungal attack which may switch on the defence mechanism of the kowhai trees. Other factors such as the age of the tree and the healthiness may also influence the result.

Also to estimate the biochemical responses in kowhai population, homogenised samples should be collected from different sites and sample size should be increased. Unfortunately, due to the budget, geographical and time limitation, a

small sample size was collected from a single site. This may not overcome the above uncontrolled factors. Hence, the result may be biased.

Theoretically, enzyme purification may be applied to increase the enzymes specific activity. The purified extract has about 108-fold increased enzyme specific activity compared to the crude extract (Wang *et al.,* 2009). For example, Kim et al., (1992) purified chitinase from green onion by affinity chromatography based on regenerated chitin. However, washing the regenerated chitin requires a relatively long period of time and the cost of the chitin column would exceed the budget in this project. Therefore, crude extract was directly used in this research. Consequently, low levels of chitinase and lysozyme activities were expected in this research.

Total phenolic content measured in 1.6. was used to represent the phenylpropanoid phytoalexins productions. Again, this was due to the budget limitation. Unfortunately, some of the phenolic compounds which are not phenylpropanoid phytoalexins may also be included in this measurement.

1.8. Organisation of the study

This thesis consists of 5 chapters. This chapter has already reviewed the extant literature and previous research that generated the research question of this thesis. Chapter 2 reviews some of the species identification methods including morphological approaches, and DNA analysis and materials that were used in this research, and discusses the results of the kowhai species identification. Following the species identification chapter, Chapter 3 reviews some of the methods which are used to determine the degree of defence system induction in the hormone treated kowhai, and shows the results and statistical analysis of the induction data. Chapter 4 introduces the reverse transcription real time quantitative PCR to measure the gene expression and last chapter is the conclusion of this study and also provides recommendations for future researchers.

Chapter 2

Species identification

As mentioned earlier at Section 1.2.3., there are 45 species within the *Sophora* genus, and only the species endemic to New Zealand are called kowhai. To ensure the trees studied are indeed New Zealand kowhai, and not an introduced foreign species of *Sophora*, identification to species level was required. Although there are lots of different methods to identify plant species, this research used a combination of morphological approaches and DNA sequence analysis (including DNA barcoding and phylogenetic analysis) to identify the sample species.

2.1. Morphology

Morphology analysis is a common way to identify the species of land plants based on size, structure, form and the configuration of living organisms. Such a taxonomic treatment has been applied to *Sophora* in New Zealand by Heenan *et al* (2001) who did a cluster analyses of 11 leaf and four growth habit characters to revise the classification of *Sophora* species. The leaf characters are leaf length (mm), leaflet number per leaf, leaflet length (mm), leaflet width (mm), leaflet thickness (mm), leaflet hair density (hairs per 1mm line), petiolule length (mm), leaf colour, leaflet overlap, leaflet taper and hair style. The growth habit characters are juvenile growth habit, growth form, adult branches and underground stems or rhizomes. The samples they used were mature leaves from the oustide of the trees which were 35 years old. Cluster analysis was undertaken to estimate the phenetic relations of the plants based on the average value of the leaf characters and four additional growth habit characters. Leaf hairs were observed by a scanning electron microscope (SEM) and viewed at -180˚C to obtain the leaf hairs density. They have classified the following species by those characters: *S. chathamica, S. longicarinata*, *S. fulvida, S. microphylla*, *S. prostrata*, *S. tetraptera S. godleyi* and *S.molloyl* by Heenan *et al* (2001). Moreover, the original species classification was *S. microphylla*, *S. prostrata* and *S. tetraptera* (Heenan *et al.,* 2001), *S. chathamica, S. longicarinata* and *S. fulvida* were divded from *S. microphylla* (Heenan et al., 2001) and *S. godleyi* and *S. molloyl* were newly discovered (Heenan et al., 2001).

2.2. Polymerase chain reaction

Polymerase chain reaction (PCR) is a DNA sequence amplification tool which involves two oligonucleotide primers that are located at the 5' and 3' ends of the region being amplified, deoxyribonucleotides (dNTPs, monomers of DNA), a heat-stable polymerase and buffer containing magnesium ions. The PCR temperature cycles are summarized in Figure 9. High temperature (95˚C) is firstly applied on the double helical DNA to melt (separate, also called denaturation) the strands, then the temperature is lowered to around 50˚C-60˚C depending on the annealing temperature of the primers, to allow the primers to stick on the strands. The final step of the cycle is elongation, which the temperature is increased to 72˚C to extend the primers by associated with dNTPs (Kubista *et al.,* 2006).

Touchdown PCR is a variation of the standard PCR method which can increase the sensitivity, specificity and yield. The principle of the Touchdown PCR is starting the annealing temperature just above the projected melting temperature at the first cycle, then decreasing the annealing temperature by 1˚C per cycle for 10-15 cycles. This method is useful for amplifying primers with an unknown annealing temperature (Korbie *et al.,* 2008). Gel electrophoresis is used to verify DNA amplification based on a method described by Brody & Kern, (2004).

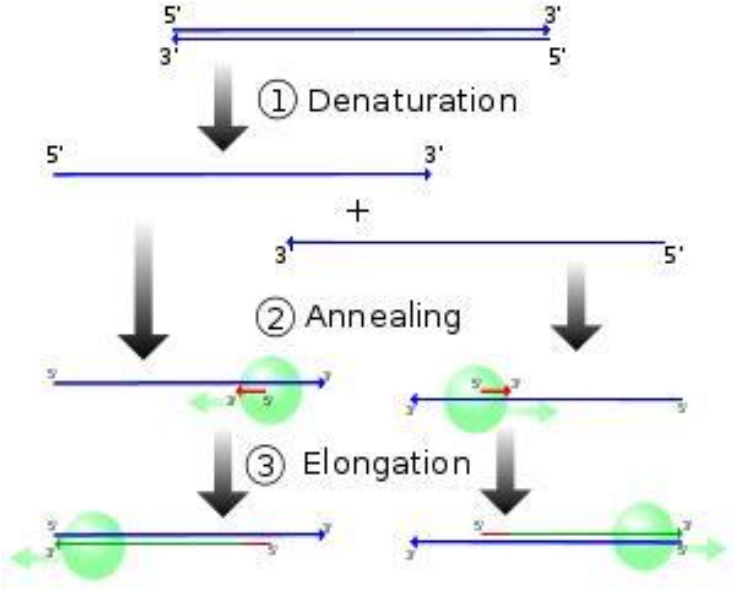


Figure 9. The PCR temperature cycle (blue lines indicate DNA templates, red arrows indicate primers, green lines indicate DNA products, green circles indicate polymerase) (Wikipedia, n.d.).

2.3. DNA barcoding

DNA barcoding is a rapid species identification tool which uses a standard region of short (400-800bp) orthologous DNA sequence (Kress *et al.,* 2005; Kress & Erickson, 2008; CBOL plant working group, 2009). The internet-based digital library of barcodes act as a standard to the DNA barcode sequence of the unknown sample, this allows users to recognize known species (Kress & Erickson, 2008). This method was first designed to identify animal species by a group of zoologists in 2003 and later applied to land plant species (Ausubel, 2009). There is no agreement on which regions of DNA sequence should be used for barcoding the land plant species but normally chloroplast genes are selected (The chloroplast genome is circular in shape, is attached to the inner organellar membrane and is not associated with proteins. The regulation and replication machinery are part of the functions of the gene). The Consortium for the Barcode of Life (CBOL) suggests that the *rbcL* gene, a chloroplast gene, is the best characterized region because it offers high universality, like cytochrome b in mitochondria. The *matK* gene, another chloroplast gene, is also preferred to use for DNA barcoding since it

is one of the most rapidly evolving plastid regions and provides high level of discrimination (CBOL plant working group, 2009). The *rbcL* and *matK* 2-locus barcode combination proposed by CBOL to be the standard barcode for land plants was used in this research. In addition, the *rpo* gene, *rpoC1* gene can also be used for DNA barcoding (CBOL plant working group, 2009). Moreover, some researchers tend to use 3-locus barcode to basically add on more non-coding region into the combination. The non-coding region can be *trnH-psbA*, *psbK-psbI* and *atpF-atpH* (Kress *et al.,* 2005; CBOL plant working group, 2009).

2.4. Phylogenetic analysis

A phylogenetic tree is created by multiple alignments from the sequences database beginning with comparison of the pairwise alignments and it is used to determine how the sample sequence is related to a sequence which is well known. The phylogenetic tree is made up by two elements, nodes (external nodes and internal nodes) and branches. Figure 10 is modified from Hall, 2008, and is an example of phylogenetic tree. The external nodes located at the end of the tree represent the taxas which are existing today (A & B) while the internal node (R) means represents an ancestral taxum. Branches connect all the nodes together and represent the amount of genetic change that occurred from the ancestral nodes to their descendants. The numbers next to the branches represent the branch lengths. For example, the number (0.02) between R and A means there are 0.02 changes per nucleoside site. Phylogenetic trees can be estimated by a few different methods. The distance method with Neighbour joining was used in this research (Hall, 2008).

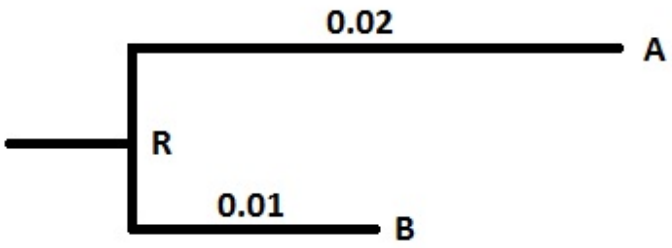


Figure 10. A sample of a phylogenetic tree (modified from Hall, 2008)

This method begins with an unresolved tree. From the original distance matrix, neighbour joining first calculates for each taxum its net divergence from other taxons. Then it uses the net divergence to calculate the new, corrected distance matrix. The pair of those taxa with the lowest distance is joined together. The distance of each of the taxa in the pair to this new node is calculated by neighbour joining followed by the calculation of the distance of all taxa outside of this pair to the new node. A new matrix is then created after those steps and the new node is substituted for those original taxa (Hall, 2008).

This study used the combination of morphology analysis, the 2-locus Barcoding of Life genes (*rbcL* and *matK*) and phylogenetic analysis based on the *atpB-rbcL* intergeneic spacer to identify the AUT kowhai samples.

2.5. Methods and materials

2.5.1. Sampling

The outer mature leaves (see Figure 11) were randomly collected from three kowhai trees growing at AUT University City campus next to the WA building (Wellesley Street East) (refer to Figure 12).

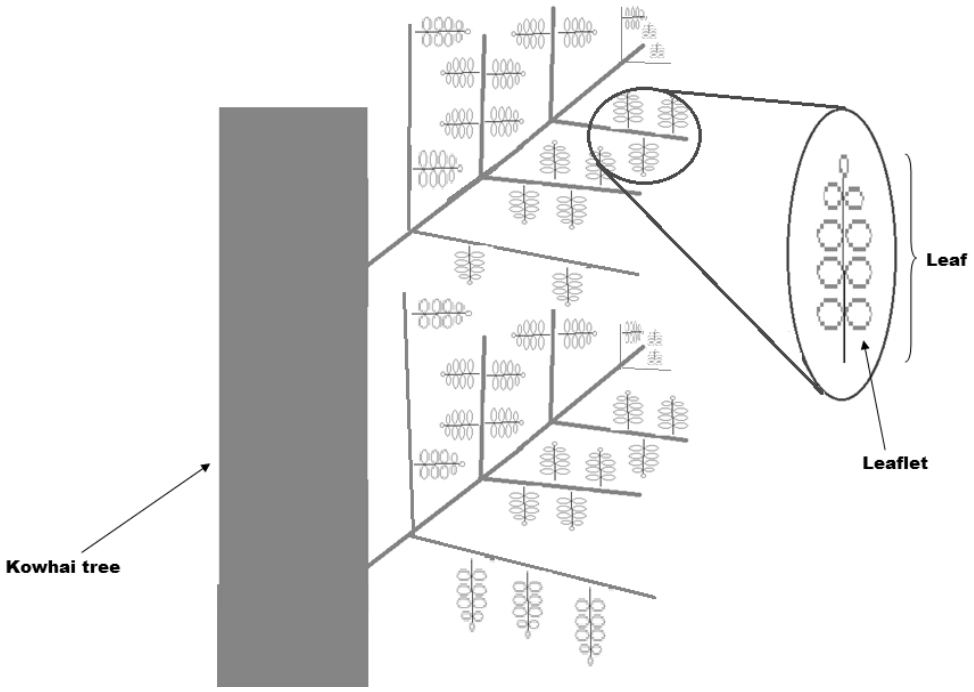


Figure 11. The outer leaves (circle) were randomly collected and the definition of leaf and leaflet

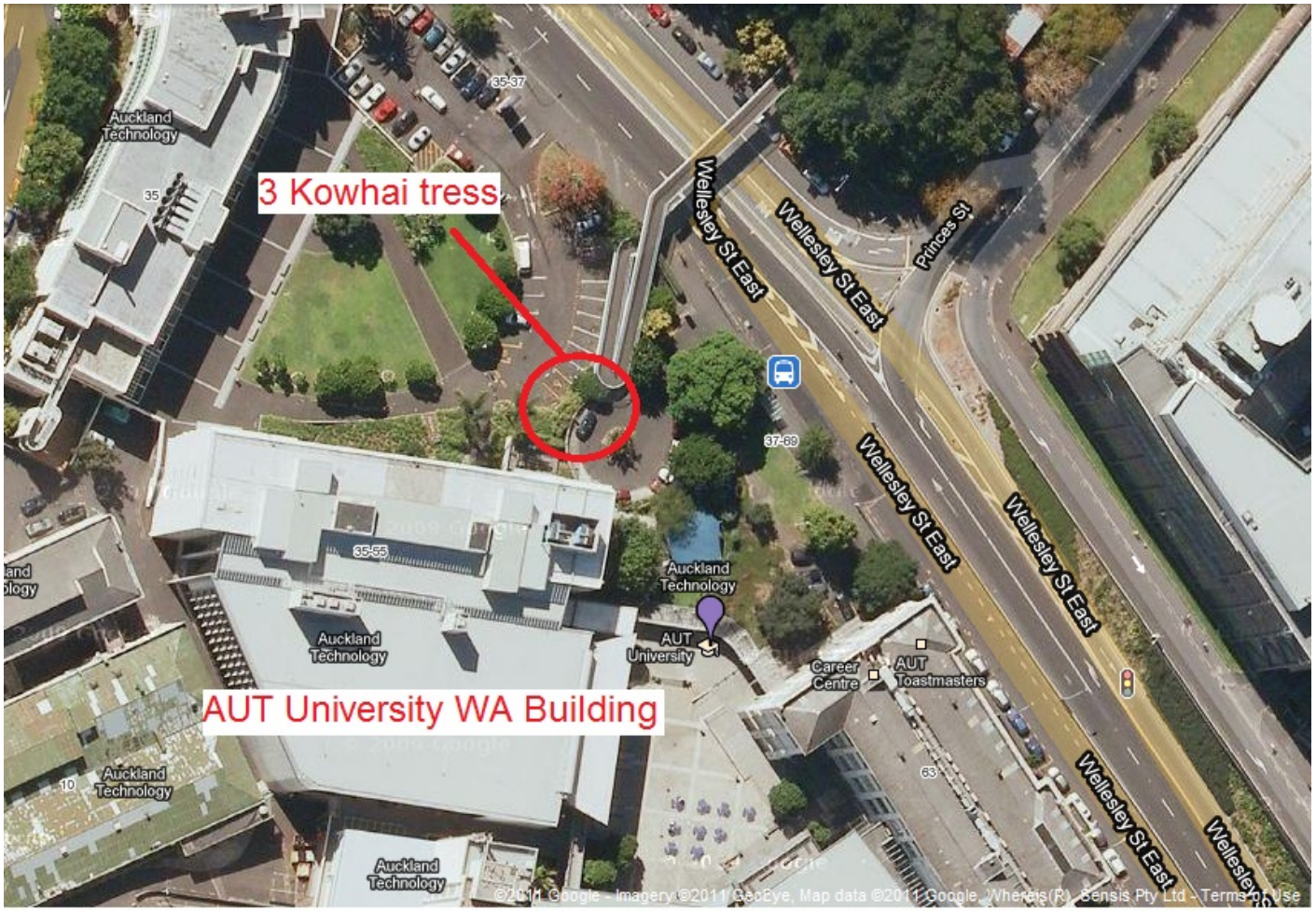


Figure 12. The kowhai trees located at AUT University (Google map New Zealand, 2011)

2.5.2. Morphological identification

2.5.2.1. Morphological observation and measurement

Six leaves from the outside of the kowhai trees were collected. The leaf length (mm), leaflet number per leaf, the leaflet length (mm) and the leaflet width (mm) were measured by Vernier calliper. For each leaf the quantitative measurements were taken from five leaflets from the middle third of the leaf (Heenan *et al.,* 2001) (see Figure 11 for the definition of leaf and leaflet).

The growth habit, leaflet shape, leaf colour and leaflet density were also collected. All the morphological characters were compared to the *Sophora (Fabaceae) in New Zealand: Taxonomy, distribution, and biogeography* tables (Table 4, and Figure 14) by Heenan *et al.,* 2001.

2.5.2.2. Statistical analysis

Morphological measurement were statistically analysed by Minitab by one sample t test. Since the sample size was larger than 30 (except leaf length and leaflet number), the normality test was not required. Hence, data can be assumed to be normal distributed. Since the sample sizes of the leaf length and leaflet number were 6, Ryan-Joiner test was applied on leaf length and leaflet number for the normality test (if $p>0.1$, accept H_0, data are normal).

2.5.3. DNA analysis

2.5.3.1. DNA extraction

A pair of scissors were rinsed with 99% ethanol and used to cut a 0.5 to 0.7cm disk of leaf tissue into a 2 mL collection tube. DNA was extracted using a REDExtract-N-Amp PCR kit (Sigma-Aldrich) according to the manufacturer's bulletin. 100 µL of extraction solution was added to the collection tube to cover the leaf tissue and mixed by vortexing. After incubating at 95˚C for 10 minutes, 100 µL of dilution solution was added and mixed by vortexing. The diluted leaf extract was stored at 4˚C.

2.5.3.2. PCR amplification

PCR amplification of barcode sequence was carried out by combining 4 µL of DNA, 10 µL REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich), 2 µL of 10 µM forward primer, 2 µL of 10 µM reverse primer and 2 µL of Milli-Q water. Primer sequences are shown in Table 3. PCR was carried out in a Mastercycler gradient Thermal Cycler (Eppendorf) using the following programmes for each gene.

Table 3. Primers used for PCR

Genes	Orientation	Sequence	Reference
rbcL	Forward	ATGTCACCACAAACAGAGACTAAAGC	CBOL plant working group, 2009
rbcL	Reverse	GTAAAATCAAGTCCACCRCG	CBOL plant working group, 2009
matK	Forward	CGTACAGTACTTTTGTGTTTACGAG	CBOL plant working group, 2009
matK	Reverse	ACCCAGTCCATCTGGAAATCTTGGTTC	CBOL plant working group, 2009
atpB-rbcL	Forward	CACTCATAGCTACAGCTCTAATTC	Hurr et al., 1999
atpB-rbcL	Reverse	ATGTTGTATATGTAAATCC	Hurr et al., 1999

rbcL:

The PCR program was started with initial melting at 95˚C for 4 mins, amplification was carried out for 35 cycles of melting at 94˚C for 30 secs, annealing at 55˚C for 1 min and elongation at 72˚C for 1 min, followed by a final elongation for 10 mins at 72˚C and held at 20˚C.

matk and *atpB-rbcL*:

The PCR program was started with initial melting at 94˚C for 3 mins, amplification was carried out for 15 cycles of melting at 94˚C for 30 secs, annealing at 60˚C for 1 min (reduced 1˚C for each cycle) and elongation at 72˚C for 1 min. After another 20 cycles of 94 C for 30 secs, annealing at 45˚C for 1 min, followed by a final elongation for 10 mins at 72˚C was held at 20˚C.

2.5.3.3. Product verification by gel electrophoresis

PCR products were analysed by gel electrophoresis at 70 V for 70 mins though a 1% agarose gel prepared with 1x TBE buffer. The PCR products were compared with 1.25 µg of DNA molecular marker X (Roche Applied Science).

2.5.3.4. DNA sequencing

The DNA samples were sent to the Waikato University DNA sequencing facility for DNA sequencing after PCR. The sequence results are shown in Appendix B.

2.5.3.5. NCBI-BLAST

Homologous sequences were identified by searching the GeneBank database (www.ncbi.nlm.nih.gov) using BLAST (Altschul et al., 1997). The results were sorted by the E-value, maximum identities, maximum score and query coverage (Claverie & Notredame, 2007). The top three homologous sequences were used for species identification of the AUT sample. The E-value is a measure of probability, the lower the value of E the better the match is between the subject and the query.

2.5.3.6. Phylogenetic analysis

Sequences from the AUT sample were aligned with *atpB-rbcL* sequences reported by Hurr et al., (1999). Multiple sequence alignment was carried out using ClusterW in Geneious Pro 5.4.2 (Hall, 2008). Distance measurements were calculated by Neighbour joining and a phylogenetic tree determined. *Clianthus puniceus* and *Carmichaelia arborea* were used as outgroups.

