# Induction of Peroxidase in Kauri (*Agathis australis*) by Hormone Treatment

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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."

Sign ……..

Date……...

# Acknowledgement

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# Abstract

Kauri dieback is a deadly kauri disease caused by a pathogen, *Phytophthora taxon Agathis* (PTA). PTA is an exotic pathogen in New Zealand and is capable of killing kauris of all ages. There is no known treatment or control tools available to manage this disease to date. Hormone induced plant resistance may give some protection to the plant against the pathogen. The objective of this research was to develop a hormone treatment option for the kauri dieback disease by monitoring the effectiveness of the dose response relationship of various plant hormones against the induction of the defensive protein, peroxidase in kauri.

Two new methods were developed for the protein extraction and enzyme assay with special reference to the peroxidase enzyme from the kauri leaf tissue. These were the first known methods of this kind specifically for the kauri peroxidase enzyme.

Optimization of the hormone doses, and the assessment of the time frame were carried out for the maximum quantisation of the target enzyme. Foliar application of different plant hormones were carried out on individual kauri trees under laboratory conditions, and changes in the activity levels of peroxidase enzyme were measured.

The trees were individually treated with different plant hormones of methyl jasmonate, ethephon, β-amino-butyric acid (BABA), salicylic acid and a combination of methyl jasmonate and ethephon and the responses of peroxidase enzyme were observed in three day intervals with a time span of 18 days. A blank treated control was also carried out in a similar way.

The individual hormone applications made an impact on the induction of the peroxidase activity in kauri trees, the increases in activity were *β*-amino-butyric acid (BABA) (84.31 %), salicylic acid (55.97%), methyl jasmonate (282.99%) and ethephon (73.62%). The synergistic combination of methyl jasmonate and ethephon showed the highest statistically significant increase in enzyme activity (379.21%) when compared to the enzyme activity level of the blank treated control plant.

In conclusion, as the foliar application of the synergistic combination of methyl jasmonate and ethephon has been shown to act as a potent inducer of the defensive protein peroxidase in kauri trees, it may be a potential treatment option for the kauri dieback disease. Further research is required to determine the effectiveness of the treatment option on the PTA infected kauri trees.

# Contents

# Abbreviations

Atmospheric pressure chemical ionization: APCI

Department of Conservation: DOC

Diphenylene iodonium: DPI

Ethephon: ET

Ethylene: ETY

Ethylenediaminetetraacetic acid: EDTA

Internal transcribed spacer: ITS

Liquid chromatography–mass spectrometry: LC-MS

Methyl jasmonate: MJ

Min: Minute

Pathogenesis-Related Proteins: PR proteins

Peroxidase enzyme: Prx

*Phytophthora taxon Agathis*: PTA

Polyvinylpolypyrrolidone: PVPP

Polyvinylpyrrolidone: PVP

Salicylic acid: SA

Sudden Oak Death: SOD

β-amino-butyric acid: BABA

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# Chapter 1. Introduction

## 1.0. Overview

Kauri dieback is a deadly kauri disease caused by the microscopic fungus-like plant pathogen *Phytophthora taxon Agathis* (PTA). It is a serious threat to the kauri population throughout Northland, Auckland and the Coromandel region. It is liable to kill trees and seedlings of all ages. The disease was first identified in the Great Barrier Island in 1972 and in 2008, PTA was declared an unwanted organism under the Biosecurity Act. The microscopic spores of the PTA infect the kauri roots and that causes the symptoms of yellowing, loss of leaves, dead branches and lesions that bleed gum at the base of the trunk and eventually the death of the entire plant (Mouldey, 2014).

In the natural environment, plants are susceptible to encounter a wide array of pathogenic microorganisms. Naturally the plants are able to produce a wide range of local and systemic resistance mechanisms towards the diverse array of the attacking microorganisms. Induction of the natural defence system by various biological agents can improve the plants resistance mechanism. At the same time it can provide a pre-pathogenic attack protection against a wide range of microorganisms.

Plant hormones play an important role in the regulation of the developmental processes and the signalling mechanism in plant responses to a wide range of biotic and abiotic stresses (Bari & Jones, 2009). Identification of the cognate or matching elicitors, either by the pathogenic attack or by the external application, leads to the activation of the defence response genes in plant species that ultimately result in the production of Pathogenesis-Related (PR) proteins (Ebrahim, Usha, & Singh, 2011). These inducible defence mechanisms are mainly controlled by three important plant hormones, salicylate (SA), jasmonate (JA),and ethylene (ET) (Tina, David De, Monica, Godelieve, & Kamrun, 2011). A non-protein amino acid, $\beta$-amino-butyric acid (BABA) was also observed to be very effective in terms of the production of PR Proteins in various plant species.

Plants produce a variety of defensive proteins and enzymes in response to attack by pathogens such as bacteria and fungi. The study of the pathogen related protein (PR Protein) or the defensive enzymes is a key to understanding the plant disease resistance mechanism. Because of the presence of the high number of isoenzymes and the versatility of the enzyme-catalysed reactions, plant peroxidases have an important role in a wide range of plant physiological processes throughout its entire life cycle (Passardi, Cosio, Penel, & Dunand, 2005).

Since hormone treatments in some plant species have been shown to offer protection against some *Phytophthora* species (Ali, Smith, & Guest, 2000; D Kone, 2009; E A G Altamiranda, 2008; Elisa Becker; Lyon, Reglinski, & Newton, 1995; Reglinski & Walters, 2009; Williams, Senaratna, Dixon, & Sivasithamparam, 2003), it is therefore possible that the treatment of kauri with plant hormones which induce defensive enzymes may offer some protection against PTA.