2.6. Result

2.6.1. Species identification based on morphology

Table 4 shows the morphological result of the AUT samples. The samples from AUT had a mean leaf length of 12.783cm (with 95% confidence interval 12.192-13.375cm), which matched with *S. chathamica*, *S. godleyi*, *S. longicarinata*, *S. microphylla* and *S. tetraptera*. The mean leaflet number was 44.83 (with 95% confidence interval 41.62-48.05), which matched with *S. chathamica*, *S. fulvida*, *S. godleyi*, and *S. longicarinata*. The mean leaflet width was 0.548cm (with 95% confidence interval 0.5252-0.5708cm), which matched with *S. cassioides* and *S. chathamica*. The mean leaflet length was 1.0937cm (with 95% confidence interval 1.0510-1.1363cm), which is only matched with *S. chathamica* (see Table 4).

Moreover, the AUT kowhai grows as a tree with no juvenile growth habit (see Figure 13). The size of the proximal leaflet is larger than the distal leaflet. The leaflets have the elliptic and broadly elliptic with ovate shape and have light green to green colours (refer to Figure 14). The leaflet growth is croded and overlapping. The species which matched all these characters could be *S. chathamica* (see Table 5).

The geographical information from Heenan *et al* (2001) suggests that *S. microphylla* and *S. chathamica* species both grow in the Auckland region (refer back to Figure 2 and Figure 3).

Table 4. The morphological result of AUT sample compared to the morphological information of the kowhai species from Heenan et al., (2001)

Species	Leaf length (cm)	Leaflet number	Leaflet length (cm)	Leaflet width (cm)
S. cassioides	9.440±1.458	29.22±5.14	0.860±0.112	0.604±0.089
S. chathamica	11.568±2.324	37.25±7.49	1.129±0.141	0.644±0.084
S. fulvida	9.760±1.452	54.33±7.49	0.645±0.141	0.358±0.063
S. godleyi	13.029±1.283	57.77±11.23	0.558±0.094	0.364±0.054
S. longicarinata	11.280±1.831	44.96±5.72	0.379±0.066	0.263±0.040
S. microphylla	11.318±1.819	35.64±4.63	0.698±0.143	0.390±0.066
S. molloyi	8.287±0.554	29.0±2.75	0.712±0.123	0.344±0.040
S. prostrata	1.510±0.446	9.45±2.26	0.368±0.080	0.203±0.021
S. tetraptera	13.970±1.240	21.70±1.63	2.952±0.393	0.724±0.078
AUT sample	12.783a±0.592	44.83a±3.215	0.548±0.023	1.094±0.043

a: (p>0.1), samples were normal

Figure 13. The kowhai trees growing at AUT University

Table 5. The morphological characters of AUT sample and the kowhai species from Henan et al., (2001)

Character	*S. chathamica*	*S. fulvida*	*S. godleyi*	*S. longicarinata*	*S. microphylla*	*S. molloyi*	AUT Sample
Growth habit	tree; juvenile growth habit absent	tree; juvenile growth habit absent	tree; juvenile growth habit absent	tree or shrub; main main stems often produced at ground level, sometimes suckering; juvenile growth habit absent	tree; juvenile growth habit present	shrub, usually broader than high; juvenile growth habit absent	tree; juvenile growth habit absent
Leaflet number	25–55	61–91	47–75	35–52	30–50	23–37	42-48
Leaflet size	6.0–16.0 × 4.0–8.0 mm; distal leaflets usually smaller than proximal leaflets	1.8–7.5 × 1.2–4.5 mm; distal leaflets usually smaller than proximal leaflets	2.0–8.0 × 2.0–5.0 mm; distal leaflets usually smaller than proximal leaflets	3.3–5.8 × 2.5–3.1 mm; distal and proximal leaflets similar in size	4.5–12.5 × 2.3–5.7 mm; distal and proximal leaflets usually similar in size	5.0–12.0 × 2.0–6.0 mm; distal and proximal leaflets similar in size	5.2-5.7 × 10.5-11.3 mm; distal leaflets usually smaller than proximal leaflets
Leaflet shape	broadly elliptic, broadly obovate, broadly ovate, obovate to ± orbicular	elliptic to elliptic-oblong, occasionally narrowly obovate	ovate to broadly elliptic, sometimes ± orbicular	orbicular, obovate, to oblong-obovate	elliptic, broadly elliptic, obovate, to ovate, sometimes ± orbicular	elliptic, elliptic-oblong, to broadly elliptic	elliptic, broadly elliptic, obovate, to ovate
Leaf colour	light green to green	green to slightly grey-green	grey to green-grey	dark green	light green to green	dark green	light green to green
Leaflet density	crowded and overlapping	often crowded and sometimes overlapping	sometimes crowded, but not overlapping	overlapping to distant	distant, not crowded or overlapping	distant, not crowded or overlapping	crowded and overlapping

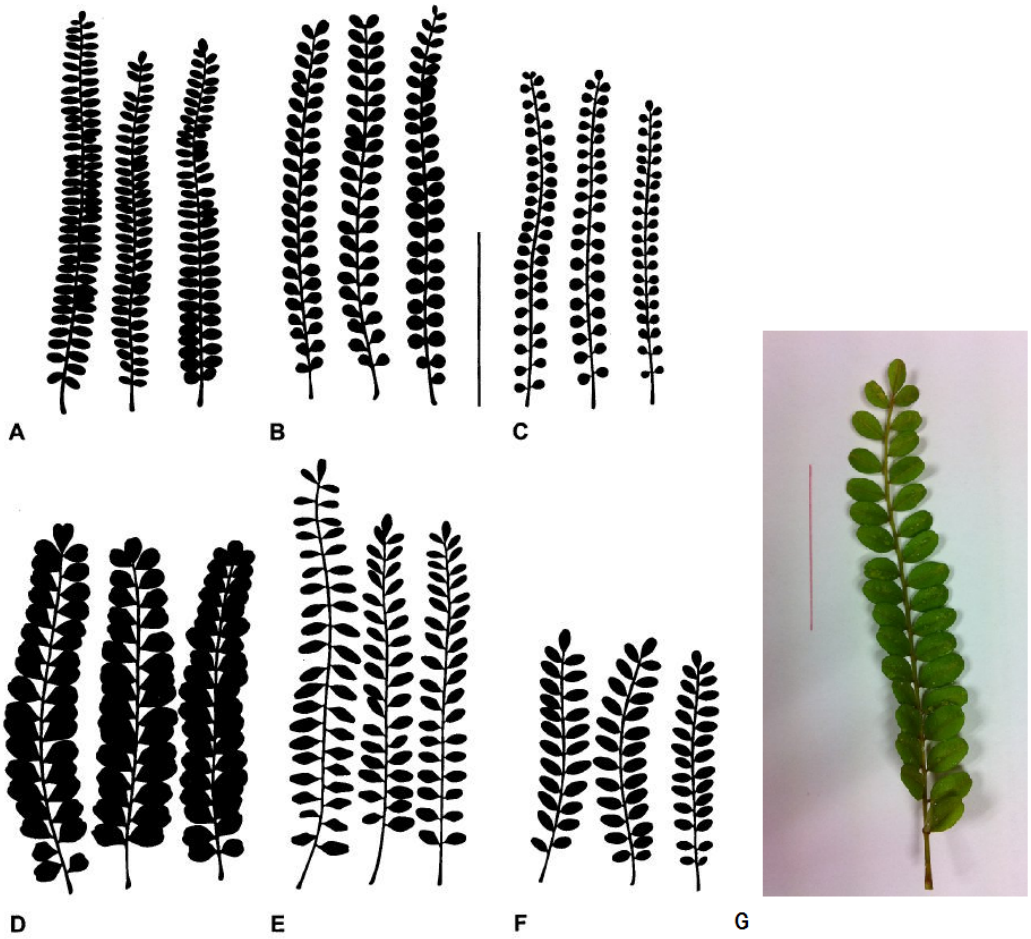


Figure 14. Leaf silhouettes of New Zealand indigenous species of *Sophora* and AUT sample (Heenan et al., 2001).

A, *S. fulvida*, from Whatipu; B, *S. godleyi*, from Taumarunui; C, *S. longicarinata*, from Leathain River valley; D, *S. chathamica*, from Chatham Island E , *S. microphylla*, from Great Island, Rakaia River; F, *S. molloyi*, from Stephens Island; G, AUT sample. Scale bar = 5 cm. All specimens from adult plants cultivated at Lincoln (except G).

2.6.2. Species identification based on DNA evidence

The electrophoresis gel (Figure 15) shows the product bands of *rbcL*, *matK* and *atpB-rbcL* after PCR. The *rbcL* primers (Lane 2) gave a product which was expected to be about 600bp, *matK* primers (Lane 3) gave a product which was expected to be about 800bp and the *atpB-rbcL* primers (Lane 4) gave a product which was expected to be about 700bp. Since each primer pair gave the expected product size, the sequencing results were reliable.

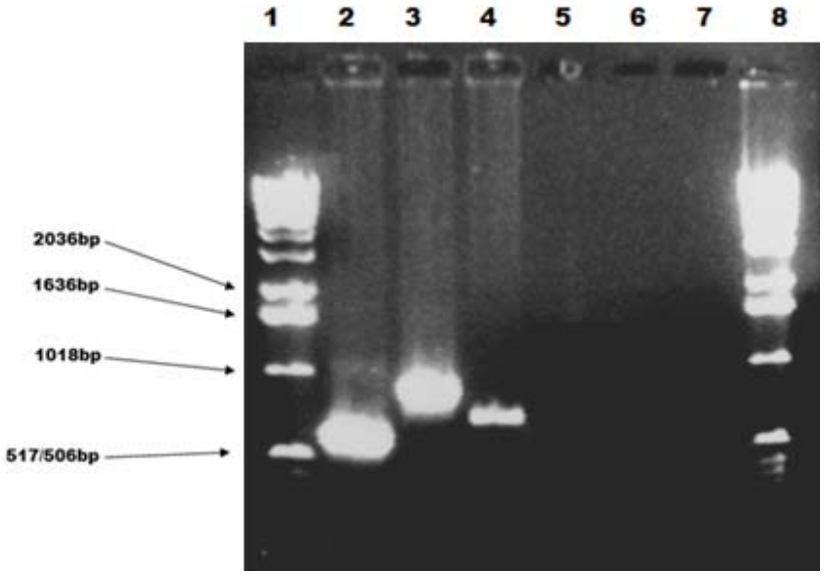


Figure 15. Agarose gel electrophoresis gel (1%, 1x TBE) showing the PCR products.

(L1: DNA molecular weight marker X, L2: *rbcL*, L3: *matK*, L4: *atpB-rbcL*, L5: *rbcL* NTC, L6: *matK* NTC, L7: *atpB-rbcL* NTC, L8: DNA molecular weight marker X)

The BLAST search showed that the closest match for the *rbcL* sequence was *S. microphylla* and *S. flavescens* (see Table 6 and Table 7), for the *matK* sequence was *S.toromiro* and *S. microphylla* (refer back to Table 8 and Table 9). On balance the DNA barcoding from BLAST search suggested that the AUT samples are *S. microphylla*.

Since the NCBI-BLAST database does not contain any *atpB-rbcL* sequences of *Sophora* species, the *atpB-rbcL* sequence of kowhai has no matches to *Sophora*

species from a BLAST search. In this case, the sequences could not be downloaded from the database or BLAST searched on the sequence database. Consequently, the sequences of different *Sophora* species from the Hurr et al., paper (1999) were used for the phylogenetic analysis (Appendix C). This analysis (see Figure 16) showed that the *atpB-rbcL* sequence from the AUT sample was most closely matched to *S. chathamica*.

Refer to Appendix B for the sequences of *rbcL*, *matK* and *atpB-rbcL* genes, Appendix C for the sequences used to create the phylogenetic tree for phylogenetic analysis of *atpB-rbcL* gene of AUT kowhai and Appendix D for the aligned sequence.

Table 6. Top three BLAST hits for *rbcL* gene sequence from forward primer

Accession	Organism	Max Score	Query coverage	E-value	Max ident
AY725480	*Sophora microphylla*	959	95%	0.0	98%
AB127038	*Sophora tomentosa*	953	95%	0.0	98%
AY725481	*Sophora tomentosa*	953	95%	0.0	98%

Table 7. Top three BLAST hits for *rbcL* gene sequence from reverse primer

Acceession	Organism	Max Score	Query coverage	E-value	Max ident
AB127037	*Sophora flavescens*	959	94%	0.0	98%
AB127038	*Sophora tomentosa*	953	94%	0.0	98%
AB127036	*Echinosophora koreensis*	948	94%	0.0	98%

Table 8. Top three BLAST hits for *matK* gene sequence from forward primer

Acceession	Organism	Max Score	Query coverage	E-value	Max ident
GQ248201	*Sophora toromiro*	1282	90%	0.0	96%
GQ248200	*Sophora microphylla*	1280	91%	0.0	95%
AY386865	*Sophora nuttalliana*	1256	96%	0.0	93%

Table 9. Top three BLAST hits for *matK* gene sequence from reverse primer

Acceession	Organism	Max Score	Query coverage	E-value	Max ident
GQ248200	*Sophora microphylla*	1295	90%	0.0	96%
GQ248201	*Sophora toromiro*	1291	90%	0.0	96%
AY386865	*Sophora nuttalliana*	1254	92%	0.0	94%

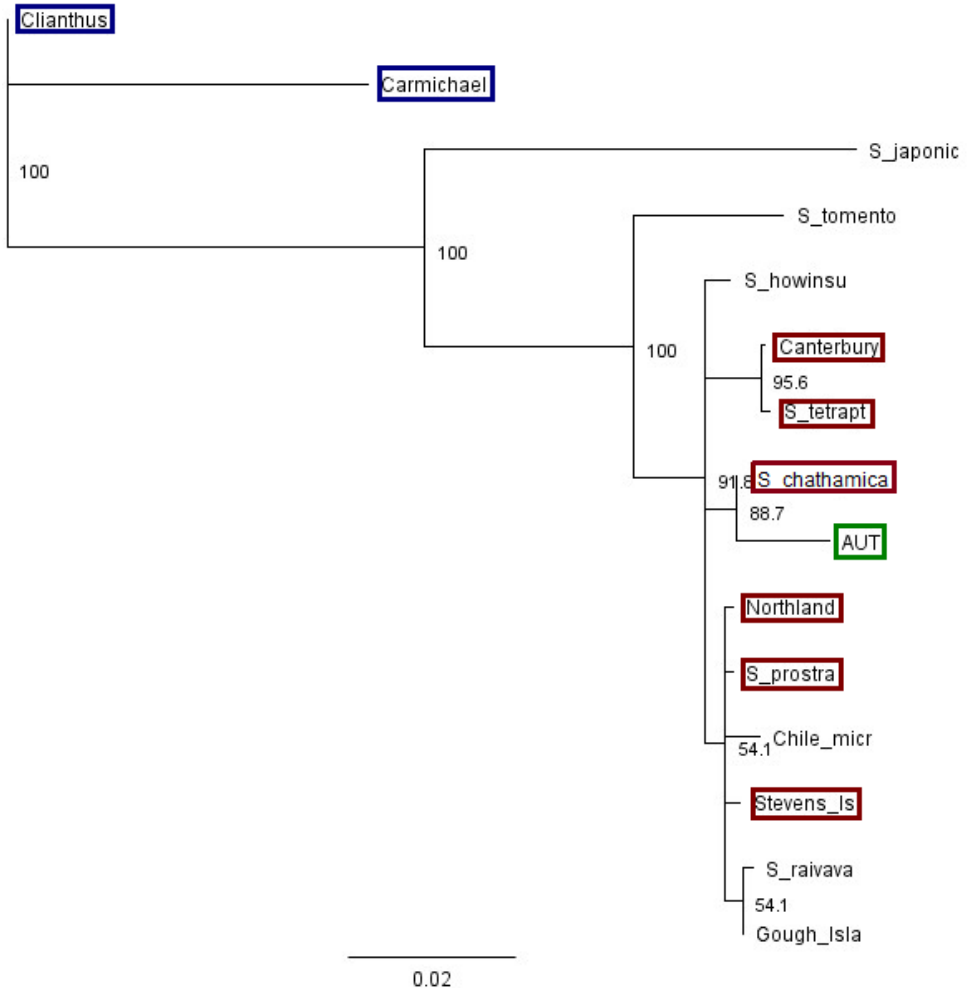


Figure 16. The phylogenetic analysis of *atpB-rbcL* gene of AUT sample (Blue indicates the outgroups, Red indicates the kowhai and green represents the AUT sample. Canterbury, Northland and Steven Is are the code of *S. microphylla* growth at Canterbury, Northland and Steven Island)

In summary, the morphological information and phylogenetic analysis strongly supported the AUT samples being closely related to *S. chathamica* and the BLAST search result shown the sequences from the AUT samples were close to *S. microphylla*. The geographical information also supported that both *S. chathamica* and *S. microphylla* are common in Auckland region. Consequently, the AUT samples were suggested to be *S. chathamica* x *S. mircrophylla* hybrids.

2.7. Discussion

The BLAST search result of the forward and reverse sequence may represent two different alleles (see Table 6 and Table 7; also in Table 8 and Table 9). These alleles are most similar to different *Sophora* species supporting the idea that the trees are hybrids. On balance, the sequences appear close to *S. microphylla*. The forward and reverse sequences were slightly different, resulting in different homologous sequences identified by BLAST. The quality of sequence was good, there was no ambiguity in base calling therefore the differences between forward and reverse sequences were true and not sequencing errors.

Trees are hybrids based on several evidences. Firstly, many morphologically characteristics of these trees are similar to both *S. chathamica* and *S. microphylla*. Secondary, BLAST showed that more than one sequences hits within the species, with *S.microphylla* as one of the hits in both the rbcL and matK results. Thirdly the phylogenetic tree supported *S.chathamica*. Furthermore *S. chathamica* x *S. microphylla* hybrids are common in the Auckland region where the species ranges overlap (Heenan 2001).

Chapter 3

Defence system induction

As mentioned in Section 1.5.1., the plant hormone ethylene is involved in many aspects of the plant life cycle, including the seeding process, root development, flowering, fruit ripening and also responses to pathogen attack (Boller et al., 1983; Wang et al., 2002). This chapter discusses the biosynthesis of ethylene by stress and how ethylene is related to the induction of certain enzymes for disease resistance. The results for ethylene induction of kowhai defence responses in the present study are then presented and discussed.

3.1. Ethylene induction

The process of ethylene production is shown in Figure 17. The first step of ethylene production is the genes and enzymes that are activated after the plant recognizes the pathogen infection. The amino acid methionine is converted by SAM synthase to *S*-adenosyl-methionine (*S*-AdoMet). Then 1-aminocyclopropane-1-carboxylic acid (ACC) is produced by ACC synthase and 5'-methylthioadenosine (MTA) by the Yang cycle. Finally, ACC is oxidized to ethylene by ACC Oxidase. The ACC synthase and ACC oxidase are the key factors in ethylene production and are activated by certain stresses such as pathogen infection (Wang *et al.,* 2002; Broekaert *et al.,* 2006).

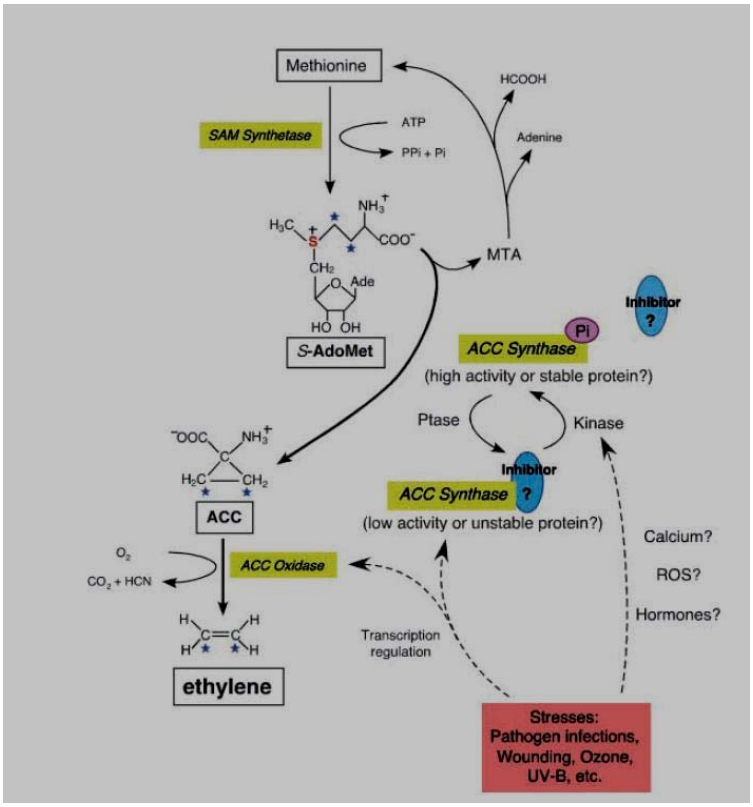


Figure 17. Ethylene production in plants (modified from Wang et al., 2002).

Ethylene acts as a signal which is transported across the plant cell wall and received by the ethylene receptors. Certain positive downstream regulators are involved in the ethylene pathway to produce the genes of ethylene response element binding protein after it receives the ethylene signal. The ethylene element binding protein is produced by transcription which has the ability to regulate gene expression by interacting with the GCC-box by ethylene-response genes production. The ethylene-response genes modulate the defence response of plants (Guo & Ecker, 2004). For example, it was shown that ethylene treatment caused an approximately 20-50 fold increase of the chitinase mRNA (*CH5B*) in the bean with a 30 fold increase in enzyme activity after ethylene treatment (Boller & Vögeli, 1984; Broglie et al., 1986; Broglie *et al.,* 1989).

3.2. Salicylic acid induction

Salicylic acid can be produced indirectly by PAL in plant (refer to Section 1.3.4.). Also as mentioned in Section 1.5.2., salicylic acid is also able to activate the Systemic Acquired Resistance (SAR) which leads to increased production of defensive proteins such as chitinase, lysozyme, phenylpropanoid

phytoalexins and acid phosphatase production in many plants (Mauch-Mani & Métraux, 1998; van Huijsduijnen *et al.,* 1986). Since hormone induction in New Zealand native plants such as kowhai has never been studied before, this research is focused on the quantitative induction by ethylene and salicylic acid of disease resistance pathways including chitinase, lysozyme, acid phosphatase and total phenolic compounds which represent the phenylpropanoid phytoalexins content.

3.3. Methods and materials

Kowhai samples were collected from three naturally grown trees located at AUT University (see Figure 12). 2,6-dichloropyridine-4-carboxylic acid (2,6 dichloroisonicotinic acid -INA) was selected to be the salicylic acid analogue (Colson-Hanks & Deverall, 2000) and ethephon was the source of ethylene (Van Kan *et al.,* 1995) since it releases ethylene gas when dissolved into buffer. Moreover, because of the inhibition of ethylene production, cobalt (II) chloride was chosen to be the negative control of the ethephon experiments (Lau & Yang, 1976; Yu & Yang, 1979; de Rueda *et al.,* 1994; Khalafalla & Hattori, 2000).

Chitinase, lysozyme and acid phosphatase were extracted by a method based on Boller *et al.,* (1983) and phenolic compounds were extracted by a method used by Ainsworth & Gillespie, (2007). Insoluble polyvinylpryrrolidone (PVP) was used to decrease the extraction of phenolic compounds in the Boller method since phenolics are known to inhibit enzymes. The binding to PVP is based on the hydrogen bond formed between phenolic compounds and PVP, (Woodhead *et al.,* 1997). The chitinase activity is presented in enzyme unit per mL of sample per micro-gram of protein (U/mL/µg) while lysozyme and acid phosphatase activities are presented in enzyme units per L of sample per micro-gram of protein (U/L/µg). The total phenolic content was used to determinate the phenylalanine ammonia-lyase activity which is presented in millimole of phenolic compounds per gram of tissue sample (Ainsworth & Gillespie, 2007). Enzyme unit (U) is defined as the amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute. The conditions also have to be specified: one usually takes a temperature of 25°C

and the pH value and substrate concentration that yield the maximal substrate conversion rate (Nelson & Cox, 2004).

Total protein content was measured by the dye binding method as developed by Bradford, (1976). This method is based on the Bradford-Coomassie blue dyes which binds to the protein and is detected by absorbance at 595nm.

The chitinase activity was measured quantitatively by a fluorimetric assay based on an artificial substrate supplied by Sigma-Aldrich. This method uses enzymatic hydrolysis of 4-methylumbelliferyl β-D-N,N',N"-triacetylchitotriose, a chitin like substrate, to produce 4-methylumbelliferone (4-MU), a product which is fluorescent under alkaline conditions and can be detected fluorimetrically under an excition wavelength at 360nm and an emission wavelength at 450nm.

The lysozyme activity was measured by a turbidimetric assay modified from the methods of McKenzie & White, (1986); Brunner *et al.,* (1998); Lee & Yang, (2002) and Wang *et al.,* (2005). *Micrococcus lysodeikticus* is a bacterium used as a "lysis meter", the bacterium cell is suspended into the buffer which causes turbidity of the solution. Lysozyme can breakdown the bacterial cell wall by breaking the bonds between the carbohydrates in the glycopeptides from the cell wall and dissolves the bacterium into the buffer which decreases the turbidity of the solution. This activity can be detected by absorbance decreases at an arbitrary wavelength, chosen to be 650nm.

Acid phosphatase activity was measured by a colorimetric assay based on the method used by dos Prazeres et al., (2004). The p-nitrophenol phosphate is a colourless phosphatase substrate which is hydrolysed by acid phosphatase to p-nitrophenol, a yellow coloured product which can be detected by absorbance at 440nm.

A colorimetric assay was also used to determine the total phenolic content of the kowhai leaf extracts using the Folin-Ciocalteu reagent as the method from Ainsworth & Gillespie, (2007). This assay is based on electrons transferred from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to

form a blue colour product which can be detected by absorbance at 765nm under alkaline condition.

The chitinase, lysozyme and acid phosphatase assay and the total phenolic determination were performed in triplicate for each sample on triplicate plant samples.

In addition, Koga et al., (1999) suggested that plant chitinase may have various optimum pH applying to different plants (can be pH 4-9). Hence, to measure the effects of pH on the chitinase activity is critical before performing the hormone treatments. A modified method from Copeland, (2000) was used for this purpose (see Appendix F.2).

3.3.1. Sampling

The kowhai samples were collected by sample method as described in Section 2.5.1. The outer leaf samples were fully developed but still relatively young and therefore it was assumed that they would contain low levels of plant hormones and enzymes.

3.3.2. Hormone treatments

The leaf samples of kowhai were incubated with hormones as shown in Figure 18 and Figure 19. The leaves were incubated in either the INA solution (8 mg INA (Sigma-Aldrich) in 0.2 mL of ethanol then to 400 mL with deionized water) or an ethephon solution (1000 ppm of ethephon (Sigma-Aldrich) in 5 mM disodium hydrogen phosphate) for 48 hours at 25ºC, exposed to light. The negative control was 0.1% (v/v) ethanol in deionized water for the INA treatment and 1mM cobalt (II) chloride (in 5mM disodium hydrogen phosphate) for the ethephon treatment.

Figure 18. Hormone treatments of kowhai leaf samples

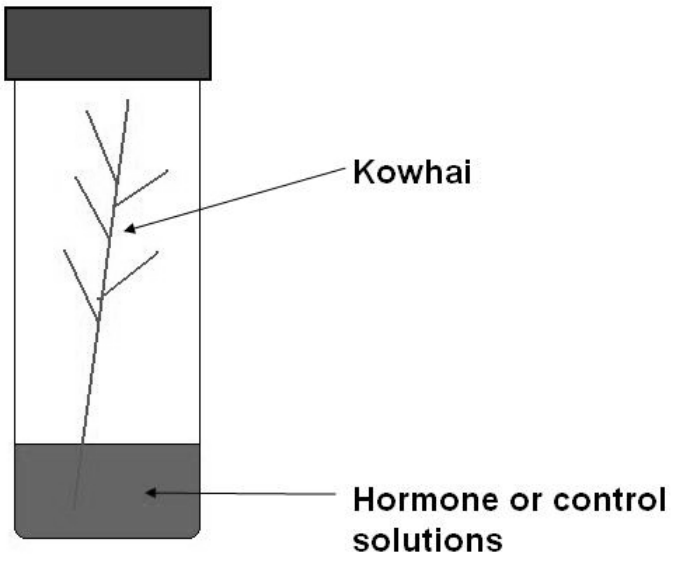


Figure 19. Hormone treatment of kowhai leaf samples

3.3.3. Extraction methods

3.3.3.1. Extraction of chitinase, lysozyme and acid phosphatase

To extract the chitinase, lysozyme and acid phosphatase from kowhai, about 200 mg of sample leaflet tissue was homogenized using a Lysing Matrix tube E (MP Biomedicals) under acceleration of 5 m/s for nine 10 sec intervals in a FastPrep 24 biological ball mill (MP Biomedicals) by adding 1g of tissue was added to 6 mL of buffer (20 mM acetate buffer at pH 4.5) with 10% (v/v) PVP (Sigma-Aldrich) to reduce the unwanted phenolic compounds which may affect the fluorescence examination. The homogenization process was completed at 5ºC. The extract was then centrifuged (Z216MK, Hermle) at 12000 g for 5 mins at 2ºC. The extract was used to perform the examination of chitinase activity, lysozyme activity, acid phosphatase activity and protein content.

3.3.3.2. Extraction of phenolic compounds

To extract the phenolic compounds from kowhai, around 20 mg of sample leaflet tissue was homogenized with 1 mL of iced 95% (v/v) methanol by Lysing Matrix tube E under acceleration of 4 m/s for 5 mins by FastPrep 24. The extract was then incubated in the dark for 48 hours and subsequently centrifuged at 13000 g for 5 mins. The clear solution used to measure total phenolic compounds.

3.4. Protein content measurement

The Bradford assay (Bradford, 1976) with a series of bovine serum albumin standards (0 to 1000ppm) was used to measure the total protein content from the kowhai sample. According to his method, the Bradford reagent was prepared by dissolving 100 mg of Brillant Blue G-250 (Sigma-Aldrich) into 50 mL 95% (v/v) ethanol and mixed with 100 mL 85% (v/v) phosphoric acid and diluted to 1L by deionized water.

10µl of sample extract and standards were pipette onto the wells of 96 Well EIA/RIA Plate. 290µl of Bradford reagent was added to all wells. After standing for 5 mins at room temperature the absorbance at 595 nm was measured by a

FLUOstar Omega microplate reader with Bradford reagent as a blank. The result was analyzed by BMG LABTECH'S MARS Data Analysis Software 3.6.2.

3.4.1. Calculation of total protein content

The protein content was detected by the absorbance at 595 nm and calculated by the equation listed in the Figure 30 at Appendix E.

3.4.2. Verification of the total protein content determination

The total protein content determination can be verified by the BSA standard.

3.5. Chitinase activity measurement

The chitinase assay was carried out by a Chitinase Assay Kit, Fluorimetric (Sigma-Aldrich). According to the manufacturer's bulletin, 100 ng of 4-methylumbelliferone was prepared in 100 µL of working buffer (in this case it was 20 mM acetic acid at pH 4.5 (refer to Appendix F.2) buffer as a standard (Hung et al., 2002)). The substrate stock solution (20 mg/mL) was made by adding 0.25 mL of dimethyl sulfoxide (DMSO) (Chitinase Assay Kit, Fluorimetric) to 5 mg of the 4-methylumbelliferyl β-D-N,N'N"-triacetylchitotriose (MU-β-$(GlcNAc)_3$) by continuous vortexing for 15 minutes to allow it dissolve completely. A working substrate solution was prepared just before the assay by diluting the substrate stock solution to 0.2 mg/mL by 20mM acetate buffer (pH 4.5).

The chitinase activity was assayed in a 96 Flat-bottom well fluorescence microplate (Greiner) with volumes as shown in Table 10, using a working buffer of 20 mM acetate buffer at pH 4.5. The plate was incubated for 1 hour at 37˚C and the reaction was stopped by adding 200 µL of glycine/NaOH buffer (pH 10.6) to each well. The fluorescence was measured at an excitation wavelength of 355 nm and an emission wavelength of 450 nm (GAIN 256) by FLUOstar Omega microplate reader (BMG labtech) directly after the reaction. The result was analyzed by BMG LABTECH'S MARS Data Analysis Software.

Table 10. The volumes required for chitinase assay

Assay	Substrate Working Solution (µL)	Sample/ Standard (µL)	Working buffer (µL)
Blank	100	-	-
Standard blank	-	-	100
Sample	90	10 of sample	-
Sample control	-	10 of sample	90

3.5.1. Calculation of chitinase activity

The chitinase activity was detected by the fluorescence intensity. To calculate the actual activity by enzyme unit per mL of sample (units/mL), Equation 1 was used with single standard concentration (100ng [1.9 µmole/mL]). Then the activity is converted to Units/mL/µg of protein.

Equation 1. Chitinase activity determination (Sigma-aldrich, n.d).

$$Units/ml = \frac{(FLUsample - FLUblank) \times 1.9 \times 0.3 \times DF}{FLU\ standard \times T \times Vsample}$$

where:

FLUsample	fluorescence of the sample
FLUblank	fluorescence of the substrate
0.3	final reaction volume in mL
DF	enzyme dilution factor
FLUstandard	fluorescence of the 100ng of standard (178)
T	time in minute
Vsample	voume of the sample in mL

3.5.2. Chitinase activity assay verification

The assay was tested using a control enzyme, chitinase from *Trichoderma viride* (Sigma-Aldrich). It was prepared by mixing with 5 mL of Dulbecco's phosphate buffered saline (Sigma-Aldrich) to give a final concentration of 0.2 mg/mL then vortex until dissolved. The rest of the procedure was the same as in Section 3.5. A positive result was observed.

3.6. Lysozyme activity measurement

Micrococcus lysodeikticus was a bacterium used as a substrate to determinate the lysozyme activity from kowhai in this study. 0.04 g of dry powder form of *M. lysodeikticus* (Sigma-Aldrich) was dissolved into 20mM acetate acid buffer (pH 5.5) and incubated at 4˚C for 24 hours for stabilization.

300µl of the *M. lysodeikticus* solution was mixed with 20µl of kowhai leaf sample extract and pipetted into a 96 Well EIA/RIA Plate (Costar). The absorbance at 650nm (with an instrument GAIN setting of 256) was measured every minute up to 30 mins at 25ºC by a FLUOstar Omega microplate reader with an empty well as a blank. The destruction of the bacterial cell wall by chitinases/ lysozyme could be observed by decrease in absorbance in 30 mins. The result was analyzed by BMG LABTECH'S MARS Data Analysis Software.

3.6.1. Calculation of lysozyme activity

The lysozyme activity was calculated as the amount of enzyme unit per litre of sample (unit/L) using Equation 2. This activity was then converted to unit/L/µg of protein based on the protein content of the sample.

Equation 2. Lysozyme activities determination (Pesce & Gendler, 1987).

$$Units/l = \frac{\Delta Abs}{T} \times \frac{Vtotal}{Vsample} \times \epsilon$$

where:

Vtotal	total volume in L
Vsample	sample volume in L
T	time in mintue
Є	lysozme: 38940 (Schultz, 1987)

3.6.2. Lysozyme activity assay verification

Lysozyme from chicken egg white (Serva) was a control enzyme for lysozyme assay. It was prepared by mixing 25 mg of chicken egg white lysozyme in 50 mL of 0.15 M NaOH (Duxbury, 2009). The rest of the procedure was same as Section 3.6. A positive result was observed.

3.7. Acid phosphatase activity measurement

The colourless substrate p-nitrophenol phosphate (Sigma) was used to determine the acid phosphatase activity. 5 mM p-nitrophenyl phosphate substrate solution was made into 100 mM acetate acid buffer (pH 5.0).

50 µL of kowhai leaf extract with 2 mL of substrate was incubated in a test tube at 37ºC in a water bath for 10 mins, the reaction was stopped by addition of 1 mL of 1M NaOH after 10mins. Enzyme activity was measured by the absorbance increase at 440nm on a plastic cuvette by a spectrophotometer (Ultrospec 2100 pro, Amersham pharmacia biotech).

3.7.1. Calculation of acid phosphatase activity

The amount of acid phosphatase activity was determined as described for lysozyme (Equation 2) using a value of 16200 for Є (Noel & Lott, 1987).

3.7.2. Acid phosphatase activity assay verification

The assay was tested by using 50 µL of extract with 2 mL of deionized water and incubated in a test tube at 37ºC water bath for 10mins. The assay was stopped by 1 mL of 1M NaOH after 10mins to stop the reaction, which gave an absorbance of almost zero at 440nm in a plastic cuvette by a spectrophotometer. This indicated that the absorbance increase in the assay is due to the acid phosphatase activity.

3.8. Phenolic compounds measurement

Total phenolic compounds of the kowhai samples were determined by Folin-Ciocalteu reagent, according to the method described by Ainsworth & Gillespie, (2007). A series of gallic acid (Sigma-Aldrich) solutions was used to as standards in this test to find the total phenolic presented in the samples. Gallic acid is a phenolic acid and each gallic acid molecule contains one benzene ring. The concentration of 0.5, 1.0, 1.5, 2.0 and 2.5mM of gallic acid was made in 95% (v/v) methanol. 200 µl of 10% (v/v) Folin-Ciocalteu reagent (Scharlau) was mixed with 100 µl of sample extract, gallic acid standards and 95% methanol (as a blank) in a 2 mL test tube followed by 800µl of 700mM Na_2CO_3. All tubes were incubated at room temperature for 2hours followed by transferring 200µl

into a 96 Well EIA/RIA Plate. The absorbance was measured at 765nm by FLUOstar Omega microplate reader. The result was analyzed by BMG LABTECH'S MARS Data Analysis Software.

3.8.1. Calculation of phenolic content

The phenolic content in the sample was measured by absorbance at 765 nm wavelength and calculated by the equation listed in the Figure 29 at Appendix E. It is presented as mg of total phenolic content/ g of sample leaflet tissues.

3.8.2. Verification of the total phenol content determination

The total phenol content determination was verified by the gallic acid standard.

3.9. Statistical analysis

All data were statistically analysed by Minitab with a Two Samples t-test used to determinate if significant differences of chitinase, lysozyme and acid phosphatase activities and phenolic content occurred with hormone treatments versus controls (if $p<0.05$, reject H_0, significantly different). The Ryan-Joiner test on residuals was used for the normality test (if $p>0.1$, accept H_0, data is normal) and Levene's test for the equal variance (if $p>0.05$m accept H_0, the variance is equal).

3.10. Results

3.10.1. INA treatment

The column charts in Figure 20, Figure 21, Figure 22 and Figure 23 in graphical form the effect of treatment of kowhai leaves with 2,6-dichloropyridine-4-carboxylic acid (INA) on defensive enzyme activities and total phenolic content. (INA is an analogue of the hormone salicylic acid). The data in Table 11 contains the information in numerical form and also indicates if the changes observed in enzyme activities and phenolic content were statistically significant.

Kowhai leaves were treated with the salicylic acid analogue INA and its effects on defence response were measured. The increase in chitinase activity from INA treatment was about 1.54 times (the difference was 3.18 U/mL/µg) higher than the control treatment. However, since the *p* value was 0.1, this result was not significant. A similar result was observed in the lysozyme assay i.e. the increase in lysozyme activity from INA treatment was about 1.07 times (the difference was 1.03 U/L/µg) higher than the control treatment and was not significantly different since the *p* value is 0.717. Hence, treatment by INA in this study had no significant effect on either chitinase or lysozyme levels. In contrast, the acid phosphatase activity and total phenolic content from the INA treatment were both significantly different ($p<0.05$) compared to the control treatment. The INA treatment induced the acid phosphatase activity by 1.77 times and the total phenolic content by 1.24 times.

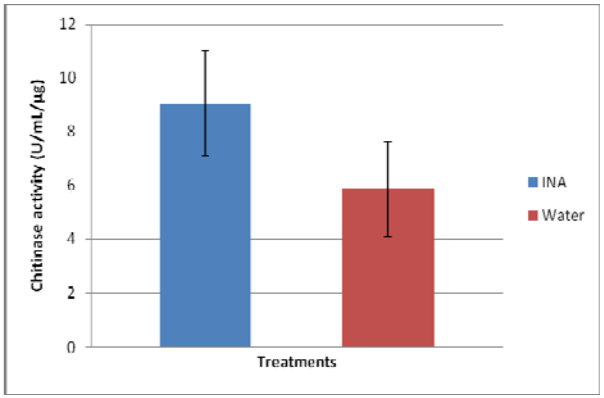


Figure 20. Chitinase activity in kowhai following treatment with INA compared to a water control (error bars show the 95% CI)

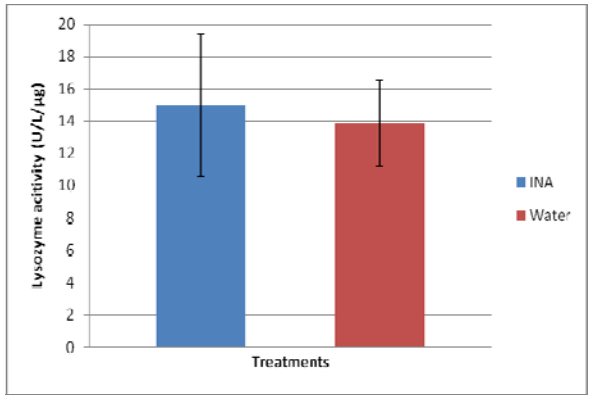


Figure 21. Lysozyme activity in kowhai following treatment with INA compared to a water control (error bars show the 95% CI)

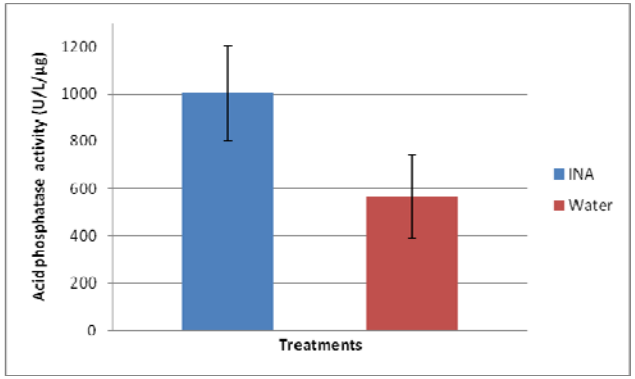


Figure 22. Acid phosphatase activity in kowhai following treatment with INA compared to a water control (error bars show the 95% CI)

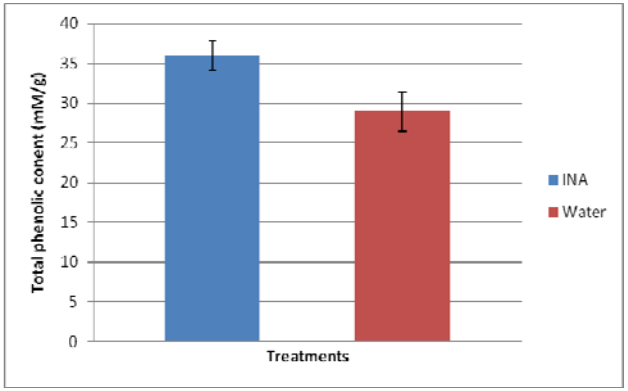


Figure 23. Total phenolic content in kowhai following treatment with INA compared to a water control (error bars show the 95% CI)

Table 11. The result of INA treatment on enzyme and phenolic induction

Test	INA treatment (Mean±SD)	Control treatment (Mean± SD)	Estimate for difference	95% CI for difference	Factor of INA induced increase
Chitinase (U/mL/µg)	9.05 ± 1.73	5.87 ± 1.57	3.18a	-1.12, 7.47	1.54a
Lysozyme (U/L/µg)	14.86 ± 3.79	13.86 ± 2.36	1.03a	-7.17, 9.23	1.07a
Acid phosphatase (U/L/µg)	1003 ± 177	568 ± 158	435.00	-1, 871	1.77b
Total phenolic content (mM/g)	36.01 ± 1.64	28.94 ± 2.15	7.07	2.10, 12.03	1.24b

n=3
a: p>0.05, no significant difference
b: p<0.05, significantly different from control

3.10.2. Ethephon treatment

The column charts in Figure 24, Figure 25, Figure 26 and Figure 27 show in graphical form the effect of treatment of kowhai leaves with ethephon on defensive enzyme activities and total phenolic content (ethephon decomposes to release the hormone ethylene). The data in Table 12 contains the ethephon induction information in numerical form and also indicates if the changes observed in enzyme activities and phenolic content were statistically significant.