The aim of the current research is to observe the extent of induction of the defensive enzyme, peroxidase, as a result of various hormone treatments options with reference to the dose response as well as the time response relationship.

The kauri seedlings were treated individually with the following plant hormones, methyl jasmonate, ethephon, salicylic acid, $\beta$-amino-butyric acid (BABA), and also a combination of methyl jasmonate and ethephon. The changes in level of the peroxidase activity at different time spans were monitored and compared with the individual treatment options.

## 1.1. Kauri (*Agathis australis*)

Scientific Name: *Agathis australis*

Family: Araucariaceae

Genus: Agathis

Species: *australis* is the only species in New Zealand (*The Mighty Kauri Tree*, 2014)

**Fig 1.a. Kauri tree**

("Kauri Dieback School Resource," 2014)

Kauri is the one of the largest and longest living endemic trees in New Zealand. It is the only indigenous member of Araucariaceae family. Kauri belongs to the genus of Agathis, branch of Pinophyta division. The Pinophytes are gymnosperms, commonly called conifers, seed bearing

plants without flowers and pistils. The *Agathis* is believed to have evolved in the New Zealand – Australia region around 20 million years ago. It can attain a diameter of 1-7 m with average height of 30–40 m, and has a lifespan of up to 1700 – 2000 years (Ahmed & Ogden, 1987).

Kauri possess thick, leathery parallel vein leaves with no petioles. The leaves are usually observed to remain on the tree from 3-6 years (Ahmed & Ogden, 1987). The young leaves usually grow to a size of 5-10 x 0.5-1.2 cm, while the adult leaves are 2.0-3.5 cm in length. The colour of the kauri seedlings varies from olive green to bronze mixed red. Presence of the higher concentration of the red pigment rhodoxanthin and the high light intensity are the main reasons of the leaf reddening (Peterson, 1963).

The newly germinated kauri seedling possess a strong taproot and a fibrous lateral root system. A single taproot can reach to a depth of 2 m unless there is no obstruction in its way. The mature trees have a well-developed root system. The lateral roots of the mature plants can even reach beyond the width of the crown. Interestingly, no taproots are observed in the mature plants, instead numerous peg roots are observed with a length of 4 m and terminating in a network of smaller roots (Steward, 2004).

The kauri exerts a significant impact on the chemical and nutritional composition of the soil beneath the canopy as its litter shed consists of high level of phenolic compounds and tannins from its leaves, bark, cones and the gum (Enright & Ogden, 1987). It eventually leads to the formation of an organic layer, which can go to a 2 m depth beneath the mature specimens (Silvester & Orchard, 1999). This organic layer usually consists of very high levels of carbon and nitrogen and typically exhibits an acidic pH of 4 (Silvester & Orchard, 1999; Wyse & Burns, 2013). The low soil pH is a growth retarding factor for many plants by various mechanisms. The lower pH can increase the concentration of $H^+$, $Al^{3+}$, and $Mn^{2+}$ ions and thereby the toxicity of the soil. At the same time the soil acidity can decrease the concentration of $Mg^{2+}$ and $Ca^{2+}$ ions, that leads to the reduction in phosphorus and molybdenum solubility and ultimately leads to the plant nutritional deficiency (Marschner, 2011). The increased $H^+$ ion concentration itself can be a growth inhibiting factor. At the same time aluminium toxicity and phosphorus deficiency can dominate (Kidd & Proctor, 2001; Wyse & Burns, 2013). The kauri possess a very effective nutritional management system by a symbiotic association with a mycorrhizal fungi. The mycorrhizal fungi, a naturally occurring soil fungi, enhances the moisture and nutrient up take by the kauri trees. It makes a symbiotic association with the kauri

roots, especially the nodule-like roots and stimulates phosphate absorption (Morrison & English, 1967).

Kauri is a monoecious plant, carrying male and female cones in the same plant. The finger shaped male cones produce pollen and fertilise the round shaped female cone during the September – October period. Usually male cones are observed above the branch that carries the female cones, but rarely are both of them observed on the same branch. The round shaped female cones usually takes 2-3 years for maturation after fertilisation, and it turns green to brown in colour as they become mature (Wassilieff, 2013). The seeds become mature around the February – March period and are dispersed by wind and gravity (Steward, 2004).

### 1.1.1. Geographic distribution

Kauri is a warm temperate species and is restricted to the natural sub-tropical rain forests in areas north of latitude 38 ºS. The kauri's natural limit is found from north of a line between Kawhia and extents towards a southern point of Tauranga. The largest remaining stands of the mature kauri are found in the north Auckland region, especially in the Waipoua State Forest region (Ecroyd, 1982; Steward, 2004). The young kauris are most common in eastern Northland and on Great Barrier Island. They are also present on little Barrier Island. Waiheke, Kawau, Rangitoto, and Ponui Islands in the Hauraki Gulf, Poor Knights Island, Great Mercury Island , Motuoruhi Island , Hen (Tauranga) , and Coppermine Island (Ecroyd, 1982).

As a lowland species, kauri is common only from sea level to 300 m, and is usually absent above 450 m. But the kauri's are found above 520 m at Mt Hobson on Great Barrier Island and are found to be consistently growing above 700 m in several parts of Coromandel Range (Ecroyd, 1982).