Following the ethephon treatment, the chitinase and acid phosphatase activities were significantly increased compared to the control treatments (p<0.05). The chitinase activity had a difference of 12.30 (U/mL/µg) higher than the control treatment which was equivalent to 1.92 times induction. The acid phosphatase activity was 398 U/L/µg higher than the control treatment corresponding to 1.76 times induction. In contrast, the lysozyme activity following ethephon treatment was only 0.96 times the lysozyme activity from the control treatment (the difference was 0.97 U/L/µg) and this difference was not significant (p=0.876). Likewise total phenolic content after ethephon treatment was 1.05 times higher than the control treatment and was not significantly different (p=0.550).

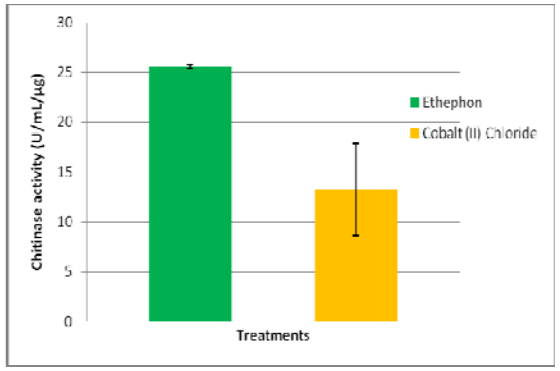


Figure 24. Chitinase activity in kowhai following treatment with ethephon compared to a cobalt (II) control (error bars show the 95% CI)

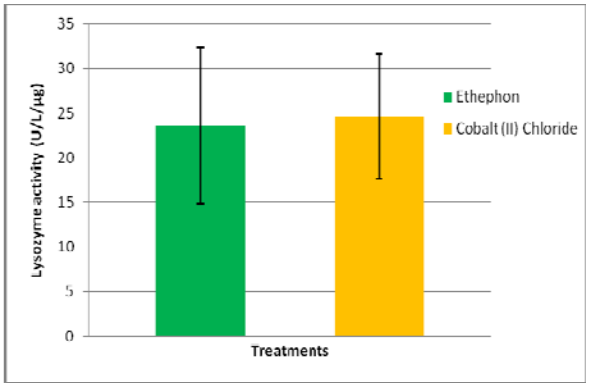


Figure 25. Lysozyme activity in kowhai following treatment with ethephon compared to a cobalt (II) control (error bars show the 95% CI)

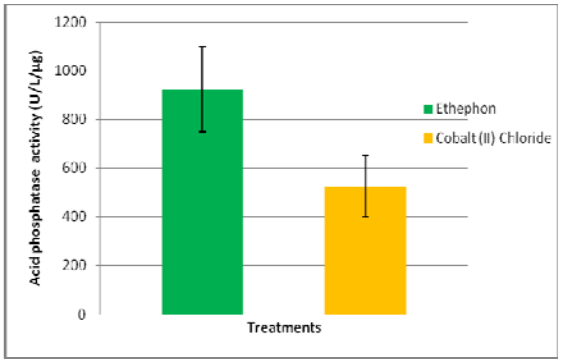


Figure 26. Acid phosphatase activity in kowhai following treatment with ethephon compared to a cobalt (II) control (error bars show the 95% CI)

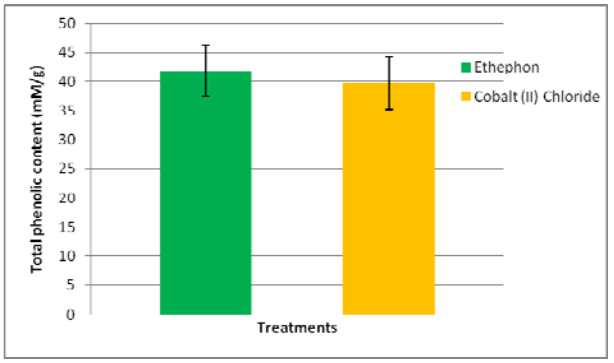


Figure 27. Total phenolic content in kowhai following treatment with ethephon compared to a cobalt (II) control (error bars show the 95% CI)

Table 12. The result of ethephon treatment on enzyme and phenolic induction

Test	Ethephon treatment (Mean±SD)	Control treatment (Mean±SD)	Estimate for different	95% CI for different	Factor of ethephon induced increase
Chitinase (U/mL/µg)	25.598 ± 0.142	13.30 ± 4.12	12.30	2.07, 22.53	1.92b
Lysozyme (U/L/µg)	23.63 ± 7.69	24.60 ± 6.21	-0.97a	-19.13, 17.19	0.96a
Acid phosphatase (U/L/µg)	924 ± 155	526 ± 112	398.00	47, 749	1.76b
Total phenolic content (mM/g)	41.88 ± 3.88	39.73 ± 3.96	2.15a	-8.05, 12.34	1.05a

n=3

a: $p>0.05$, no significant difference

b: $p<0.05$, significantly different from control

Comparing the chitinase response from ethylene treatment for kowhai to other plants (see Table 13), the response lies within the range normally seen for plants (*Triticum aestivum* (wheat) has 0.9 fold to *Phaseolus vulgaris* (common bean) which has 20 fold increase). However, compared to other legumes, the chitinase result is at the low end of the range and is similar to *Pisum sativum* (pea), which has 2.9 fold induction while the increase was1.92 for kowhai.

Table 13. Effect of ethylene on chitinase activity. The listed plant species were treated with the hormone ethylene and the chitinase activity was determined (modified from Boller et al., 2002).

Species	Factor of ethylene induced increase
AUT sample	1.92b
Phaseolus vulgaris	20.00
Pisum sativum	2.90
Glycine max	5.20
Lycopersicon esculentum	3.60
Helianthus annus	14.00
Cucumis sativus	10.00
Gossypium hirsutum	4.70
Zea mays	1.70
Triticum aestivum	0.90

b: significant increase in this study

In conclusion the acid phosphatase activity and total phenolic content were induced 1.77 times and 1.24 times by INA treatment and the chitinase and acid phosphatase activities were induced 1.92 and 1.76 times by ethephon treatment.

3.11. Discussion

Moreover, although chitinase and acid phosphatase activities were significantly induced by ethephon treatment, the total phenolic content was not. Agrios (2005) suggested that ethylene becomes toxic to many kinds of plants when the concentration is higher than 0.05 ppm. In this study, the ethylene content may have been higher than 0.05 ppm since the leaf sample become dark brown and the leaflets fell off readily. This may have been due to intoxication by ethylene or it may have been due to induction of the hypersensitive response which also causes leaf necrosis (refer back to Section 1.4.1.). Leaf necrosis could occur within 24-36 hrs from the pathogen infection. Gorvin & Levine, (2000) observed that the *Arabidopsis* leaf became extensively necrotic after 24-36 hrs by the *Botrytis cinerea* infection.

Since the ethylene level was not quantified in the present study either possibility is valid. This observation may explain why the kowhai faded when exposed to high level of ethylene and did not activate the phenylpropanoid phytoalexins production. Alternatively however it may be that ethylene does not activate the enzyme PAL in kowhai.

In addition lysozyme is mainly found at the roots and flowers of higher plant (Audy *et al.,* 1990). As a result, a low level of lysozyme activity can be expected in leaves (see Section 1.3.3.).

Comparison of chitinase induction in kowhai with other plants showed it responded to a similar level as most plant species (refer back to Table 13). This raises the question of why common bean gives a very high induction level (20 fold), compared to the other legumes soybean (5.2 fold), pea (2.9 fold) and kowhai (1.92 fold). One explanation may be that kowhai (Heenan et al., 2004), pea (Zohary & Hopf, 2001) and soybean (Liu, 2004) originate from Eurasia, whereas common bean originates from America (Singh *et al.,* 1991). The

environmental stresses in Eurasia and America may differ. This may also explain why kowhai is giving a similar result to Eurasian legumes since it is believed to originate from Eurasia (see Section 1.2.3.1.). In support of this a BLAST search result (see Table 20) of a partial class I chitinase nucleotide sequence of kowhai (Appendix B) has the closet match with pea, then soybean (Ha, 2009).

Previous research has shown that treatment of various plants with salicylic acid or its analogue (INA) induces an increase in chitinase activity (refer back to Section 1.5.2.). In contrast, in the present study a 1.54 fold increase was observed but that was not statistically significant.

There may be several explanations for this. The first may be that insufficient time was allowed between treatment and measurement for a change to occur. Dann et al., (1996) observed that 1 day of INA treatment only gave a slight increase in chitinase activity, then up to 9 days of INA treatment gave a significant change.

The weak response of kowhai to the hormones used in this study may have been due to it being a woody perennial legume rather than a herbaceous annual legume (e.g. pea and soybean). Since no publication was found which studies all the above defence enzymes and phenolic phytoalexinIn on both woody perennial legumes and herbaceous annual legumes, the response relationship between two kinds of legumes remain unknown. In contrast a statistically significant increase in acid phosphatase activity is consistent with another study (Beβer et al., 2000) but that was in barley, a non-leguminous plant.

In conclusion, the ethylene intoxication or hypersensitive response may influence the response level followed by ethephon treatment. The low level of lysozyme activity observed may have been due to lysozyme accumulating at root and flower but not the leaves. The low level of induction in chitinase activity by ethephon treatment may have been due to kowhai originating from Eurasia. An insufficient time frame of INA treatment may have been a factor that the chitinase activity was not significantly increased.

Chapter 4

Gene expression measurement

The previous chapter only focused on measuring the *total* chitinase activity. However, chitinase is a large group of enzymes which has five classes and belongs to two families (refer back to Section 1.3.2.). Moreover, Taira, (2010) concluded that not all classes of chitinases have disease resistance properties. Referring back to Figure 6, only class I chitinase was shown to have anti-fungal activity but the activity was not observed in class III and class V chitinases. Different classes of chitinase have specific functions and may respond differently with regard to hormones. Also the induction level of chitinase may have been or may not been due to RNA expression.

The induction of these genes at RNA level might be reverse transcription quantified by real time quantitative PCR (RT-qPCR) which is a modern quantitative and senitive approach for measuring mRNA abundance (Wong & Medrano, 2005; VanGuilder *et al.,* 2008). This would determine if the enzymatic induction is due to an increase in transcription and also identify which classes are induced. No publications were found about the relative gene expression by hormones on GH-19 chitinases and GH-18 chitinases. Hence, no data can be used for comparison.

It is suggested that the RNA expressions of class I and class III chitinase genes can be examined using RT-qPCR, since these classes represent the two major families of chitinases, (Class I chitinase belongs to GH-19 and class III chitinase belongs to GH-18 (Henrissat, 1999). A partial start was made toward this goal with the class I kowhai gene as outlined below, but could not be taken to completion because of time constraints in the present study:

For this purpose, the partial internal nucleotide sequence of a kowhai class I chitinase gene (Ha, 2009 and Appendix B) was used to design specific primers for testing as outlined in Material and Methods below. It is also recommended that specific class III chitinase primers be similarly developed in the future using the degenerate primers of Salzer et al., (2000) (see Table 14).

Table 14. Degenerate primer sequences can be used for chitinase III genes (Salzer et al., 2000)

Genes	Orientation	Sequence
Class III chitinase	Forward	TGGGTCTGGGTICARTTYTAYAAYAAYCC
Class III chitinase	Reverse	GAACGCTHCCAIARCATIACNCCNCCRTA

R=A or G, Y=C or T, B=not A, H=not G, N=A,C,G or T, W=A or T, S=G or C, K=G or T, M=A or C

4.1. Methods and materials

4.1.1. Class I chitinase specific primer design

Specific internal primers were designed (refer to Table 15 for sequences) in the present study from the partial Kowhai class I chitinase sequence (Ha, 2009 and Appendix B) for the use of RT-q PCR. These primers were obtained using Primer3Plus software (http://biotools.umassmed.edu/cgi-bin/primer3plus/primer3plus.cgi) (Claverie & Notredame, 2007).

Table 15. Specific Kowhai Class I chitinase primer sequences tested

Genes	Orientation	Sequence
Class I chitinase	Forward	GAGCAGCAGCAATAAAAGCA
Class I chitinase	Reverse	CTTCAACTCCCACACCCAGT

4.1.2. Sophora Reference Genes for RT-qPCR

According to the MIQE guidelines for developing RT-qPCR methods, reference genes are required to act as internal controls to normalize the RT-qPCR assays. This is because the normalization by internal standard controls the variations in extraction yield, reverse transcription yield and efficiency amplification which can allow the comparison of mRNA concentration across different samples (Bustin et al., 2009).

Housekeeping genes such as *GAPDH*, *β-actin* and *18s* were used as the reference genes (refer to Table 16 for the sequences) for the normalization of quantitative expression analysis in *S. tetraptera* (Song et al., 2008). Although this is a different species than the AUT kowhai, the *Sophora* species in New Zealand are closely related, so it is likely that the housekeeping gene sequences may be the same. Therefore primers for the *β-actin* housekeeping

gene used by Song et al., (2008) in *S. tetraptera* were tested on the AUT kowhai (*S.microphylla* x *S.chathamica* hybrid) in the present study to see if they would amplify the correctly sized PCR product.

Table 16. Real-time PCR primers for housekeeping (Song et al., 2008)

Genes	Orientation	Sequence
β-actin	Forward	GAGCTATGAGTTACCTGATGGACA
β-actin	Reverse	GTAATCTCCTTGCTCATCCTATCA
GAPDH	Forward	ATGACAGATTTGGCATTGTTGA
GAPDH	Reverse	TGCCCTCAGACTCTTCCTTGA
18s	Forward	TACCGTCCTAGTCTCAACCATAA
18s	Reverse	AGAACATCTAAGATCACA

4.1.3. Testing the specific class I chitinase primers and *β-actin* housekeeping primers

The specific class I chitinase primers and *β-actin* primers were tested using endpoint PCR on AUT kowhai genomic DNA with annealing temperatures from 52-58ºC (data not shown) , 54ºC resulted in the optimum PCR performance. The extraction, PCR amplification and gel electrophoresis method described in Section 2.5.3.1., 2.5.3.2. and 2.5.3.3. with the following PCR programme and gel electrophoresis was compared with a 0.25 µg of EZ load precision molecular mass standard (Bio-Rad).

Class I chitinase and *β-actin*:

The PCR program was started with initial melting at 95˚C for 4 mins, amplification was carried out for 35 cycles of melting at 94˚C for 30 secs, annealing at 54ºC for 1 min and elongation at 72˚C for 1 min, followed by a final elongation for 10 mins at 72˚C and held at 20˚C.

4.2. Result

Figure 28 shows the result of PCR products. After 35 cycles, a 250 bp and 170 bp product were resolved on the gel. The specific class I chitinase primers gave a product of about 170 bp and *β-actin* primers gave a product of about 250 bp. This suggested that the specific class I chitinase primers and *β-actin* primers successfully bind on the kowhai DNA.

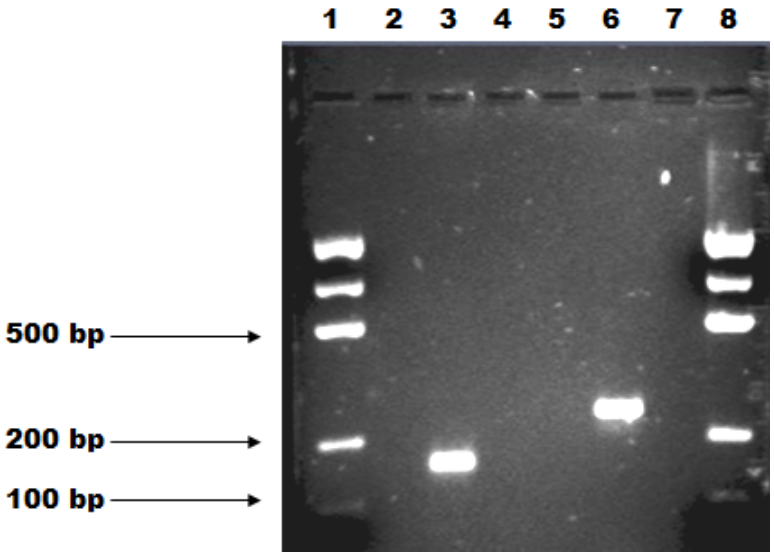


Figure 28. Agarose gel electrophoresis (1%, 1x TBE) showing the PCR product as a result of amplification of genomic DNA.

(L1: EZ load precision molecular mass standard, L2: class I chitinase NTC, L3: class I chitinase product, L4: empty, L5: β-actin NTC, L6: β-actin product, L7: empty, L8: EZ load precision molecular mass standard)

4.3. Discussion

The step in analyzing the response of kowhai to pathogens is to study the mRNA response to the relevant genes. This part of the project was carried out to initiate this work. Specially, primers were designed and tested on the AUT sample genomic DNA to determine their usefulness.

The primers were show to be appropriate for amplifying the class I chitinase and a candidate reference gene (*β-actin*) since a PCR product of the expected size resulted from the AUT sample genomic DNA. However, to be used for mRNA expression measurement, the primers have to be tested on mRNA and RT-qPCR conditions need to be optimized.

Chapter 5
Conclusion & recommendations

5.1. Conclusion

Since the germination period of kowhai is long and the seedling growth process is slow, the kowhai samples were collected directly from the trees growing at AUT University instead of being cultivated from seed. A series of tests on species identification was performed to ensure the samples were kowhai (only those *Sophora* species natively grown in New Zealand are called kowhai, see Section 1.2.2.).

The morphology of kowhai leaf samples which included leaf length, leaflet number, leaflet length and width and also the morphological characteristics of kowhai trees supported the judgement that the kowhai trees located at AUT University were *Sophora chathamica*. The phylogenetic tree suggested that the *atpB-rbcL* gene from the AUT sample was in the group with the *S. chathamica.* The DNA barcode gave more species identity information, suggesting that the kowhai samples were *Sophora microphylla*. Combining both morphological and genetic results, there is strong evidence that the samples that were used in this research were hybrids between *S. chathamica* and *S. microphylla*. This conclusion is supported by the fact that hybrids of *S. chathamica* x *S. microphylla* are known to grow across the Auckland region (Heenan 2001).

There are some uncontrollable factors which may have influenced the result since the trees are naturally growing i.e. not in controlled laboratory conditions (see Section 1.7.). To minimize those effects, both hormone treatment and control treatment were performed at the same time after the samples were collected.

Chitinase, lysozyme and acid phosphatase assays and tests of total phenolic content were undertaken to see if the level of those enzymes and compounds were induced by hormones. INA and ethephon were selected to be the sources of the hormones during treatment. INA is an analogue of salicylic acid and ethephon is the source of ethylene. Water (with 0.1% (v/v) of ethanol) was used to be the control of the INA treatment and cobalt (II) chloride was the control of the ethephon treatment since it can inhibit ethylene production by plant (refer back to Section 3.3.). Protein assays were also performed on the

samples which were used to minimize the protein denatured by either heat or other factors during the enzyme activity calculation

The results showed that the acid phosphatase activity and total phenolic content were significantly induced about 1.77 and 1.24 times by the salicylic acid analogue. Chitinase activity and lysozyme activity did not show significant increases by INA. On the other hand, chitinase and acid phosphatase activitites were significant induced 1.92 and 1.76 times by ethylene (since ethephon transforms to ethylene). Lysozyme activity and total phenolic content did not significantly increase on ethylene treatment. Moreover, low levels of lysozyme activity were found from both control treatments. The chitinase activity was compared to other legumes (see Table 2), *Phaseolus vulgaris* (common bean) had 20 times the induction rate by ethylene treatment, *Pisum sativum* (pea) had 2.9 times and *Glycine max* (soybean) had 5.2 times.