The climate and the soil type are the major influential factors for the growth and development of the kauri population. The kauri usually grow in warm and humid conditions with an average range of rainfall of 1000 to 2500 mm and an annual temperature range of 13° to 16 °C. As discussed above, the kauri usually grow in a low fertility soil, and make it even less fertile as it grows. According to Clayton-Greene (1978), lack of suitable soils and terrain is the key factor that prevents the extension of the kauri population further south rather than any climatic conditions (Clayton-Greene, 1978).


**Fig 1.b. Geographic distribution of kauri in New Zealand**

(Orwin, 2013)

### 1.1.2. Kauri and the Māori culture

The kauri forests always had cultural significance with the Māori population. As a native plant kauri is considered as a taonga species by many Māori - a spiritual connection to the life of the Māori ancestors. The kauri gum was called "kapia" by early Māori, and was used as chewing gum in older ages. The Tā moko or the permanent body tattoo is an inevitable part of Māori culture. The black and bluish pigments made by oil burned kauri gum was used as an essential ingredient for the tattoo ink (Steward, 2004). Moreover kauri is closely integrated in many other aspects to the early Māori culture, in creation mythology, rituals, war, art and everyday life (About Kauri, 2014).

During the early period of European settlement, the kauri forests were logged and cleared for various purposes. The kauri timber had a very high economic value as it was one of the main export items. They used the kauri timber for building houses, ships, fences, railway sleepers, dams, furniture etc. The kauri gum was widely used as a base for hard varnishes, paints and for making Linoleum. At present, only less than one percent of the total kauri forest remains, largely protected and remains as a part of our national identity ("Kauri Dieback School Resource," 2014).

## 1.2. *Phytophthora*

*Phytophthora* belongs to a class of plant - damaging oomycetes and it has received considerable attention because of its capability to pose significant threats to the natural ecosystem and the agricultural industry. The name *Phytophthora* came from the ancient Greek, meaning plant destroyer. The genus was first described by a German mycologist Heinrich Anton de Bary in 1875 (Lamour, 2013). According to Brasier (2008), there are more than 600 potential *Phytophthora* species that are well known for their role in creating various diseases in fruit, vegetable, agricultural and tree crops (Brasier, 2008).

Many species of *Phytophthora* are plant pathogens, having considerable economic importance. With its wide host range, polycyclic nature and long term survival in the soil this results in enormous economic losses to the worldwide agricultural industry, as well as the natural eco system. During the mid-19th century the potato blight fungus, *Phytophthora infestans*, caused a major threat for the European potato industry. Recently, the *Phytophthora cinnamomi* root rot resulted in the entire dieback of the eucalypt forest ecosystem in Australia. The continuing black pod disease by *P. palmivora* and *P. megakarya* is major threat for the cocoa industry in the tropics (Brasier, 1992). The Sudden Oak Death (SOD) disease caused by *Phytophthora ramorum* resulted in devastating effects on the oak populations in California and the Oregon region during 1995 (Sudden Oak Death, 2010).

The genus *Phytophthora* comes under the family of Pythiaceae and the class of oomycetes, in the kingdom of Chromista (Hawksworth D.L, 1995). The taxonomic classification of oomycetes is given below,

| Kingdom | Class | Order | Family | Genus |
|---|---|---|---|---|
| Chromista | Oomycetes | Lagenidiales | | |
| | | Leptomitales | | |
| | | Saprolegniales | Saprolegniaceae | *Achlya*<br>*Saprolegnia* |
| | | Peronosporales | Pythiaceae | *Pythium*<br>***Phytophthora*** |
| | | | Peronosporaceae | *Bremia*<br>*Peronospora* |
| | | | Albuginaceae | *Albugo* |

**Table 1.I. Taxonomic classification of oomycetes** (Hawksworth D.L, 1995)

Oomycetes are fungus like eukaryotic microorganisms sharing certain biological characteristics with the true fungi. Because of these reasons the oomycetes have been considered as fungi for a long time. As in fungi, the oomycetes obtain their nutrients by absorption and many of them produce a mycelium, a characteristic filamentaneous thread-like structure commonly seen in fungi (Rossman & Palm, 2006). Even though the oomycetes possess certain similarities with fungi they are distinctly classified as a separate class under the kingdom of Chromista based on the following unique biochemical and morphological characteristics.

| **Character** | **Oomycetes** | **True Fungi** |
|---|---|---|
| Sexual reproduction | Heterogametangia. Fertilization of oospheres by nuclei from antheridia forming oospores. | Oospores not produced; sexual reproduction results in zygospores, ascospores or basidiospores |
| Nuclear state of vegetative mycelium | Diploid | Haploid or dikaryotic |
| Cell wall composition | Beta glucans, cellulose | Chitin. Cellulose rarely present |
| Type of flagella on zoospores, if produced | Heterokont, of two types, one whiplash, directed posteriorly, the other fibrous, ciliated, directed anteriorly | If flagellum produced, usually of only one type: posterior, whiplash |
| Mitochondria | With tubular cristae | With flattened cristae |

**Table 1.II. Major biochemical and morphological distinctions between oomycetes and true fungi** (Rossman & Palm, 2006)

Most oomycetes, especially the *Phytophthora*, exhibits a remarkable characteristics to adapt chemical and genetic resistance by the development of new resistant strains. The metalaxyl resistant strain of *Phytophthora infestans* is a classic example of the chemical and genetic resistance strain of *Phytophthora*, which resulted in the potato late blight epidemics in the United States (*Oomycetes*, 2014).

The oomycetes can propagate either sexually or asexually. The most important feature of the sexual spore is, it can survive in the soil for a long time without the help of the host plant. The

sexual reproduction allows the pathogen to easily adapt to more adverse conditions (Govers, 2001).

The mycelium of the oomycetes produces branched sporangiophores containing asexual sporangia. The sporangium releases the motile zoospores. The taxonomy and the pathogenicity of the oomycetes mainly depend on the characteristics of the motile zoospores (Islam, 2005). The zoospores are attracted towards the host plant by chemotactic stimuli by a wide range of chemicals including vitamins, phenolic compounds, nitrogen bases of nucleic acids, sugars and amino acids (Khew & Zentmyer, 1973; Tyler, 2002). Once it contacts with the host tissue the zoospores become immotile and encyst followed by the development of an appressorium, a typical intermediate cell structure formed during the infectious stage. The appressorium results in the formation of an infection vesicle in the host plant (Grenville-Briggs & van West, 2005). The mycelium grows from the infection vesicle in between the host cells, which in turn results in the death of the host tissue because of the nutrient acquisition by the infected oomycetes for its growth, development and reproduction. The cycle repeats with a new and healthy host tissue (Hardham, 2007).

The sexual reproduction of the oomycetes takes place when the male reproductive organ, the antheridium, fertilizes the female reproductive organ, the oogonium. After fertilization the oogonium matures to an oospore. The oospore gradually matures and develops the sporangium and repeats the cycle. The oospores can survive outside the host body for 3-4 years (Fugelstad, 2008).

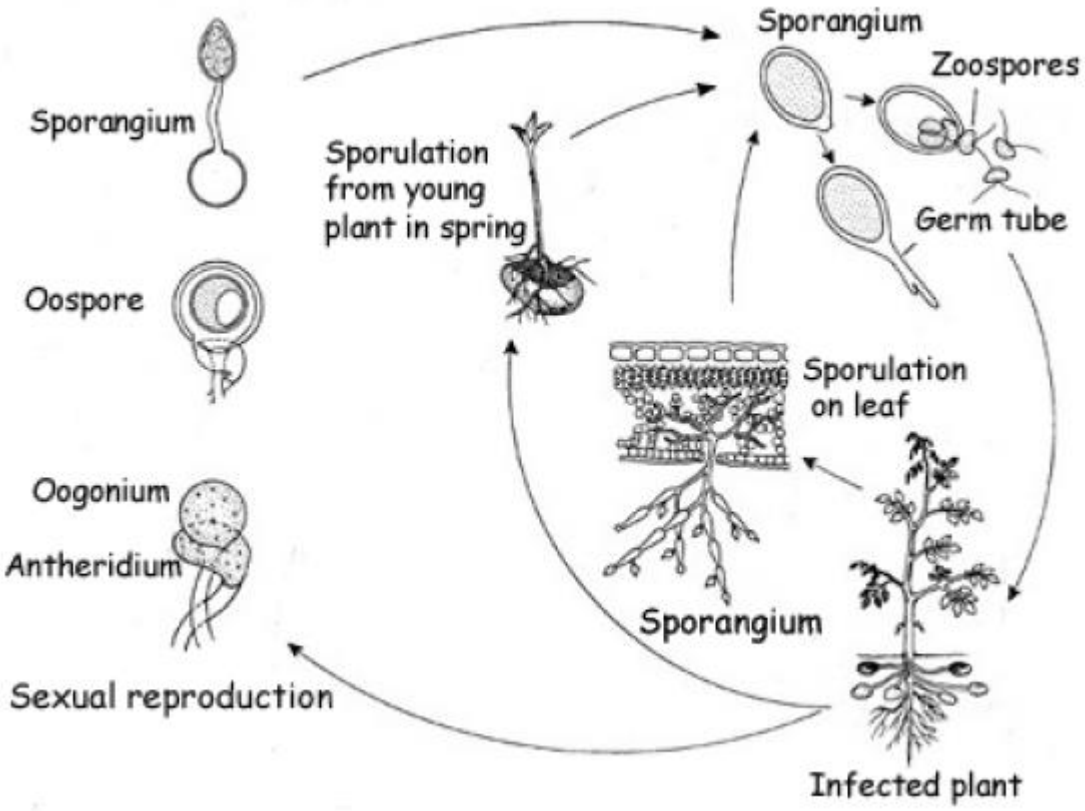



**Fig 1.c. Sexual and asexual reproduction cycle of oomycetes**

(Fugelstad, 2008; Schumann, 1991)

### 1.2.1. *Phytophthora Taxon Agathis*

*Phytophthora Taxon Agathis* is a soil and water borne oomycete that only affects the lower risk/conservation dependent kauri. The main symptoms of the disease include foliage yellowing, canopy thinning, and development of lesions on the lower trunk and roots and that ultimately leads to the tree death. The recent studies in this field suggests that the PTA is a distinct and previously misidentified *Phytophthora* species (*Phytophthora taxon Agathis* (PTA)).

PTA was originally misidentified as the morphologically similar *P. heveae* by Gadgil in 1974 (Gadgil, 1974; Stanley E. Bellgard, 2011). It was first found in New Zealand at the Great Barrier State forest in early 1972 (Gadgil, 1974). The isolations from the basal cankers and the soil samples from the vicinity of the affected trees consistently yielded the presence of the genus *Phytophthora*. This was confirmed as *Phytophthora heveae* by the Commonwealth Mycological Institute (Gadgil, 2009). The pathogenicity test conducted by using both wound

and soil inoculation methods revealed that the pathogen is highly capable of killing the kauri seedlings (Gadgil, 1974).