5.2. Recommendations

The specific classes of chitinase e.g. the class I chitinase might be purified by either chitin affinity chromatography on a column of regenerated chitin (Molano et al., 1977; Boller et al., 1983) or washed chitin from clam shells (Bloch & Burger, 1974) and SDS-PAGE (Trudel & Asselin, 1989; Gijzen et al., 2001) to increase the specificity of chitinase in future research. Moreover, the level of ethylene present after 48 hours incubation should also be monitored. The ethylene determination can be performed by either gas-chromatography (GC) or colormetric assay (LaRue & Kurz, 1973). Further research and development from this research may applied on agriculture and horticulture commerically.

The gene expression measurement by RT-qPCR should be progressed further. The present study demonstrated that the specific class I chitinase and *β-actin* primers were working on the AUT kowhai sample. Specific class III chitinase can be designed based on the sequence from the PCR product of the degenerate class III chitinase primers suggested at Table 14. Other housekeeping genes (*GADPDH* and *18s*) were suggested at Table 16.

This research is the first study about hormone induction of specific disease resistance enzymes on New Zealand native plants. To understand more about New Zealand biota, similar research should be done on other New Zealand native plant species such as golden sand sedge (also known as Pikar or Pingao, *Desmoschoenus spiralis*), Kauri (*Agathis australis*) and cabbage tree (also know as ti kouka, *Cordyline australis*) (Department of Conservation, n.d.).

Other research should also be done on comparing the hormones response in terms of the disease resistance between the woody perennial legume and herbaceous annual legumes, because the relationships between the two types are not well known.The results of the present research may indicate that woody plants have a smaller induction response to hormones than herbaceous plants. Further research should be done to confirm this.

References

Agrios, G. N. (2005). *Plant pathology* (5th ed.). Amsterdam: Elsevier Academic Press.

Ainsworth, E. A., & Gillespie, K. M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nature Protocols, 2*(4), 875-877.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acid Research, 25*(17), 3389-3402.

Audy, P., Quéré, D. L., Leclerc, D., & Asselin, A. (1990). Electrophoretic forms of lysozyme activity in various alant species. *Phytochemistry, 29*(4), 1143-1159.

Ausubel, J. H. (2009). A botanical macroscope. *Proceedings of the National Academy of Sciences, 106*(31), 12569-12570.

Beintema, J. J., & van Scheltinga, A. C. T. (1996). Plant lysozymes. In P. Jollès (Ed.), *Lysozymes--model enzymes in biochemistry and biology* (pp. 75-86). Basel: Birkhäuser Verlag.

Beβer, K., Jarosch, B., Langen, G., & Kogel, K. (2000). Expression analysis of genes induced in barley after chemical activation reveals distinct disease resistance pathways. *Molecular Plant Pathology, 1*(5), 227-286.

Bioscience. (n.d.). *Acid phosphatase*. Retrieved 15th October, 2010, from http://www.gbiosciences.com/PhosphataseAssay-desc.aspx

Bloch, R., & Burger, M. M. (1974). Purification of wheat germ agglutinin using affinity chromatography on chitin. *Biochemical and Biophysical Research Communications, 58*(1), 13-19.

Boller, T., Gehri, A., Mauch, F., & Vögeli, U. (1983). Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. *Planta, 157*, 22-31.

Boller, T., & Vögeli, U. (1984). Vacuolar localization of ethylene-induced chitinase in bean leaves. *Plant Physiology, 74*, 442-444.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248-254.

Brameld, K. A., & Goddard III, W. A. (1998). The role of enzyme distortion in the single displacement mechanism of family 19 chitinase. *Proceeding of the National Academy of Science, 95*, 4276-4281.

Bravo, J. M., Campo, S., Murrillo, I., Coca, M., & San Segundo, B. (2003). Fungus- and wound-induced accumulation of mRNA containing a class II chitinase of the pathogenesis-related protein 4 (PR-4) family of maize. *Plant Molecular Biology, 52*, 745-759.

Brody, J. R., & Kern, S. E. (2004). Review: History of principles of conductive media for standard DNA electorphoresis. *Analytical Biochemistry, 333*, 1-13.

Broekaert, W. F., Delauré, S. L., De Bolle, M. F. C., & Cammue, B. P. A. (2006). The role of ethylene in host-pathogen interactions. *Phytopathology, 44*, 393-416.

Broglie, K. E., Biddle, P., Cressman, R., & Broglie, R. (1989). Fuctional analysis of DNA sequences responsible for ethlyene regulation of a bean chitinase gene in transgenic tobacco. *The Plant Cell, 1*, 599-607.

Broglie, K. E., Gaynor, J. J., & Broglie, R. M. (1986). Ethylene-regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. *Proceedings of the National Academy of Sciences, 83*, 6820-6824.

Brooker, S. G., Cambie, R. C., & Cooper, R. C. (1989). Economic native plants of New Zealand. *Economic Botany, 43*(1), 79-106.

Brooker, S. G., & Cooper, R. C. (1959). New Zealand medicinal plants. *Economic Botany*, *15*(1), 1-10.

Brunner, F., Stintzi, A., Fritig, B., & Legtand, M. (1998). Substrate specificities of tobacco chitinase. *The Plant Journal, 14*(2), 225-234.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative Real-time PCR experiments. *Clinical Chemistry, 55*(4), 611-622.

Cabello, F., Jorrín, J. V., & Tena, M. (1994). Chitinase and *β*-1,3-glucanase activities in chickpea (*Cicer arietinum*). Induction of different isoenzymes in response to wounding and ethephon. *Physiological Plantarum, 91*(654-660).

CBOL Plant Working Group. (2009). A DNA barcode for land plants. *Proceedings of the National Academy of Sciences, 106*(31), 12794-12797.

Chakraborty, S., & Pangga, I. B. (2004). Plant disease and climate change. In M. Gillings & A. Holmes (Eds.), *Plant microbiology* (pp. 175-193). London: BIOS Scientific Publishers.

Chatterjee, A., & Ghosh, S. K. (2008). Alterations in biochemical components in mesta plants infected with yellow vein mosaic disease. *Brazilian Society of Plant Physiology, 20*(4), 267-275.

Claverie, J., & Notredame, C. (2007). *Bioinformatics FOR DUMMiES* (Vol. 2nd). Indianapolis: Wiley.

Colson-Hanks, E. S., & Deverall, B. J. (2000). Effect of 2,6-dichloroisonicotinic acid, its formulation materials and benzothiadiazole on systemic resistance to alternaria leaf spot in cotton. *Plant Pathology, 49*, 171-178.

Cooper, R. C. (1991). *New Zealand's economic native plants*. Auckland: Oxford University Press.

Copeland, R. A. (2000). *Enzymes: a practical introduction to structure, mechanism, and data analysis* (2nd ed.). New York: John Wiley and Sons, Inc.

da Cunha, A. (1987). The Estimation of L-phenylalanine ammonia-lyase shows phenylpropanoid. *Phytochemistry, 26*(10), 2723-2727.

da Cunha, A. (1988). Purification, characterization and induction of L-phenylalanine ammonia-lyase in *Phaseolus vulgaris*. *European Journal of Biochemistry, 178*, 243-248.

Dangl, J. L., & Jones, J. D. G. (2001). Plant pathogens and integrated defence responses to infection. *Nature, 411*, 826-833.

Dann, E. K., Meuwly, P., Métraux, J. P., & Deverall, B. J. (1996). The effect of pathogen inoculation of chemical treatment on activities of chitinase and β-1,3-glucanase and accumlation of saliclyic acid in leaves of green bean, *Phaseolus vulgaris* L. *Physiological and Molecular Plant Pathology, 49*, 307-319.

Davies, R. C., Neuberger, A., & Wilson, B. M. (1969). The defendence of lysoyme activity on pH and ionic strength. *Biochemica et Biophysica Acta, 178*, 294-503.

de Rueda, P. M., Gallardo, M., Sánchez-Calle, I. M., & Matilla, A. J. (1994). Germination of chick-pea seeds in relation to manipulation of the ethylene pathway and polyamine biosynthesis by inhibitors. *Plant Science, 97*, 31-37.

Department of Conservation. (n.d.). *New Zealand native plants: Conservation*. Retrieved 11th June, 2011, from http://www.doc.govt.nz/conservation/native-plants/

Derckel, J. P., Legendre, L., Audran, J., Haye, B., & Lambert, B. (1996). Chitinases of the grapevine (*Vitis vinifera* L.): five isoforms induced in leaves by salicylic acid are constitutively expressed in other tissues. *Plant Science, 119*, 31-37.

Dixon, R. A. (1986). The Phytoalexin Response: Elicitation, signalling and control of host gene expression. *Biological Reviews of the Cambridge Philosophical Society, 61*, 239-291.

Dixon, R. A., & Palva, N. L. (1995). Stress-induced phenylpropanoid metabolism. *The Plant Cell, 7*, 1085-1097.

dos Prazeres, J. N., Ferreira, C. V., & Aoyama, H. (2004). Acid phosphatase activities during the germination of *Glycine max* seeds. *Plant Physiology and Biochemistry, 42*, 15-20.

Düring, K. (1993). Can lysozymes mediate antibactierial resistance in plants? *Plant Molecular Biology, 23*, 209-214.

Duxbury, M. (2009). *Biochemistry 6 laboratory manual*. Unpublished manuscript. School of applied science, Auckland University of Technology. Auckland.

Ebel, J. (1986). Phytoalexin synthesis: The biochemical analysis of the induction process. *Annual Review of Phytopathology, 24*, 235-264.

Enyedi, A. J., Yalpani, N., Silverman, P., & Raskin, I. (1992). Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. *Proceedings of the National Acadmy of Sciences, 89*, 2480-2484.

Esaka, M., & Teramoto, T. (1998). Short communication: cDNA cloning, gene expression and secretion of chitinase in winged bean. *Plant Cell Physiology, 39*(3), 349-356.

Fan, J., Wang, H., Feng, D., Liu, B., Liu, H., & Wang, J. (2007). Molecular characterization of plantain class I chitinase gene and its expression in response to infection by *Gloeosporium musarum* Cke and Massee and other abiotic stimuli. *Journal of Biochemistry, 142*, 561-570.

Frohman, M. A., Dush, M. K., & Martin, G. R. (1988). Rapid production of full-length cDNAs from rare transcripts: Amplification using single gene-specific oligonucleotide primer. *Proceeding of the National Acadmy of Science, 85*(23), 8998-9002.

Gijzen, M., Kuflu, K., Qutob, D., & Chernys, J. T. (2001). A class I chitinase from soybean seed coat. *Journal of Experimental Botany, 52*(365), 2283-2289.

Goodman, R. N., & Novacky, A. J. (1994). *The hypersensitive reaction in plants to pathogens: a resistance phenomenon*. St Paul: American phytopathological society.

Google map New Zealand. (2011). Retrieved 16th April, 2011, from http://maps.google.co.nz/

Govrin, E. M., & Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Current Biology, 10*(13), 751-757.

Guo, H., & Ecker, J. R. (2004). The ethylene signaling pathway: new insights. *Current opinion in Plant Biology, 7*, 40-49.

Ha, Q. C. (2009). *Identification of kowhai plant and its chitinase through PCR amplification and DNA barcoding*. Unpublished research project. Auckland University of Technology, Auckland, New Zealand.

Hahlbrock, K., & Scheel, D. (1989). Physiology and molecular biology of phenylpropanoid metabolism. *Plant Molecular Biology, 40*, 347-369.

Hall, B. G. (2008). *Phylogenetic trees made easy* (3rd ed.). Massathusetts: Sinauer Associates.

Hamel, F., Boivin, R., Tremblay, C., & Bellemar, G. (1997). Structural and evolutionary relationships among chitinases of flowering plants. *Journal of Molecular Biology, 44*, 614-624.

Hatch, G. M. (2007). *Mitochondrial phospholipid synthesis and incorporation* [Online talk]. Retrieved from http://hstalks.com.ezproxy.aut.ac.nz/main/browse_talk_view.php?t=166&s=166&s_id=28&c=252

Heath, M. C. (2000). Hypersensitive response-related death. *Plant Molecular Biology, 44*, 321-334.

Heenan, P. B. (1998). Reinstatement of *Sophora longicarinata* (Fabaceae-Sophoreae) from northern South island, New Zealand, and typification of *S. microphylla*. *New Zealand Journal of Botany 36*, 369-379.

Heenan, P. B., Dawson, M. I., & Wagstaff, S. J. (2004). The relationship of *Sophora* sect. *Edwardsia* (Fabaceae) to *Sophora tomentosa,* the type species of the genus *Sophora*, observed from DNA sequence data and morphological characters. *Botanical Journal of the Linnean Society, 146*(4), 439-446.

Heenan, P. B., de Lange, P. J., & Wilton, A. D. (2001). *Sophora* (Fabaceae) in New Zealand: taxonomy, distribution, and biogeography. *New Zealand Journal of Botany, 39*, 17-53.

Henrissat, B. (1999). Classification of chitinase modules. In P. Jollès & R. A. A. Muzzarelli (Eds.), *Chitn and chitinase* (pp. 137-156). Basel: Birkhäuser Verlag.

Hubbard centre for genome studies. (n.d.). *Degenerate primer design*. Retrieved 17th March, 2010, from http:/hcgs.unh.edu/proctocol/basic/pcrgegenpri.html

Huet, J., Rucktooa, P., Clantin, B., Azarkan, M., Looze, Y., Villerey, V., & Wintjens, R. (2008). X-ray structure of Papaya chitinase reveals the substrate binding mode of glycosyl hydrolase family 19 chitinases. *Biochemistry, 47*, 8283-8291.

Hung, T., Chang, Y., Sung, H., & Chang, C. (2002). Purification and characterization of hydrolase with chitinase and chitosanase activity from commercial stem bromelain. *Journal of Agricultural and Food Chemistry, 50*, 4666-4673.

Hurr, K. A., Lockhart, P. J., Heenan, P. B., & Penny, D. (1999). Evidence for the recent dispersal of *Sophora* (Leguminosae) around the Southern oceans: Molecular data. *Journal of Biogeography, 26*, 565-577.

Jakobek, J. L., & Lindgren, P. B. (1993). Generalized induction of defense responses in bean Is not correlated with the induction of the hypersensitive reaction. *The Plant Cell, 5*, 49-56.

Jakobek, J. L., & Lindgren, P. B. (2002). Expression of a bean acid phosphatase cDNA is correlated with disease resistance. *Journal of experimental Botany, 53*(367), 387-389.

Jollès, P. (1996). From the discovery of lysozyme to the characterization of several lysozyme families. In Jollès (Ed.), *Lysozymes--model enzymes in biochemistry and biology* (pp. 3-5). Basel: Birkhauser Verlag.

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature, 444*, 323-329.

Kasprzewska, A. (2003). Plant chitinase-regulation and function. *Cellular & Molecular Biology Letters, 8*, 809-824.

Kataria, H. R., Wilmsmeier, B., & Buchenauer, H. (1997). Efficacy of resistance inducers, free-radical scavengers and an antagonistic strain of *Pseudomonas fluorescens* for control of *Rhizoctonia solani* AG-4 in bean and cucumber. *Plant Pathology, 46*, 897-909.

Kawase, T., Yokokawa, S., Saito, A., Fujii, T., Nikaidou, N., Miyashita, K., & Watanabe, T. (2006). Comparison of enzymatic and antifungal properties between family 18 and 19 Chitinases from *S. coelicolor* A3(2). *Bioscience, Biotechnolgy, and Biochemistry, 70*(4), 988-998.

Khalafalla, M. M., & Hattori, K. (2000). Ethylene inhibitors enhance *in vitro* root formation on faba bean shoots regenerated on medium containing thidiazuron. *Plant Growth Regulation, 32*, 59-63.

Khan, W., Prithiviraj, B., & Smith, D. L. (2003). Chitosan and chitin oligomers increase phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities in soybean leaves. *Journal of Plant Physiology, 160*, 859-863.

Kim, Y. S., Lee, M. Y., & Park, Y. M. (1992). Purification and characterization of chitinase from green onion. *Korean Biochemical Journal, 25*(2), 171-177.

Koga, D., Mitsutomi, M., Kono, M., & Matsumiya, M. (1999). Biochemistry of chitinase. In P. Jollès & R. A. A. Muzzarelli (Eds.), *Chitin and chitinase* (pp. 111-123). Basel: Birkhäuser Verlag.

Korbie, D. J., & Mattick, J. S. (2008). Touchdown PCR for increased specificity and sensitivity in PCR amplification. *Nature Protocols, 3*(9), 1452-1456.

Kress, W. J., & Erickson, D. L. (2008). DNA barcodes: Genes, genomics, and bioinformatics. *Proceedings of the National Academy of Sciences, 105*(8), 2761-2762.

Kress, W. J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A., & Janzen, D. H. (2005). Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences, 102*(23).

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A., & Zoric, N. (2006). The real-time polymerase chain reaction. *Molecular Aspects of Medicine, 27*, 92-125.

LaRue, T. A., & Kurz, W. G. (1973). Estimation of nitrogenase using a colorimetric determination for ethylene. *Plany Physiology, 51*, 1074-1075.

Lau, O. L., & Yang, S. F. (1976). Inhibition of ethylene production by cobaltous ion. *Plant Physiology, 58*, 114-117.

Lee, Y. C., & Yang, D. (2002). Determination of lysozyme activities in a microplate format. *Analytical Biochemistry, 310*, 223-224.

Lewis, G., Schrire, B., Mackinder, B., & Lock, M. (2005). *Legumes of the world*. Richmond: Royal Botanic Gardens.

Liu, K. (2004, 12th June 2011). *Soybeans as a powerhouse of nutrients and phytochemicals* [e-Book]. Retrieved from http://www.crcnetbase.com.ezproxy.aut.ac.nz/doi/pdf/10.1201/9781439822203.fmatt

Liu, Y., Ahn, J., Datta, S., Salzman, R. A., Moon, J., Huyghues-Despointes, B., Murdock, L. L., Koiwa, H., &Zhu-Salzman, K. (2005). Arabidopsis vegetative storage protein is an anti-insect acid phosphatase. *Plant Physiology* 1-12.

Löffert, D., Seip, N., Karger, S., & Kang, J. (1998). PCR optimization: Degenerate primers. *Qiagen News, 2*, 3-6.

MacDonald, M. J., & D'Cunha, G. B. (2007). A model view of phenylalanine ammonia lyase. *Biochemistry and Cell Biology, 85*, 273-282.

Mauch-Mani, B., & Métraux, J. (1998). Saliclyic acid and systemic acquired resistance to pathogen attack. *Annals of Botany 82*, 535-540.

Mckenzie, H. A., & White, F. H. (1986). Determination of lysozyme activity at low levels with emphasis on the milk enzyme. *Analytical Biochemistry, 157*, 367-374.

Metraux, J. P., Burkhart, W., Moyer, M., Dincher, S., Middlesteadt, W., Williams, S., Payne, G., Carnes, M., & Ryals, J. (1989). Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. *Proceedings of the National Aceademy of Science, 86*, 896-900.

Mitchell, A. D., & Heenan, P. B. (2002). *Sophora* sect. *Edwardsia* (Fabaceae): Further evidence from nrDNA sequence data of a recent and rapid radiation around the Southern oceans. *Botanical Journal of the Linnean Sociey, 140*, 435-441.

Molano, J., Durán, A., & Cabib, E. (1977). A rapid and sensitive assay for chtitinase using tritiated chitin. *Analytical Biochemistry, 83*, 648-656.

Moore, T. C. (1989). *Biochemistry and physiolology of plant hormones* (2nd ed.). New York: Springer.

Nelson, D. L., & Cox, M. M. (2004). *Lehninger principles of biochemistry* (4th ed.). New York: W. H. Freeman.

Noel, S. A., & Lott, J. A. (1987). Acid phosphatase, total. In A. J. Pesce & L. A. Kaplan (Eds.), *Methods in clinical chemistry* (pp. 683-690). St louis: The C. V. Mosby Company.

O'Gara, F., & Shanmugam, K. T. (1976). Regulation of nitrogen fixation by *Rhizobia* export of fixed N_2 as NH_4^+. *Biochimica et Biophysica Acta, 437*, 313-321.

Osswald, W. F., McDonald, R. E., Niedz, R. P., Shapiro, J. P., & Mayer, R. T. (1992). Quantitative fluorometric analysis of plant and microbial chitosanases. *Analytical Biochemistry, 204*, 40-46.

Pesce, A. J., & Gendler, S. M. (1987). Gamma-glutamyl transferase. In A. J. Pesce & L. A. Kaplan (Eds.), *Methods in clinical chemistry* (pp. 1120-1124). St Louis: The C. V. Mosby Company.

Pontier, D., Balague, C., & Roby, D. (1998). The Hypersensitive response. A programmed cell death associated with plant resistance. *Molecular Biology and Genetics, 321*, 721-734.

Proud, C. (2010). *Protein phosphorylation and the control of protein synthsis* [Online talk]. Retrieved from http://hstalks.com.ezproxy.aut.ac.nz/main/browse_talk_view.php?t=1888&s=1888&s_id=531&c=252

Raskin, I. (1992). Role of salicylic acid in plants. *Annual Review of Plant Physiology and Plant Molecular Biology, 43*, 439-463.