As the *P. heveae* was also isolated in the soil samples where healthy kauri was growing, Gadgil made a conclusion that the fungi is only pathogenic at specific combinations of environmental conditions. As most of the affected trees recovered in the following summer, Gadgil observed that the wet soil conditions also favoured the pathogenic activity (Gadgil, 1974)..

Following an enquiry from the public about unusual kauri deaths in the Waitakere Ranges in 2006, investigations on the pathogen revived a special interest by scientists from Landcare Research. The symptoms and the findings of their recent investigations were very similar to those of Gadgil (1974). The morphology of the isolates from the Waitakere Ranges were closely identical to the Great Barrier island isolates. The taxonomic analysis at the DNA level revealed that all the isolates had an identical internal transcribed spacer (ITS) which was distinct from *P. heveae* in the ITS clade 5 of Cooke and others (2000) (Beever, Waipara, Ramsfield, Dick, & Horner, 2009; Cooke, Drenth, Duncan, Wagels, & Brasier, 2000).

*Phytophthora katsurae* is a Japanese chestnut pathogen, which has subsequently been identified in natural forest soil in Taiwan (Ko & Chang, 1979). The closest PTA ITS sequence observed to match the ITS sequence of *P. katsurae* was soil isolates from Taiwan (ICMP16915) (Beever et al., 2009). Even though the PTA possess the identical ITS sequence with the *P. katsurae*, the PTA was not classified under the same species based on its distinct oogonial ornamentation as well as the differences in cardinal temperatures (Beever et al., 2009).

Because of the ITS similarities with the foreign isolates, the PTA is considered as an exotic species. There are no isolates reported from anywhere in the world with both similar spore orientation and the same ITS sequencing as PTA (Beever et al., 2009).

**Fig 1.d. PTA infected kauri tree**

(Wairepo, 2013)

### 1.2.2. Occurrence

Based on the symptomatology, occurrence in the soil samples, rate of spread at distinct localities, the *Phytophthora Taxon Agathis* is considered as a soil borne species and is spread mainly through water and soil movements (Beever et al., 2009).

The presence of PTA has been confirmed in Huia and Maungaroa Ridge in the Waitakere Ranges Regional Park and at Department of Conservation reserves at Great Barrier and Trounson kauri park in Northland (Kauri die back - The facts on phytophthora taxon agathis (PTA), 2012).

The kauri dieback has also been confirmed at cascade kauri in the Waitakere ranges and other sites in Rodney, Franklin, Waitakere and the North Shore, as well as Pakiri, Albany, Okura and the Waipoua Forest (Waipara et al., 2013).

In October 2008 the PTA was classified as an unwanted organism under the biosecurity act, and a national response and management programme was initiated by Ministry for Primary Industries, Tangata Whenua, Department of Conservation, and regional councils within the natural range of kauri (Waipara et al., 2013).

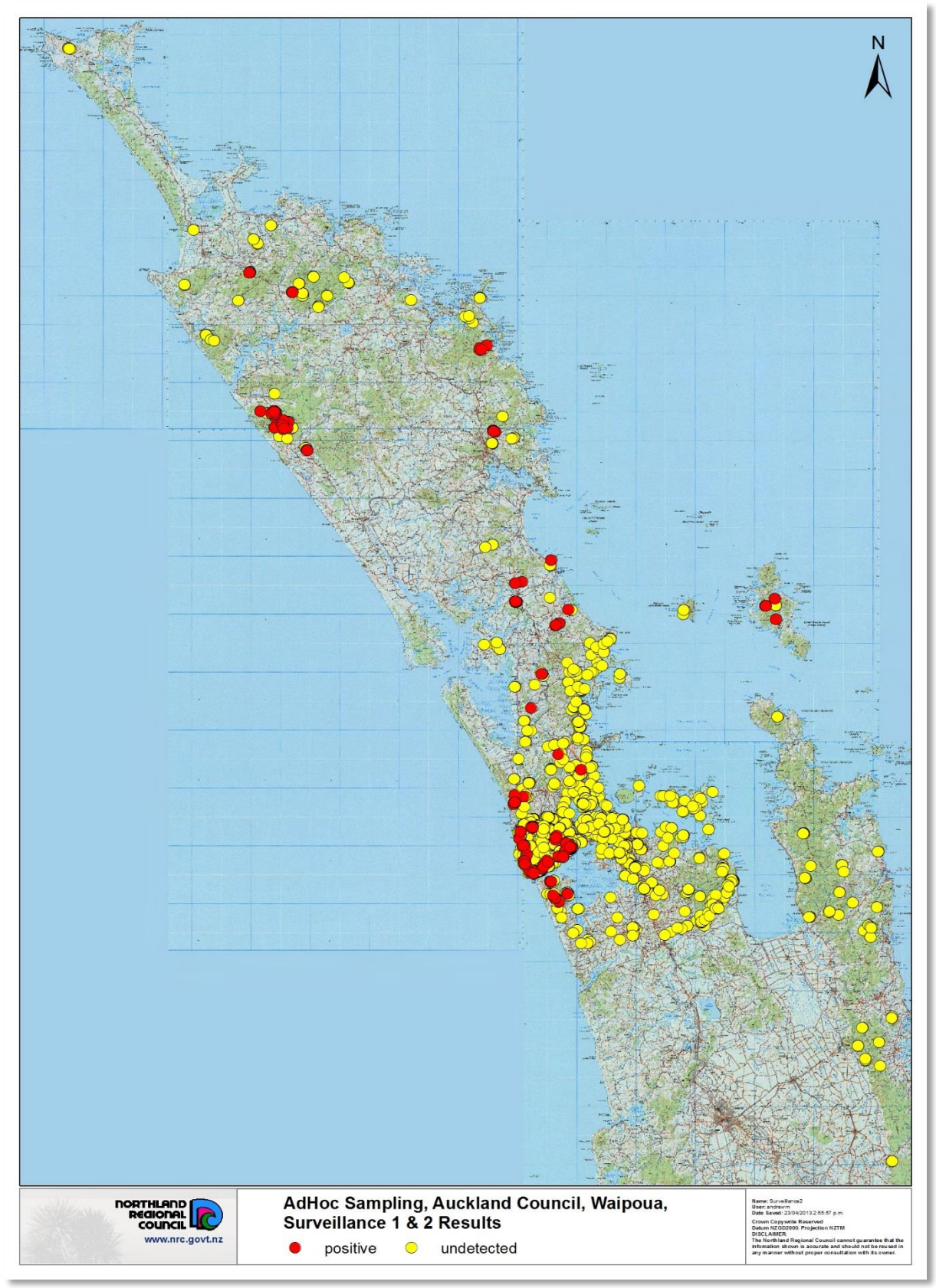



**Fig 1.e. Location map of kauri dieback surveillance**

*(Location map from all survelliance to date, 2014*)

## 1.3. PR Proteins

Pathogenesis Related (PR) proteins is the collective term for all the plant proteins induced by pathogens and their homologues derivatives. The PR proteins such as phenylalanine ammonia-lyase, peroxidases, and the polyphenoloxidases are constitutively occurring protein subunits and usually increase during pathogenic attack. Based on the biochemical and molecular biological properties, the pathogenesis-related (PR) proteins and their homologues are broadly classified into 17 sub families (Van Loon, Rep, & Pieterse, 2006). Most of the PR proteins are induced due to the action of the endogenous growth hormones such as salicylic acid , jasmonic acid and ethylene (Durrant & Dong, 2004).

Peroxidases are a well-known class of the pathogenesis-related (PR) proteins and belong to the PR protein 9 subfamily (Van Loon et al., 2006). The presence of the multiple potential stress responsive *cis* elements in the 5' flanking regions of the peroxidase gene is the basis of the biotic and abiotic gene expression due to the pathogenic attack (Sasaki et al., 2007).

## 1.4. Peroxidases

### 1.4.1. Structure and chemistry of peroxidases

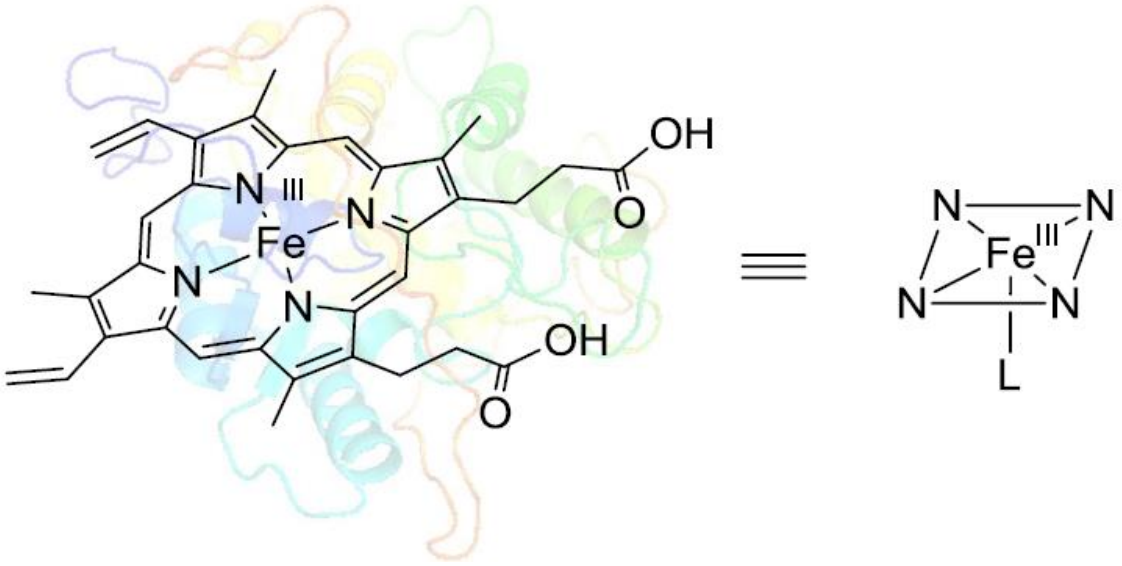



**Fig. 1.f. Resting state of heme peroxidases**

(Hollmann & Arends, 2012)

Peroxidases are an assorted group of oxidative enzymes occurring in plants, animals and microbes. Peroxidases consists of a protoporphyrin IX as a prosthetic group with a low-spin FeIII at its resting state. In most of the cases the L constitutes a histidine ligand (Hollmann &

Arends, 2012). Out of five coordinated positions of the iron ion, four are coordinated to the pyrrole nitrogens of the haem and to nitrogen from an axial histidine. The sixth free coordination position determines the high spin state for the iron (Banci, 1997). The prosthetic group of plant peroxidases usually consists of 10 - 12 conserved $\alpha$ helices, 2 short $\beta$-strands, and four conserved disulphide bridges (Barceló, Ros, & Carrasco).