Regalado, A. P., Pinheiro, C., Vidal, S., Chaves, I., Ricardo, C. P. P., & Rodrigues-Pousada, C. (2000). The *Lupinus albus* class-III chitinase gene, *IF3*, is constitutively expressed in vegetative organs and developing seeds. *Planta, 210*, 543-550.

Robertus, J. D., & Monzingo, A. F. (1999). The structure and action of chitinase. In P. Jollès & R. A. A. Muzzarelli (Eds.), *Chitin and chitinase* (pp. 125-135). Basel: Birkhäuser Verlag.

Robertus, J. D., Monzingo, A. F., Marcotte, E. M., & Hart, P. J. (1998). Structural analysis shows five glycohydrolase families diverged from a common ancestor. *The Journal of Experimental Zoology, 282*, 127-132.

Salmon, J. T. (1991). *Native New Zealand flowering plants*. Auckland: Reed.

Salzer, P., Bonanomi, A., Beyer, K., Vögeli-Lange, R., Aeschbacher, R. A., Lange, J., Wiemken, A., Kim, D., Cook, D. R., & Boller, T. (2000). Different expression of eight chitinase genes in *Medicago truncatula* roots during mycorrhiza formation, nodulation, and pathogen infection. *Molecular Plant-Microbe Interactions, 13*(7), 763-777.

Sampson, M. N., & Gooday, G. W. (1998). Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. *Microbiology, 144*, 2189-2194.

Schultz, A. L. (1987). Lysozyme. In A. J. Pesce & L. A. Kaplan (Eds.), *Methods in clinical chemistry* (pp. 742-746). St louis: The C.V. Mosby Company.

Sharma, D. P. (2005). *Environmental microbiology*. Harrow: Alpha science.

Sigma-Aldrich. (n.d.). *Chitinase Assay Kit, Fluorimetric*. Unpublished product information. Sigma-Aldrich. St louis.

Singh, A., Kirubakaran, I., & Sakthivel, N. (2007). Heterologous expression of new antifungal chitinase from wheat. *Protein Expression & Purification, 56*, 100-109.

Singh, S. P., Gepts, P., & Debouck, D. G. (1991). Races of common bean (*Phaseolus vulgaris*, Fabaceae). *Economic Botany, 45*(3), 379-396.

Song, J., Clemens, J., & Jameson, P. E. (2008). Quantitative expression analysis of the ABC genes in *Sophora tetraptera*, a woody legume with an unusual sequence of floral organ development. *Journal of Experimental Botany, 59*(2), 247-259.

Swiss institute of bioinformatics. (n.d.). *Acid phosphatases* Retrieved 15th October, 2010, from http://www.expasy.org/cgi-bin/nicezyme.pl?3.1.3.2

Taira, T. (2010). Structures and antifungal activity of plant chitinase. *The Japanese Society of Applied Glycoscience, 57*, 167-176.

Trudel, J., & Asselin, A. (1989). Detection of chitinase activity after polyacrylamide gel electrophoresis. *Analytical Chemistry, 178*, 362-366.

van Huijsduijnen, A. M. H., Alblas, S. W., de Rijk, R. H., & Bol, J. F. (1986). Induction by salicylic acid of pathogenesis-related proteins and resistance to alfalfa mosaic virus infection in various plant species. *Journal of General Virology, 67*, 2135-2143.

Van Kan, J. A. L., Cozijnsen, T., Danhash, N., & De Wit, P. J. G. M. (1995). Introduction of tomato stress protein mRNAs by ethephon, 2,6-dichloroisonicotinic acid and salicylate. *Plant Molecular Biology, 27*, 1205-1213.

van Loon, L. C., Rep, M., & Pieterse, C. M. J. (2006). Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology, 44*, 135-162.

VanGuilder, H. D., Vrana, K. E., & Freeman, W. M. (2008). Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques, 44*, 619-626.

Verburg, J. G., & Huynh, Q. K. (1991). Purification and characterization of an antifungal chitinase from *Arabidopsis thaliana*. *Plant Physiology, 95*, 450-455.

Wang, K. L. C., Li, H., & Ecker, J. R. (2002). Ethylene biosynthesis and signaling networks. *The Plant Cell*, 131-151.

Wang, S., Ng, T. B., Chen, T., Lin, D., Wu, J., Rao, R., & Ye, X. (2005). First report of a novel plant lysozyme with both antifungal and antibacterial activities. *Biochemical and Biophysical Research Communications, 327*, 820-827.

Wang, S., Shao, B., Fu, H., & Rao, P. (2009). Isolation of a thermostable legume chitinase and study on the antifungal activity. *Applied Microbiology and Biotechnology, 85*, 313-321.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Metraux, J. P., & Ryals, J. A. (1991). Coordinate gene activity in response to agents that induce sysmtemic acquired resistance. *The Plant Cell, 3*, 1085-1094.

White, J. S., & White, D. C. (1997). *Source book of enzymes*. Boca Raton: CRC

Wikimedia. (n.d.). *Kowhai*. Retrieved 31th October, 2010, from http://commons.wikimedia.org/wiki/File:Kowhai_flowers.jpg

Wikipedia. (n.d.). *Polymerase chain reaction*. Retrieved 14th April, 2011, from http://en.wikipedia.org/wiki/PCR

Witmer, X., Nonogaki, H., Beers, E. P., Bradford, K. J., & Welbaum, G. E. (2003). Characterization of chitinase activity and gene expression in muskmelon seeds. *Seed Science Research, 13*, 167-178.

Wong, M. L., & Medrano, J. F. (2005). Real-time PCR for mRNA quantitation. *Biotechniques, 39*, 75-85.

Woodhead, M., Taylor, M. A., Davies, H. V., Brennan, R. M., & McNicol, R. J. (1997). Isolation of RNA from blackcurrant (*Ribes nigrum* L.) fruit. *Molecular Biotechnology, 7*, 1-4.

Yu, Y. B., & Yang, S. F. (1979). Auxin-induced ethylene production and its inhibition by aminoethoxyvinyiglycine and cobalt ion. *Plant Physiology, 64*, 1074-1077.

Zhakhia, F., HJeder, H., Domergue, O., Williems, A., Cleyet-Marel, J., Gillis, M., Dreyfus, B., & de Lajudie, P. (2004). Characterisation of wild legume nodulation bacteria (LNB) in the infra-arid zone of Tuisia. *Systematic and applied Microbiology, 27*, 380-395.

Zohary, D., & Hopf, M. (2001). *Domestication of plants in the old world-the origin and spread of cultivated plants in West, Asia, Europe, and the Nile Valley*. Oxford: Oxford University Press.

Appendices

Appendix A. Morphological information

Table 17. The morphological measurement of AUT kowhai samples.

Leaf length (cm)	Leaflet number	Leaflet length (cm)	Leaflet width (cm)
11.9	47	1.03	0.5
12.8	46	1.1	0.5
12.7	49	1.15	0.48
13.1	41	1.13	0.5
13.6	44	1.13	0.55
12.6	42	1.03	0.52
		0.95	0.5
		1.04	0.45
		1.02	0.45
		1	0.51
		1.31	0.51
		1.3	0.51
		1.25	0.64
		1.04	0.6
		1.34	0.7
		1.01	0.6
		0.96	0.57
		1.16	0.56
		1.01	0.55
		1	0.6
		0.95	0.48
		0.97	0.52
		1.14	0.6
		1.25	0.56
		1	0.6
		1.03	0.66
		1.14	0.54
		1	0.58
		1.21	0.6
		1.16	0.5

Appendix B. Sequences information

matK from forward primer:

ATGCCTCACGGAAGAGGATAATATTTTATTCGATACAAACTCTTTTTTTTTGAGGACCATTGT
AATAATGAGAAAGTTTTCTACATATCCGCAAAAATCGGTCAATAATATCAAAATCAGATGAAT
CGGCCCAGACCGGCTTACTAATGGGGTGCCCTAATACATTACAAAATTTCGCTTTAGCCAA
TGATCTAATTAGAGGAATAATTGGAACTATTGTATCAGGCTTTTTCATAACAATTTCTATTAA
AAATGAATTTTCCAACATTTGACTCCGTACTGCTGAAAGATTTAACCGCACACTTGAAAAAT
AGCCCAAAAGGTGAAATGAATGTTCGGAGAATTGGTTTATTTGGATCATTCCTGGTTGAGA
CCAAACATCATAATGACATTGCCATAAATGGATAAGATAGCATTTCCAGTTATTCATCATAAA
GGGCGCATTCTTTGAAGCCAAAATGGATTTTCCTTGATATCGCACATAATGAATGACAGTAT
CTGTGAAGAATGATAAGGGAGACGAAAAATCCTTAGCAAAGACTTCGACAAAACCTTCCGT
TTTTGCATAGAAATAGATTCGCTCAAAAAAAACACTGAAAGATTTTAATCGTAAATGAGAGA
ATTTGTTACGCAGAAAAAGGAAGATAGATTCGTATTCACATACGTAAAAATGATATAGGAAT
AAGAGAAATCTTGCATTACTTTTTGAAATAGTAGAAATCGATTTTTTTTGAAGTAATAAGACT
ATTCCCAATTATATACTGATACAGCAAACAAACATTGATATATGATAGAAAGGGGTATCTTTC
ACCGCCGTATCGGGAAGGTTTAGAACCGGGATTTCAAGATGGAAACTGGGGCTAATAC

matK from reverse primer:

CCTGTCGACTGGGTGAAGAACCCTTTCTTTCATTTATTAAGGTTGTTTCTTTATGAGTATTGT
AATTGGAATAGTCTTATTACTCCAAAAAAATCGATTTCTACTTTTTCAAAAAGTAATCCAAGA
TTTTTCTTATTCCTATATCATTTTTATGTATGTGAATACGAATCTATCTTCCTTTTTCTGCGTA
ACAAATTCTCTCATTTACGATTAAAATCTTTTAGTGTTTTTTTTGAGCGAATCTATTTCTATGC
AAAAACGGAAGGTTTTGTCGAAGTCTTTGCTAAGGATTTTTCGTCTACCTTATCATTCTTCA
CAGATACTTTCATTCATTATGTTCGATATCAAGGAAAATCCATTTTGGCTTCAAAGAATGCG
CCCTTTTTGATGAATAAATGGAAATACTATCTTATCCATTTATGGTAATGTCATTTTGATGTTT
GGTCTCTACCAGGAATGATCCATATAAACGAATTCTCCGAACATTCATTTCACCTTTTGGGC
TATTTTTCAAGTGTGCGGTTAAATCTTTCAGCAGTACGGAGTCAAATGTTGCAAATTTCATTT
CTAATAGAAATTGTTATGAAAAAAGCTTGATACAATAGTTCCCGATTATTCCTCTAATTAGAT
CATTGGCTAAAGCGAAATTATGTAATGTATCAGGGCACCCCATTAGTAAGCCGGTCTGAGC
CGATGCATCTGATTTTGATAATAATAGACCGATTTTTGCTTGATATGTAGAAATCTATCTCAT
TATACTATGGATCCTCAAACAACAATGAGTTAGTGTCGAATAGAGATATATACTCCCCGCTC
GTACCTCTAGGATAGCATTGGCTCGTCAAGACGGAACGTTGCCTAAGAACCGGA

rbcL from forward primer:

GAGCCGATTCAGCTGGTGCTAAGATATAAACAGACTTATTATACTCATGACTATGAAACCAA
AGATACTGATATCTTAGCAGCATTCCGAGTAACTCCTCAACCCGGAGTTCCGCCTGAAGAA
GCAGGTGCCGCGGTAGCTGCCGAATCTTCTACTGGTACATGGACAACTGTGTGGACCGAT
GGACTTACCAGTCTTGATCGTTACAAAGGACGATGCTATCACATCGAGCCTGTTGCTGGAG
AAGAAAGTCAATTTATTGCTTATGTAGCTTATCCCTTAGACCTTTTTGAAGAAGGTTCTGTTA
CTAACATGTTTACTTCCATTGTAGGTAATGTATTTGGGTTCAAGGCCCTGCGCGCTTTACGT
CTGGAAGATTTGCGAATCCCTACTTCTTATGTTAAAACTTTCCAAGGCCCGCCTCACGGCA
TCCAAGTTGAGAGAGATAAATTGAACAAGTATGGCCGTCCCCTATTGGGATGCACTATTAA
ACCTAAATTGGGGTTATCCGCTAAGAATTATGGTAGAGCAGTTTATGAAAAGACTCCGCGG
TGGATGGGAATTTTACA

rbcL from reverse primer:

CTGCTCGCTCATAGCTCTTAGCGGCTACCATCTATTTAGAGTTTAATAGTGCATCCCAATAG
GGGACGGCCATACTTGTTCAATTTATCTCTCTCAACTTGGATGCCGTGAGGCGGGCCTTGG
AAAGTTTTAACATAAGAAGTAGGGATTCGCAAATCTTCCAGACGTAAAGCGCGCAGGGCCT
TGAACCCAAATACATTACCTACAATGGAAGTAAACATGTTAGTAACAGAACCTTCTTCAAAA
AGGTCTAAGGGATAAGCTACATAAGCAATAAATTGACTTTCTTCTCCAGCAACAGGCTCGAT
GTGATAGCATCGTCCTTTGTAACGATCAAGACTGGTAAGTCCATCGGTCCACACAGTTGTC
CATGTACCAGTAGAAGATTCGGCAGCTACCGCGGCACCTGCTTCTTCAGGCGGAACTCCG
GGTTGAGGAGTTACTCGGAATGCTGCTAAGATATCAGTATCTTTGGTTTCATAGTCAGGAG
TATAATAAGTCAATTTATAATCTTTAACACCAGCTTTGAACCCAACACTTGCTTTAGTCTCTG
TTTGTGGGTGACATAAA

atpB-rbcL from forward primer:

ATCTATATTGCTGTCTTCACAAGTACGTTAATTTGTTGACCAATAGTATCTTGGCCCTTAACT
ACTAGAGCATTGTAAATATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCGGAC
CAATTATTTGAGCGATACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGACCAAAA
GGGGAAGGATTTATTCCCATATTAAAATATATCCATTTTTTTTTCTAAAATTTTGGAAATCAAA
AATAGAAAGGTTCGAAAACAAAGCAAGTAAATCGGTTAATTCAATAATAAAGGGGAGTAAGA
ATTCAATTTTCTTGGTACCATCCAACCAATTCAATTGGTTACTTATTCAATTTCAAGGATTTA
ATTTTCAAGTCCAACCAAGAAAGTAAGTTTTAAAATATAACATTGGAGGAAATCTTTCATTTG
TCTATTATTATGGACAATTATAGACAATACCATCTATTTTACAATATTAGGGGAATTCGAACT
TTCACTTTATTTCCGAGTCAGTTTTTCTATTTCATTAGCCCTAATTTCATATTTTACCAGCTTA
CGATTTATACCTAGTATATTTTTTTTAACCAATTTATTTTTTTTCCGGGGGGGCAATTTCCTGG
CCAAATAAAAAAAATCAGGGATTTACA

atpB-rbcL from reverse primer:

GGGGCTATATCGCTGTGTTCACAAGTACGTAGATTTGTTGACCAACAGGATCTTGGCCTTA
ACTACTAAAGCATGGAAAATATTAGGAAGCTTCACAGGAGGAAAAGCGACATCCAGGAGC
GGGCCAATTATTTGAGCGATACGTCCCAGGTATTTTTTTGCCAATAATTGACTTTGAGAACA
GAAAGAGGAAAGATTTCTTCCCATGTTAAATTTTAAAACTGTTTTTTTTGAAAGAACTTGAAA
ATTAAATCATTGAAATTGAATAAGTAAACAATTGAATTGGTTGTACGGTACCAAGAGAATAG
AATACTACCTCCCATTTATTACTGAATTAACCGATTAACTTGCTTTGTTATCGAACATTTCTA
TTTTTGATTTCCAAAATTTTAGAAAAAAAAATGTAAAAATTTAAAATTGAAAATAATTCCTACC
ACTTCTGGTCCCCAAGTTTCTGCGCTTGAAAAAAAAAACCGGGGAGGTATCCCCCAAATTC
TGGGCCCGGTTTGATTTCCAAGTTTTCCCCCGGGGAAAAGGCCCCTTTTTCCAAATTTTAT
CAAGTTAAGGGCCAAACCACGATTGTTTTTTTAATCAATTTATTTTGTGAGGGGGGGCAATT
TTTGCCAATTTTTCCATCTAGGGTTTAAATTTTAAAAAATAA

Class I chitinase partial sequence from forward degenerate primer (Ha, 2009):

TTGGCAGTTGTGCCACGAAGAGCACGCTAACTAATGTGGCTGCAGTAGTCAATAGTTGTGT
CATGTCCATTGAAAGAGCGAGCAGCAGCAATAAAAGCATCATGGGTGTAGAATCCATGGC
CAACACACCTTGCATCATTTCGATATTTAAGCATTTGTTCGAAGAGGGAGGAACTAACGAG
CCTCCCAATATCACCACCACCACCACCACTGGGTGTGGGAGTTGAAGTAGAAGCATTA
CATTGACTCTGGCACCGTGATGTTGTTGAGCAGTCTGTGTCAGCGCACCCACACAACTCG
CTGTGGCTGCTCCTAGTTGTATCTTGTTCCTGAACC

Class I chitinase partial sequence from reverse degenerate primer (Ha, 2009):

ATTGCTGATGTGGCTCCGCATCATACACTCACAGATGCCGAGTGGATTTAAAGCTGGTGGA
CAACTCCCACACCCACACCTGTGTGTGTGTTGATATTGATGTTGTCATTATCTCCTCCTTCT
TCGTCTTCATGCTTATGCATAGATATGATAATGGTGGTGGTGGGCGATGGCCACTACACCT
ATGATGCTGACATTGCTGCTGCTCGTGCTTGTCTATGTCTTTGGAACAGCCGGAGATGACA
CCGTCCCCAACAGAGAGCGTGATGTTGTTGTTTTCTAGACACAAAATGCAACGCAGGTGGA
GTTGCCGTCGTTTACATTAC

Appendix C. atpB-rbcL sequences from Hurr et al., (1999). Note: these are not presently archived in NCBI-BLAST

The codes below are definite as follow: Canterbury is *S. microphylla* from Canterbury, *S. howinsu* is *S.howinsula* Oliv., *S. chathamica* is *S. chathamica*, Chile micr. is *S. microphylla* from Chile, *S. tetrapt* is *S. tetraptera* J. Mill., *S. prostra* is *S. prostrate* Buchan., Stevens Is. is *S. microphylla* from Stephens Island, Gough Isla. is *S. microphylla* Ait. from Gough Island, Northland is *S. microphylla* from North Island, *S. raivava* is *S. raivavaeensis* St John from Austral Ridge, *S. tomento* is *S. tomentosa* from Vanuatu, *S. japonic* is *S. japonica* from Japan, *Clianthus* is *Clianthus puniceus* and *Carmichael* is *Carmichaelia arborea*.

Canterbury:

AAGTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-
AACTACTAGAGCATTGTAAATATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACC
GGACCAATTATTTGAGCGATACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC
CAGAA------GTGGTAGGATTTATTCTCATATTAAA------CCA-------
TTTTTTTTCTAAAATTTTGGAAATCAAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCGG
TTAATTCAATA--ATAAAT--------GGGAGTT-
AGTATTCTATTTTCTTGGTACCATCCAACCAATTCAA----
TTGTTTACTTATTCAATTTCAATGATTTAATTTTCAAGTTCAACCAAGTAAGTC----
AGTTTTAAA-ATATAACATTGGATGAAA-
TCTTTCATTTGTCTATTATTATAGACAATTATAGACAATACCATCTATATTA-
CTATATTAAGGGAATTCGAACTTTCACTTTATTTCCGAGTCAGTTTTTCTATTTCATTAGCCC
TTATGTCATATTTTATCAGCATACGATTTAAAACCTAGTATATTTTTTTTATCTATTTATTTTTT
TTTTCGT

***S. howinsu*:**

AAGTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-
AACTACTAGAGCATTGTAAATATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACC
GGACCAATTATTTGAGCGATACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC
CAGAA------GTGGTAGGATTTATTCTCATATTAAA------CCA-------
TTTTTTTTCTAAAATTTTGGAAATCAAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCGG
TTAATTCAATA--ATAAAT--------GGGAGTT-
AGTATTCTATTTTCTTGGTACCATCCAACCAATTCAA----
TTGTTTACTTATTCAATTTCAATGATTTAATTTTCAAGTTCAACCAAGTAAGTC----
AGTTTTAAA-ATATAACATTGGATGAAA-
TCTTTCATTTGTCTATTATTATAGACAATTATAGACAATACCATCTATATTA-
CTATATTAAGGGAATTCGAACTTTCACTTTATTTCCGAGTCAGTTTTTCTATTTCATTAGCCC
TTATTTCATATTTTATCAGCATACGATTTAT-
ACCTAGTA????????????????????????????????

***S. chathamica*:**

AAGTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-
AACTACTAGAGCATTGTAAATATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACC
GGACCAATTATTTGAGCGATACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC
CAGAA------GTGGTAGGATTTATTCTCATATTAAAATATATCCA------
TTTTTTTTTCTAAAATTTTGGAAATCAAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCG
GTTAATTCAATA--ATAAAT--------GGGAGTT-
AGTATTCTATTTTGTTGGTACCATCCAACCAATTCAA----
TTGTTTACTTATTCAATTTCAATGATTTAATTTTCAAGTTCAACCAAGTAAGTC----
AGTTTTAAA-ATATAACATTGGATGAAA-
TCTTTCATTTGTCTATTATTATAGACAATTATAGACAATACCATCTATATTA-
CTATATTAAGGGAATTCGAACTTTCACTTTATTTCCGAGTCAGTTTTTCTATTTCATTAGCCC
TTATTTCATATTTTATCAGCATACGATTTATTACCTAGTATA??????????????????????????
????

Chile micr:

AAGTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-
AACTACTAGAGCATTGTAAATATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACC
GGACCAATTATTTGAGCGATACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC
CAGAA------GTGGTAGGATTTATTCTCATATTAAAATATATCCA------
TTTTTTTTTCTAAAATTTTGGAAATCAAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCG
GTTAATTCAATA--ATAAAT--------GGGAGTT-
AGTATTCTATTTTCTTGGTACCATCCAACCAATTCAA----
TTGTTTACTTATTCAATTTCAATGATTTAATTTTCAAGTTCAACCAAGTAAGTC----
AGTTTTAAA-ATATAACATTGGATGAGA-
TCTTTCATTTGTCTATTATTATAGACAATTATAGACAATACCATCTATATTA-
CTATATTAAGGGAACTCGAACTTTCACTTTATTTCCGAGTCAGTTTTTCTATTTCATTAGCCC
TTATGTCATATTTTATCAGCATACGATTTAT-
ACCTAGTATATTTTTTTTATCTATTTATTTTTTTTT-CGT

***S. tetrapt*:**

AAGTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-
AACTACTAGAGCATTGTAAATATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACC
GGACCAATTATTTGAGCGATACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC
CAGAA------GTGGTAGGATTTATTCTCATATTAAA------CCA-------
TTTTTTTTCTAAAATTTTGGAAATCAAAAATAGAA-
TGTTCGATAACAAAGCAAGTTAATCGGTTAATTCAATA--ATAAAT--------GGGAGTT-
AGTATTCTATTTTCTTGGTACCATCCAACCAATTCAA----
TTGTTTACTTATTCAATTTCAATGATTTAATTTTCAAGTTCAACCAAGTAAGTC----
AGTTTTAAA-ATATAACATTGGATGAAA-
TCTTTCATTTGTCTATTATTATAGACAATTATAGACAATACCATCTATATTA-
CTATATTAAGGGAATTCGAACTTTCACTTTATTTCCGAGTCAGTTTTTCTATTTCATTAGCCC
TTATCTCATATTTTATCAGCATACGATTTAA-
ACCTAGTATATTTTTTTTATCTATTTATTTTTTTTTTCG?

***S. prostra*:**

AAGTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-
AACTACTAGAGCATTGTAAATATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACC
GGACCAATTATTTGAGCGATACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC

CAGAA------GTGGTAGGATTTATTCTCATATTAAAATATATCCA------
TTTTTTTTTCTAAAATTTTGGAAATCAAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCG
GTTAATTCAATA--ATAAAT--------GGGAGTT-
AGTATTCTATTTTCTTGGTACCATCCAACCAATTCAA----
TTGTTTACTTATTCAATTTCAATGATTTAATTTTCAAGTTCAACCAAGTAAGTC----
AGTTTTAAA-ATATAACATTGGATGAAA-
TCTTTCATTTGTCTATTATTATAGACAATTATAGACAATACCATCTATATTA-
CTATATTAAGGGAAGTCGAACTTTCACTTTATTTCCGAGTCAGTTTTTCTATTTCATTAGCCC
TTATGTCATATTTTATCAGCATACGATTTAT-
ACCTA??????????????????????????????????

Stevens Is:

AAGTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-
AACTACTAGAGCATTGTAAATATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACC
GGACCAATTATTTGAGCGATACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC
CAGAA------GTGGTAGGATTTATTCTCATATTAAAATATATCCA------
TTTTTTTTTCTAAAATTTTGGAAATCAAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCG
GTTAATTCAATA--ATAAAT--------GGGAGTT-
AGTATTCTATTTTCTTGGTACCATCCAACCAATTCAA----
TTGTTTACTTATTCAATTTCAATGATTTAATTTTCAAGTTCAACCAAGTAAGTC----
AGTTTTAAA-ATATAACATTGGATGAAA-
TCTTTCATTTGTCTATTATTATAGACAATTATAGACAATACCATCTATATTA-
CTATATTAAGGGAATTCGAACTTTCACTTTATTTCCGAGTCAGTTTTTCTATTTCATTAGCCC
TTATGTCATATTTTATCAGCATACGATTTAT-
ACCTAGTATATTTTTTTTATCTATTTATTTTTTTTT-CGT

Gough Isla:

AAGTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-
AACTACTAGAGCATTGTAAATATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACC
GGACCAATTATTTGAGCGATACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC
CAGAA------GTGGTAGGATTTATTCTCATATTAAAATATATCCA------
TTTTTTTTTCTAAAATTTTGGAAATCAAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCG
GTTAATTCAATA--ATAAAT--------GGGAGTT-
AGTATTCTATTTTCTTGGTACCATCCAACCAATTCAA----
TTGTTTACTTATTCAATTTCAATGATTTAATTTTCAAGTTCAACCAAGTAAGTC----
AGTTTTAAA-ATATAACATTGGATGAAA-
TCTTTCATTTGTCTATTATTATAGACAATTATAGACAATACCATCTATATTA-
CTATATTAAGGGAAGTCGAACTTTCACTTTATTTCCGAGTCAGTTTTTCTATTTCATTAGCCC
TTATGTCATATTTTATCAGCATACGATTTAT-
ACCTAGTATATTTTTTTTATCTATTTATTTTTTTTT-CGT

Northland:

?AGTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-
AACTACTAGAGCATTGTAAATATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACC
GGACCAATTATTTGAGCGATACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC
CAGAA------GTGGTAGGATTTATTCTCATATTAAAATATATCCA------
TTTTTTTTTCTAAAATTTTGGAAATCAAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCG
GTTAATTCAATA--ATAAAT--------GGGAGTT-
AGTATTCTATTTTCTTGGTACCATCCAACCAATTCAA----
TTGTTTACTTATTCAATTTCAATGATTTAATTTTCAAGTTCAACCAAGTAAGTC----

AGTTTTAAA-ATATAACATTGGATGAAA-
TCTTTCATTTGTCTATTATTATAGACAATTATAGACAATACCATCTATATTA-
CTATATTAAGGGAACTCGAACTTTCACTTTATTTCCGAGTCAGTTTTTCTATGTCATTAGCCC
TTATGTCATATTTTATCAGCATACGATTTAT??????????????????????????????????????
??

S. raivava:

??GTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-
AACTACTAGAGCATTGTAAATATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACC
GGACCAATTATTTGAGCGATACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC
CAGAA------GTGGTAGGATTTATTCTCATATTAAAATATATCCA------
TTTTTTTTTCTAAAATTTTGGAAATCAAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCG
GTTAATTCAATA--ATAAAT--------GGGAGTT-
AGTATTCTATTTTCTTGGTACCATCCAACCAATTCAA----
TTGTTTACTTATTCAATTTCAATGATTTAATTTTCAAGTTCAACCAAGTAAGTC----
AGTTTTAAA-ATATAACATTGGATGAAA-
TCTTTCATTTGTCTATTATTATAGACAATTATAGACAATACCATCTATATTA-
CTATATTAAGGGAAGTCGAACTTTCACTTTATTTCCGAGTCAGTTTTTCTATTTCATTAGCCC
TTATATCATATTTTATCAGCATACGATTTAT-ACCTAGTATATTTTTTTTAT-
TATTTATTTTTTTT—-CGT

S. tomento:

???TAACGTTAATTTGTTGACCAATAGTATCTTGACCCTT-
AACTACCAGAGCATTGTAAATATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACC
GGACCAATTATTTGAGCG
ATACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGTACCAGAACCAGAAGTGGTAG
GATTTATTCTCATATTAAAATATATCCA------
TTTTTTTTTCTAAAATTTTGGAAATCAAAAAGAGAAATGTTCGATAACAAAGCAAGTTAATCG
GTTAATTCAATA--ATAAAT--------GGGAGTT-
AGTATTCTATTTTCTTGGTACCATCCAACCAATTCAA----
TTGTTTACGTATTCAATTTCAATGATTTAAGTTTCTAGTTCAACCAAG-AAGTC----
AGTTTTAAA-ATATAACATTGGATGAAAG-
CTTTCAGTTGTCTATTATTATAGACAAGTATAGACAATACCATCTATAGTA-
CTATATTAAGGGAAGTCGAACTTTCACTTTATTTCCGAGTCAGTTTTTCTATCTCATAAGCCC
TTATCTCATATTTTATCAGCATACGACTTAT-
ACCTAGTATATTTTTTTT??????????????????????

S. japonic:

??????????????????GACCAACAGTATCTCGACCATT-
AACTACCAGAGCGTTGTAAATATTGGGCATCTTCCCTGGGGGAAAAGCTACATCCAGTACC
GGACCAATTATTTGAGCGATACGTCCCAGGTTTTTTTTTTCAAGCGCAGAAACCTCAGGAC
CAGAA------GTGGTAGGATTTATTCTCATACTAAAATATATCCA------
TTTTTTTTTCGAAAATTTT-GAAATAAAAAAGA--
AATGTTCGATAACAAAGCAAGTTGATCGGTTAATTCAATA--
ATAAATAAGAAACTGGGAGTTCAGTATTCTATTTTCTTGGTACCATCCAACCAATACAATTCA
TTGTTTACTTATTCAATTTAAATGATTGAATTTTCAAGTTCAACCAAG----TC----ATTTTGAAA-
ATATCACCTGGGATGAAA-TCGTTC-GAAGTCTATT------
GACTATTATAGACAATACCATCTATATTA-C----
TCTATGGAATTCGAACCTGAACTTTATTTACGATTCCGTTTTTCTATCTCATTGGCCCTTATT
TCATATTTCATCAGCATACGATTTAT-ACCTAGCATATTTTTTTTACC-----TTTTTCTTTT-CGT

Clianthus:

??????????????????????CAACAGGATCTAGACCTTT-
CACTACCAGAGCGTTGTAAATATTAGGCATCTTCCCAGGTGGAAAAGCTACATCGAGTACC
GGACCAATTATTTGGGAGATACGCCCCAGATTTTTGTTTTCAAGTGCAGAAATCTCAGTATC
AGA----------G--TCAGTTATTCTCATATTAAAATCTATCCGTTTTTTTTTTTTTTTCTAAAATTT-
GGAAATCAAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCGGTTAATTCAATA--
ATAAAT--------GGGAGTT-AGTATTCTATTTTCTTGGTACCATCCAACCAAGTCAA----
TTGTTTACTTATTCAATTTCAATGATTTAATTTTCAAGTTCAACCAA-TAAGTC----
AGTTTTAAA-ATATAACATTGGATGAA-
GTCTTTCATGTGTCTATTATTATAGACAATTATAGACAATACCATCTATATTA-
CTATATTAAGGGAATTCGAACTTTCACTTTATTTCCGAGTCAGTTTTTCTATGTCATTGGCCC
TTATTTCATATTTCATCAGCATACGATATTATACCTAGCATATTTTTTTTACCGA---
TTTTTCTTTT-CGT

Carmichael:

??????????????????????CAACAGGATCTAGACCTTT-
CACTACCAGAGCATTGTAAATATTAGGCATCTTCCCTGGTGGAAAAGCTACATCGAGTACC
GGACCAATTATTTGGGAGATACGCCCCAGATTTTTGTTTTCAAGTGCAGAAATCTCAGTATC
AGA----------G--TCAGTTATTCTC-TATTAAAATCTATCCGTTTTTTTTTTTTTTTCTAAAATTT-
GGAAATCTGGAAAAAAAATGTTCGA??
??
??
??
??TTTTTTTTATGATTCTAGACAATACTATCTATATTA------
TCTATGTAATTCGAACTTTCACTTTATTTCCGAGTCCGTTTTTCTATGTCATTGGCCCTTATT
TCATATTTCATCAGCATACGATATTATACCTAGCATATTTTTTTTACCGA---TTTTTCTTTT-
CGT

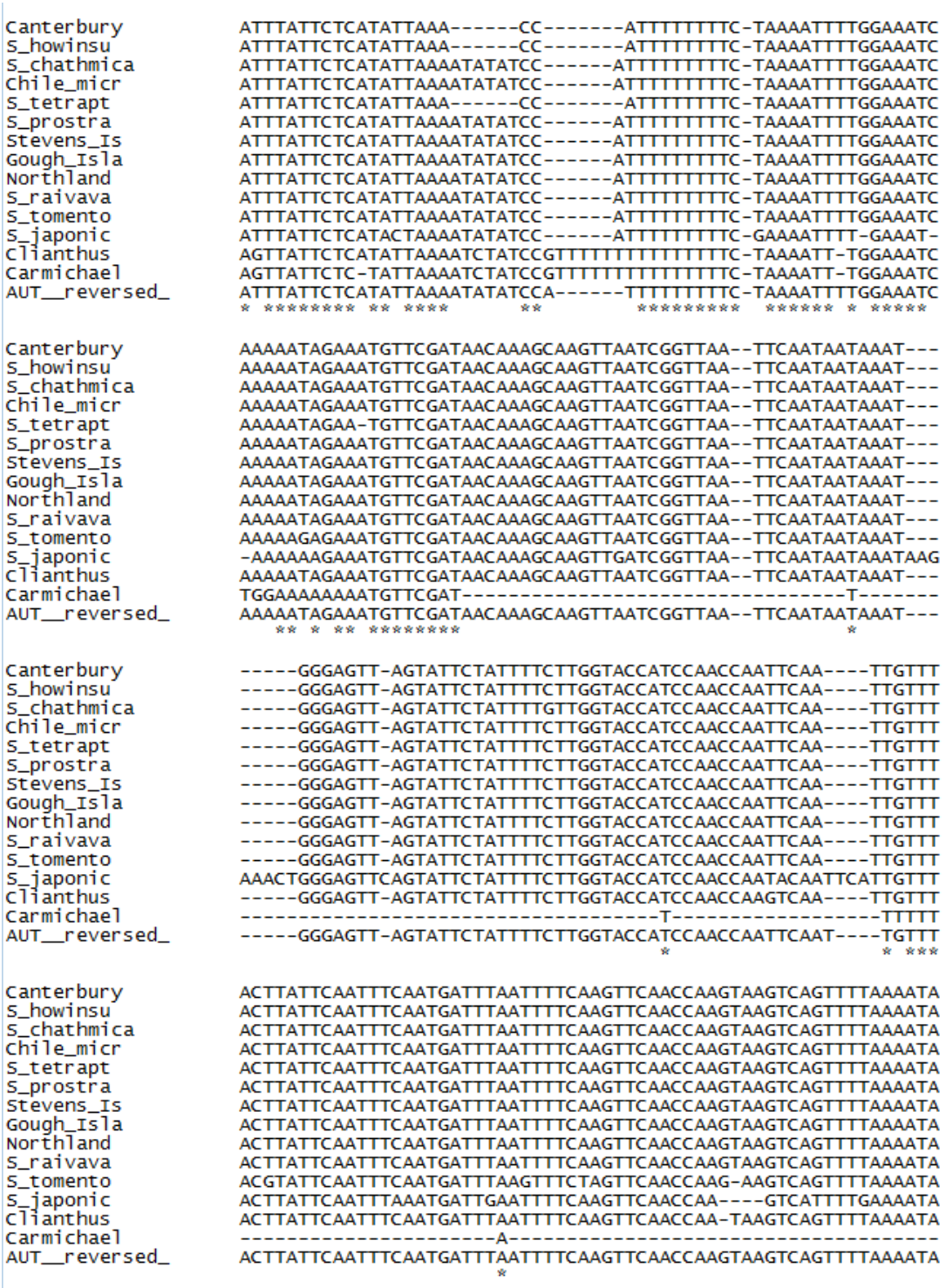
Appendix D. Aligned sequence data

```
Canterbury          ----------------------------------------------------------AA
S_howinsu           ----------------------------------------------------------AA
S_chathmica         ----------------------------------------------------------AA
Chile_micr          ----------------------------------------------------------AA
S_tetrapt           ----------------------------------------------------------AA
S_prostra           ----------------------------------------------------------AA
Stevens_Is          ----------------------------------------------------------AA
Gough_Isla          ----------------------------------------------------------AA
Northland           ----------------------------------------------------------A
S_raivava           -----------------------------------------------------------
S_tomento           -----------------------------------------------------------
S_japonic           -----------------------------------------------------------
Clianthus           -----------------------------------------------------------
Carmichael          -----------------------------------------------------------
AUT__reversed_      TCACTTCAAAAGCTACAGCTCTAAATCGATTATTTCCTAATAATTGGCTGTACTTCACAA


Canterbury          GTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-AACTACTAGAGCATTGTAAAT
S_howinsu           GTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-AACTACTAGAGCATTGTAAAT
S_chathmica         GTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-AACTACTAGAGCATTGTAAAT
Chile_micr          GTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-AACTACTAGAGCATTGTAAAT
S_tetrapt           GTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-AACTACTAGAGCATTGTAAAT
S_prostra           GTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-AACTACTAGAGCATTGTAAAT
Stevens_Is          GTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-AACTACTAGAGCATTGTAAAT
Gough_Isla          GTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-AACTACTAGAGCATTGTAAAT
Northland           GTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-AACTACTAGAGCATTGTAAAT
S_raivava           GTAACGTTAATTTGTTGACCAATAGTATCTTGGCCCTT-AACTACTAGAGCATTGTAAAT
S_tomento           -TAACGTTAATTTGTTGACCAATAGTATCTTGACCCTT-AACTACCAGAGCATTGTAAAT
S_japonic           ----------------GACCAACAGTATCTCGACCATT-AACTACCAGAGCGTTGTAAAT
Clianthus           ------------------CAACAGGATCTAGACCTTTC-ACTACCAGAGCGTTGTAAAT
Carmichael          ------------------CAACAGGATCTAGACCTTTC-ACTACCAGAGCATTGTAAAT
AUT__reversed_      GTAACGTTAATTTGTTGAACAAAAGTATCTTGGCCCTT-AACTACTAGAGCATTGTAAAT
                                    *** ** **** * ** **  ***** ***** ********

Canterbury          ATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCGGACCAATTATTTGAGCGAT
S_howinsu           ATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCGGACCAATTATTTGAGCGAT
S_chathmica         ATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCGGACCAATTATTTGAGCGAT
Chile_micr          ATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCGGACCAATTATTTGAGCGAT
S_tetrapt           ATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCGGACCAATTATTTGAGCGAT
S_prostra           ATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCGGACCAATTATTTGAGCGAT
Stevens_Is          ATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCGGACCAATTATTTGAGCGAT
Gough_Isla          ATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCGGACCAATTATTTGAGCGAT
Northland           ATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCGGACCAATTATTTGAGCGAT
S_raivava           ATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCGGACCAATTATTTGAGCGAT
S_tomento           ATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCGGACCAATTATTTGAGCGAT
S_japonic           ATTGGGCATCTTCCCTGGGGGAAAAGCTACATCCAGTACCGGACCAATTATTTGAGCGAT
Clianthus           ATTAGGCATCTTCCCAGGTGGAAAAGCTACATCGAGTACCGGACCAATTATTTGGGAGAT
Carmichael          ATTAGGCATCTTCCCTGGTGGAAAAGCTACATCGAGTACCGGACCAATTATTTGGGAGAT
AUT__reversed_      ATTAGGCATCTTCCCTGGAGGAAAAGCTACATCCAGTACCCGACCAATTATTTGAGCGAT
                    *** ************ ** *************** ****** ************ * ***

Canterbury          ACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC------CAGAAGTGGTAGG
S_howinsu           ACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC------CAGAAGTGGTAGG
S_chathmica         ACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC------CAGAAGTGGTAGG
Chile_micr          ACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC------CAGAAGTGGTAGG
S_tetrapt           ACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC------CAGAAGTGGTAGG
S_prostra           ACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC------CAGAAGTGGTAGG
Stevens_Is          ACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC------CAGAAGTGGTAGG
Gough_Isla          ACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC------CAGAAGTGGTAGG
Northland           ACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC------CAGAAGTGGTAGG
S_raivava           ACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC------CAGAAGTGGTAGG
S_tomento           ACGTCCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGTACCAGAACCAGAAGTGGTAGG
S_japonic           ACGTCCCAGGTTTTTTTTTTCAAGCGCAGAAACCTCAGGAC------CAGAAGTGGTAGG
Clianthus           ACGCCCCAGATTTTTGTTTTCAAGTGCAGAAATCTCAGTAT------CAGA----GTC--
Carmichael          ACGCCCCAGATTTTTGTTTTCAAGTGCAGAAATCTCAGTAT------CAGA----GTC--
AUT__reversed_      ACGTTCCTGGTTTTTTTTTTCAAGCGCAGAAACTTGGGGAC------CAGAAGTGGTAGG
                    ***  ** * ***** ******** *******  *  * *       ****    **
```