Peroxidases have a high affinity toward compounds containing a hydroxyl group or groups attached to an aromatic ring. Peroxidases catalyse dehydrogenative oxidations or hydrogen abstraction reactions of compounds containing hydroxyl groups attached to an aromatic ring. This generates the corresponding phenoxy radicals. Subsequent coupling of these unstable radicals will initiate further non enzymatic polymerization of monomers (Hiraga, Sasaki, Ito, Ohashi, & Matsui, 2001).

### 1.4.2. Mechanism of action of peroxidase-catalyzed radical formation reactions

As shown in fig. 1.g., during the catalytic mechanism, an intermediate peroxo complex generates as a result of the substitution of the water ligand by $H_2O_2$ or other organic hydroperoxides. A following heterolytic cleavage of the O - O bond results in the formation of an intermediate compound I. The intermediate compound I further undergoes two individual hydrogen abstractions from the reducing substrates (In-H) leads to the formation of the two radical species (In·), and also the compound II returns to the resting state. The resulting radical species initiates the polymerization reactions (Hollmann & Arends, 2012).

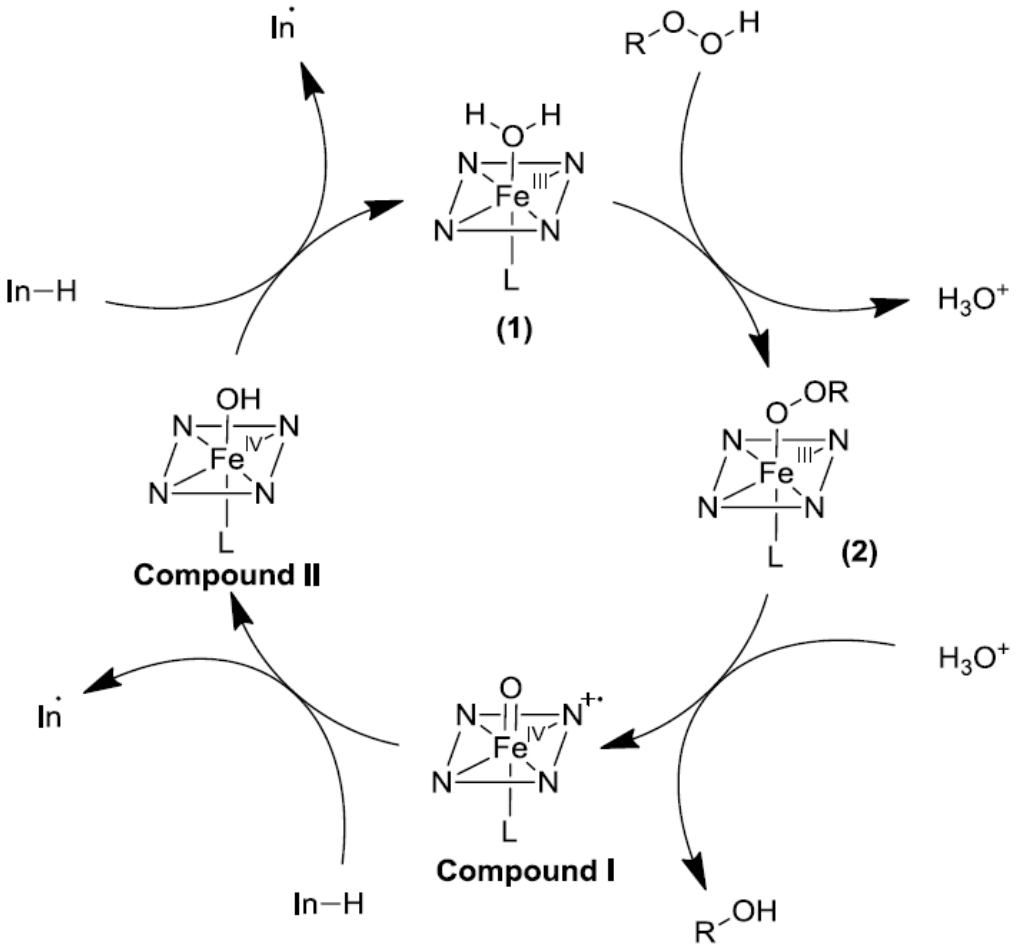



**Fig. 1.g. Mechanism of action of peroxidase-catalyzed radical formation reactions**

(Hollmann & Arends, 2012)

### 1.4.3. Action of peroxidases in the plant defensive mechanism

Because of the presence of the high number of isoenzymes and the versatility of the enzyme-catalysed reactions, plant peroxidases have an important role in a wide range of plant physiological processes throughout its entire life cycle (Passardi et al., 2005).

The involvement of peroxidases as part of the plant defence mechanism can be considered as follows:

1. Reinforcement of cell wall physical barriers
    a. Lignin
    b. Suberin
    c. Feruloylated polysaccharides
2. Metabolism of reactive oxygen species (ROS) (Almagro et al., 2009)

### 1.4.3.1. Reinforcement of cell wall physical barriers

In response to different stimuli, such as pathogen invasion, wounding or by means of the artificial activation of the signalling pathways, peroxidases create a physical barrier against the invading pathogen by the activation of cross linking of cell wall components by the $H_2O_2$ dependant cross linking pathway of cell wall components (Almagro et al., 2009).