Canterbury ATTTATTCTCATATTAAA------CC-------ATTTTTTTTC-TAAAATTTTGGAAATC
S_howinsu ATTTATTCTCATATTAAA------CC-------ATTTTTTTTC-TAAAATTTTGGAAATC
S_chathmica ATTTATTCTCATATTAAAATATATCC------ATTTTTTTTC-TAAAATTTTGGAAATC
Chile_micr ATTTATTCTCATATTAAAATATATCC------ATTTTTTTTC-TAAAATTTTGGAAATC
S_tetrapt ATTTATTCTCATATTAAA------CC-------ATTTTTTTTC-TAAAATTTTGGAAATC
S_prostra ATTTATTCTCATATTAAAATATATCC------ATTTTTTTTC-TAAAATTTTGGAAATC
Stevens_Is ATTTATTCTCATATTAAAATATATCC------ATTTTTTTTC-TAAAATTTTGGAAATC
Gough_Isla ATTTATTCTCATATTAAAATATATCC------ATTTTTTTTC-TAAAATTTTGGAAATC
Northland ATTTATTCTCATATTAAAATATATCC------ATTTTTTTTC-TAAAATTTTGGAAATC
S_raivava ATTTATTCTCATATTAAAATATATCC------ATTTTTTTTC-TAAAATTTTGGAAATC
S_tomento ATTTATTCTCATATTAAAATATATCC------ATTTTTTTTC-TAAAATTTTGGAAATC
S_japonic ATTTATTCTCATACTAAAATATATCC------ATTTTTTTTC-GAAAATTTT-GAAAT-
Clianthus AGTTATTCTCATATTAAAATCTATCCGTTTTTTTTTTTTTTC-TAAAATT-TGGAAATC
Carmichael AGTTATTCTC-TATTAAAATCTATCCGTTTTTTTTTTTTTTC-TAAAATT-TGGAAATC
AUT__reversed_ ATTTATTCTCATATTAAAATATATCCA------TTTTTTTTC-TAAAATTTTGGAAATC
* ******** ** **** ** ********* ****** * *****
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Stevens_Is AAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCGGTTAA--TTCAATAATAAAT---
Gough_Isla AAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCGGTTAA--TTCAATAATAAAT---
Northland AAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCGGTTAA--TTCAATAATAAAT---
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Clianthus AAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCGGTTAA--TTCAATAATAAAT---
Carmichael TGGAAAAAAAAATGTTCGAT--------------------------------T-------
AUT__reversed_ AAAAATAGAAATGTTCGATAACAAAGCAAGTTAATCGGTTAA--TTCAATAATAAAT---
** * ** ******** *
Canterbury -----GGGAGTT-AGTATTCTATTTTCTTGGTACCATCCAACCAATTCAA----TTGTTT
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Carmichael ------------------------------------T------------TTTTT
AUT__reversed_ -----GGGAGTT-AGTATTCTATTTTCTTGGTACCATCCAACCAATTCAAT----TGTTT
* * ***
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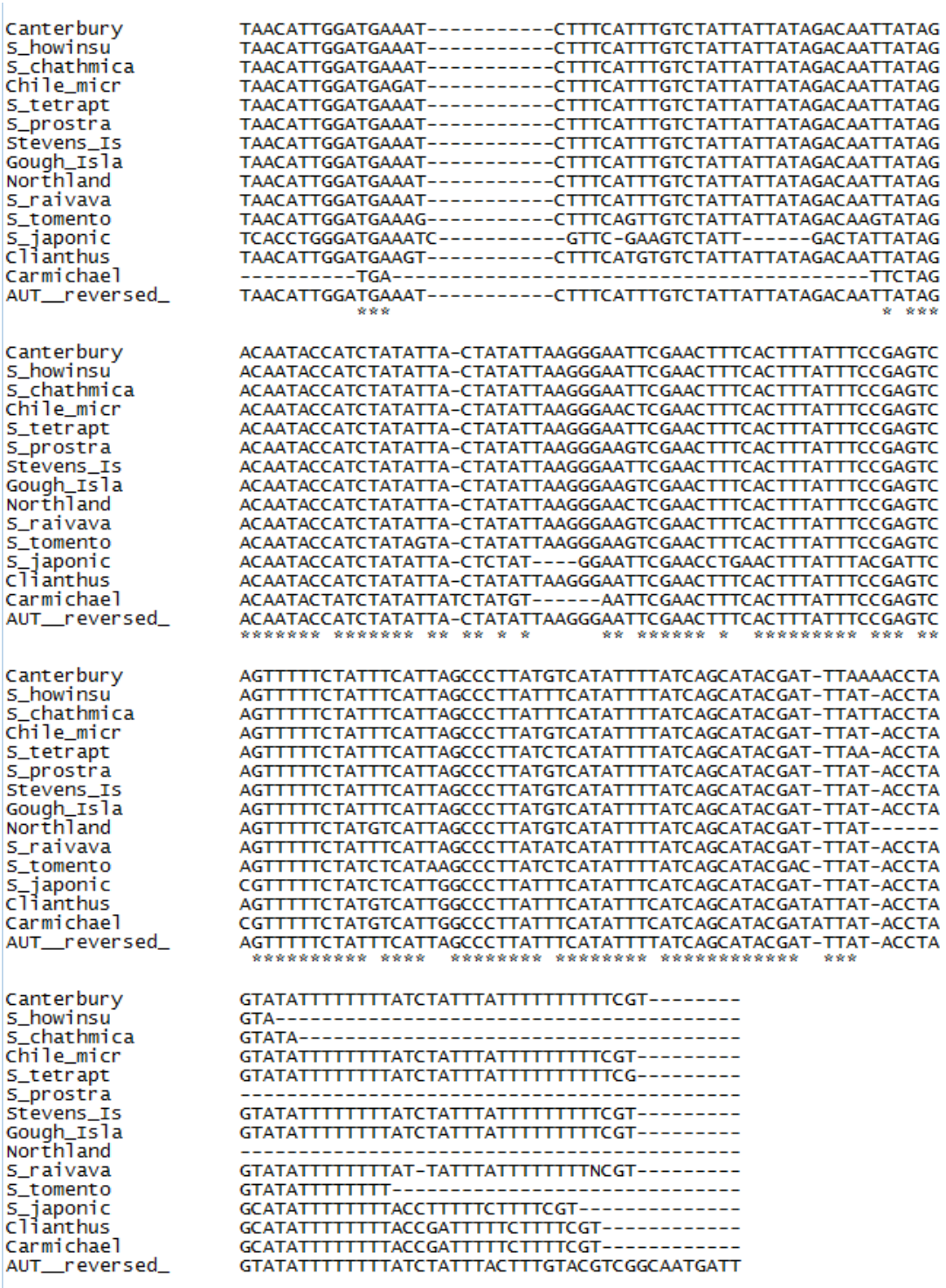
*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Carmichael ----------TGA----------TTCTAG
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Chile_micr AGTTTTTCTATTTCATTAGCCCTTATGTCATATTTTATCAGCATACGAT-TTAT-ACCTA
S_tetrapt AGTTTTTCTATTTCATTAGCCCTTATCTCATATTTTATCAGCATACGAT-TTAA-ACCTA
S_prostra AGTTTTTCTATTTCATTAGCCCTTATGTCATATTTTATCAGCATACGAT-TTAT-ACCTA
Stevens_Is AGTTTTTCTATTTCATTAGCCCTTATGTCATATTTTATCAGCATACGAT-TTAT-ACCTA
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Northland AGTTTTTCTATGTCATTAGCCCTTATGTCATATTTTATCAGCATACGAT-TTAT------
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S_tomento AGTTTTTCTATCTCATAAGCCCTTATCTCATATTTTATCAGCATACGAC-TTAT-ACCTA
S_japonic CGTTTTTCTATCTCATTGGCCCTTATTTCATATTTCATCAGCATACGAT-TTAT-ACCTA
Clianthus AGTTTTTCTATGTCATTGGCCCTTATTTCATATTTCATCAGCATACGATATTAT-ACCTA
Carmichael CGTTTTTCTATGTCATTGGCCCTTATTTCATATTTCATCAGCATACGATATTAT-ACCTA
AUT__reversed_ AGTTTTTCTATTTCATTAGCCCTTATTTCATATTTTATCAGCATACGAT-TTAT-ACCTA
Canterbury GTATATTTTTTTTATCTATTTATTTTTTTTTTCGT--------
S_howinsu GTA----------
S_chathmica GTATA----------
Chile_micr GTATATTTTTTTTATCTATTTATTTTTTTTTTCGT---------
S_tetrapt GTATATTTTTTTTATCTATTTATTTTTTTTTTCG---------
S_prostra ----------
Stevens_Is GTATATTTTTTTTATCTATTTATTTTTTTTTTCGT---------
Gough_Isla GTATATTTTTTTTATCTATTTATTTTTTTTTTCGT---------
Northland ----------
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S_tomento GTATATTTTTTTT----------
S_japonic GCATATTTTTTTTACCTTTTTCTTTTCGT--------------
Clianthus GCATATTTTTTTTACCGATTTTTCTTTTCGT------------
Carmichael GCATATTTTTTTTACCGATTTTTCTTTTCGT------------
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Appendix E. Standard curves

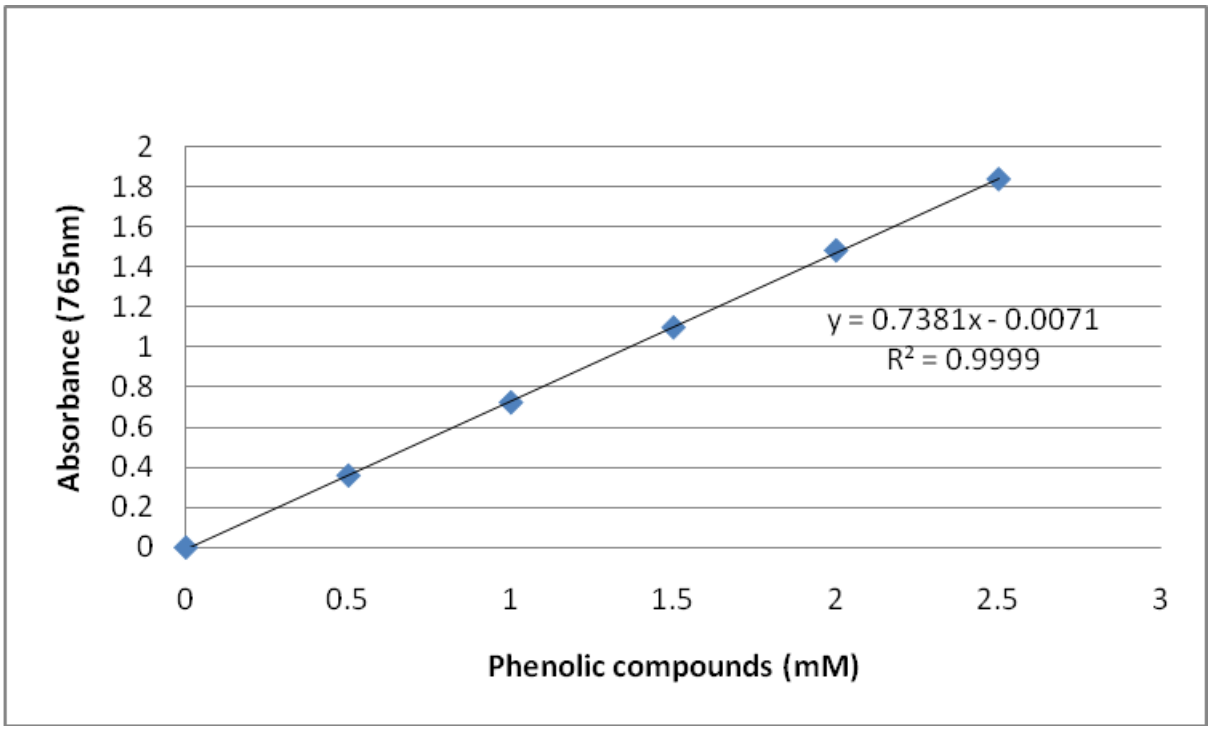

Figure 29. The standard curve of phenolic content (mM)

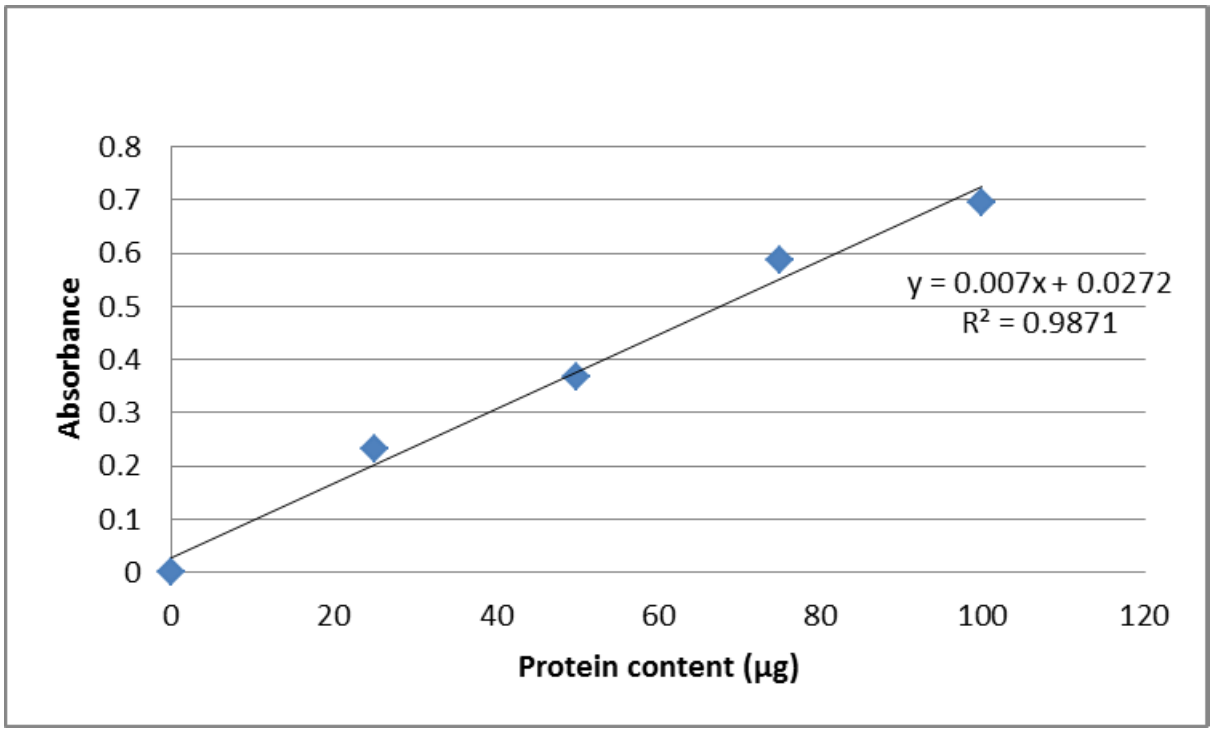

Figure 30. The standard curve of protein content (µg)

Appendix F. Method development

This appendix Section mainly discusses how the method used in this study was developed based on different trials to reach optimum performance and efficiency. In theory, to minimize the uncontrollable factors which are mentioned at Section 1.7., the kowhai sample should be cultivated instead of being collected from the trees which are naturally growing. However, the kowhai has a low growth rate, so sample collection was done on kowhai trees which are growing in nature. Cultivation method modified from Boller *et al.,* (1983) was shown at Appendix E.1. As mentioned before, the optimal pH of chitinase from different plants are variable (see Section 3.3.), Appendix E.2 was the method used to determinate the optimal pH of chitinase from kowhai.

In the original protocol, the sample was homogenized by Ultra-turrax (T 25 basic, IKA) to extract chitinase, lysozyme and acid phosphatase and by mortar and pestle to extract the phenolic compounds. Unfortunately, the Ultra-turrax generated a high amount of heat which can denature the enzymes and both Ultra-turrax and mortar and pestle were time consuming when dealing with a large amount of samples, so instead a biological ball mall method using commercial lysing tube was introduced to provide a faster method with less heat generation during extraction.

Many studies suggest that purified chitinase (refer back to Section 1.7.) can provide a much higher activity compared to the crude extract. Alternative methods based on concentrating the sample were tested for this purpose. Centricon tubes (Millipore) for concentrating the extract was introduced during method development. However, the crude centrifuged extract treated with PVP was still used in this study after method development in preference to the concentrate. Appendix F.3 discusses the concentration protocol and the result of chitinase activity comparison between concentrated extract and the crude extract.

F.1. Cultivation of kowhai

Kowhai seeds from the seed pod were sterilised by 2% hypochlorite for 15 minutes and washed with running tap water for 4 hours. The seeds were scarred on the surface and soaked in water for 2 days. Then the beans were transferred to the Perti dish and surrounded by a wet filter paper. All the beans were kept in the dark until they germinated. The germinated beans were transferred to a container and buried by moist Vermiculite under room temperature with natural sunlight and watered daily with tap water.

The germination process took at least two weeks and the first leaflets were growing after three weeks from the seeds transferred to Vermiculite.

F.2. Optimal pH of chitinase from kowhai

Citrate buffer for pH 4.0-5.5 was used in this measurement. All the procedures are the same as described as Section 3.5. except the working solution was replaced by the above buffer system. Since all the extractions were completed from one sample, protein conversion is not necessary. U was calculated by Equation 1.

One way ANOVA by Minitab (if $p<0.05$, reject H_0, not all means are equal) with Tukey test was used to determinate the optimal pH of kowhai chitinase with Ryan-Joiner test on residual was used for the normality test (if $p>0.1$, accept H_0, data is normal) and Levene's test for the equal variance (if $p>0.05$, accept H_0, the variance is equal).

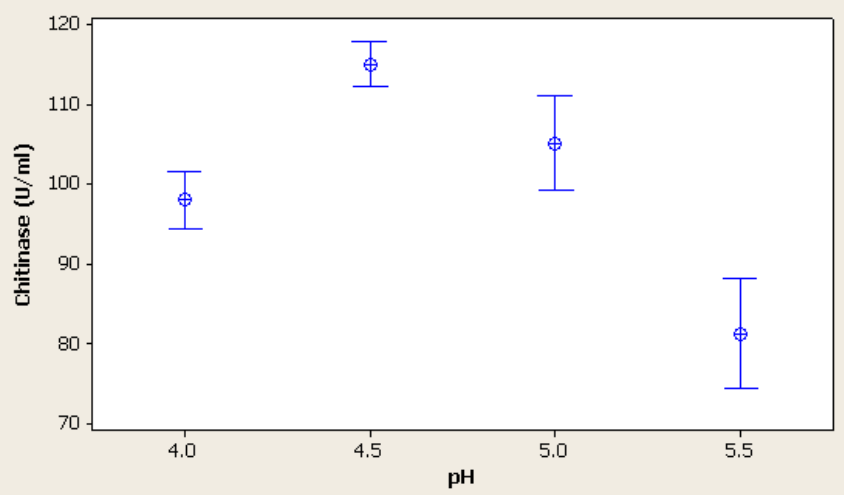

Figure 31. The interval plot of fluorescence intensity under pH 4.0-5.5

Table 18. The chitinase activity at different pH with one way ANOVA

pH	Chitinase (U/mg) Mean ± SD	95% CI for mean based on Pooled SD (2.04)
		-+---------+---------+---------+---------
4.0	98.02 ± 1.42	(--*--)
4.5	115.12 ± 1.16	(--*--)
5.0	105.19 ± 2.40	(--*--)
5.5	81.28 ± 2.74	(-*--)
		-+---------+---------+---------+---------
		80 90 100 110

n=3

Figure 31 and Table 18 are the result of the chitinase activities at pH 4.0-5.5. The one way ANOVA result showed that the activities were significant different (p=0.000), Tukey test was supported pH 4.5 is having the significant highest chitinase activity from kowhai. For this purpose, the chitinase assays (refer back to sction 3.5) were performed at pH 4.5. This is similar to the pH used by Boller et al., (1983) when assessing the chitinase activity of crude *Phaseolus* bean extracts.

F.3. Concentration of chitinase by Centricon

The crude extract was transferred to the Amicon Centricon (Millipore) molecular weight cut off filters and centrifuged (Eppendorf) at 3000g at 10ºC after the extraction step described in Section 3.3.3.1.). The chitnase assay was performed on both the concentrated extract and the crude extract by the same

method as in Section 3.5. and compared to the crude extract which was prepared as same as Section 3.3.3.1..

The two sample t test by Minitab (if $p<0.05$, reject H_0, means are not equal) with Ryan-Joiner test on residual was used for the normality test (if $p>0.1$, accept H_0, data is normal) and Levene's test for the equal variance (if $p>0.05$m accept H_0, the variance is equal).

Table 19. The result of the comparison of chitinase activity by concentrated and crude extract

Test	Crude extract (Mean±SD)	Concentrated extract (Mean± SD)	Estimate for different	95% CI for different
Chitinase (U/mL/µg)	4.1487 ± 0.01347	1.7926 ± 0.0744	2.36	2.1682, 2.5439

n=3

Table 19 are the result of chitinase assay based on both crude extract and concentrated extract. The chitinase activity from the crude extract was significant higher (2.36 U/mL/µg) than the chitinase activity from the concentrated extract. Hence, the crude extract was decided to use in this study since it has a higher performance in the chitinase assay.

Appendix G. Class I chitinase gene

Table 20. Results of a BLAST search of Kowhai Class I chitinase gene from forward primer

Accession	Organism	Max Score	Query coverage	E-value	Max ident
X63899	*Pisum sativum*	156	60%	9.00E-35	76%
AF335589	*Glycine max*	141	44%	2.00E-30	80%
AF202731	*Glycine max*	141	44%	2.00E-30	80%