### 1.4.3.1.a. Participation in the lignification process

Lignin is a complex polymer of aromatic alcohols - the monolignols. It is an essential secondary cell wall component of the plant cells. Lignin provides a physical barrier against the pathogenic attack and their colonization in the host plants (Buendgen, Coors, Grombacher, & Russell, 1990).

### 1.4.3.1.a.1. Mechanism of action of peroxidases in lignification process - Radical dehydrogenation of monolignols

Peroxidase plays a vital role in lignin biosynthesis. It activates the oxidation of phenol and phenolic derivatives by $H_2O_2$, which are mainly responsible for the dehydrogenation of the phenylpropane units – the monolignols. The electron transfer during the peroxidase catalysed dehydrogenation process yields a reactive monolignol species with free radicals. The monolignols will undergo the free radical coupling that leads to the polymerization process, especially the sinapyl, coniferyl and *p*-coumaryl alcohol to lignin during the lignin biosynthesis. The coniferyl, sinapyl and *p*-coumaryl alcohol will give rise to respective guaiacyl (G), syringyl (S) and the *p*-hydroxyphenyl (H) subunits of the Lignin biopolymer (Ales Lebeda 1999; EvaMiedes, August 2014).

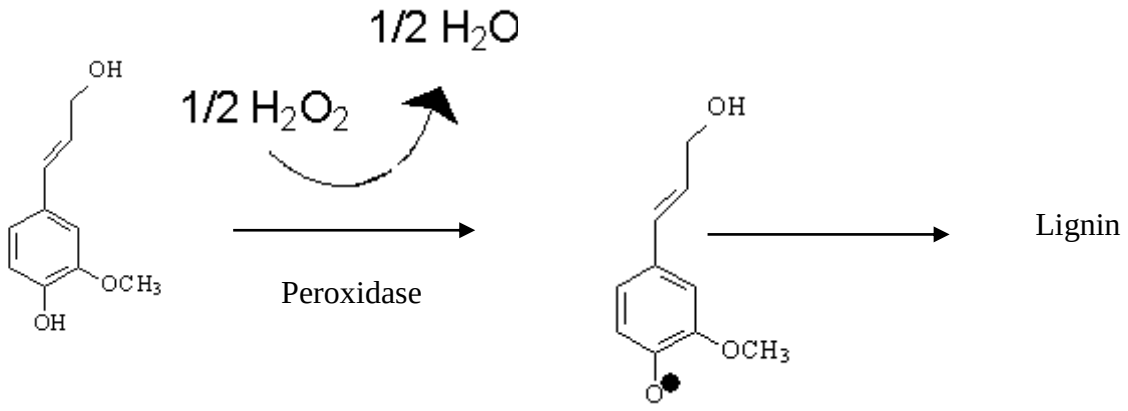



**Fig 1.h. Peroxide catalysed polymerization of coniferyl alcohol to lignin**

### 1.4.3.1. b. The role of peroxidase in suberization process

Research in biosynthesis of suberin suggests that the suberization by the polymerization of its aromatic monomers is analogous to that of lignin biosynthesis (Gross, 1977). The exact chemistry of the suberin monomers and the direct involvement of peroxidases in the suberization process is still under investigation.

According to Bernards et al, (2004) during suberization the cells develop a distinct polyaliphatic layer as a polyester connected through the primary ester bonds between the major aliphatic components (Almagro et al., 2009; Bernards, Summerhurst, & Razem, 2004). The Diphenylene iodonium (DPI) - sensitive NADPH oxidase-like enzyme is considered as the primary source for the $H_2O_2$, that is necessary for the peroxidase mediated oxidation of phenolics during the Suberization process (Almagro et al., 2009; Bernards et al., 2004; Razem & Bernards, 2003).

The low yield of aromatic aldehydes on nitrobenzene oxidation by the highly or partially condensed *p*-coumaric and ferulic acids during the polymerization in potato parenchyma cells suggests the presence of peroxidases in the formation of the phenolic matrixes during the suberization process.(BLAND & LOGAN, 1965). The observations of Borchert 1974, suggests the presence of peroxidases specifically at the suberizing cells during wound healing process of the potato tissues, enhances the finding of the involvement of peroxidase in the Suberizing cell wall reinforcement process (Borchert, 1974).

### 1.4.3.1.c. Feruloylated Polysaccharides

In the presence of $H_2O_2$, peroxidases activate the oxidative coupling of feruloyl residues by the formation of a covalent bond, resulting in the corresponding dehydrodiferulate residues, which in turn form a cross link between the cell wall polysaccharides to which it is esterified (Almagro et al., 2009; Ralph et al., 2004).

The research results of Ralph et al., 1992, 1994 & 2004 reveals the presence of several other isomers of 5–5-coupled dehydrodimer during the saponification process (Ralph et al., 2004; Ralph, Helm, Quideau, & Hatfield, 1992; Ralph, Quideau, Grabber, & Hatfield, 1994). These peroxidase catalysed cross linked products of ferulate determines the cell wall assembly and its digestibility (Grabber, Hatfield, & Ralph, 1998).

### 1.4.3.2 Metabolism of reactive oxygen species (ROS)

The peroxidases play a key role in limiting the cellular spreading of infections by the generation of a highly toxic environment due to the massive production of radical species (Passardi et al., 2005).

The peroxidase mediated production of ROS is mainly located at the level of the cell wall matrix of the plant cells. The enzyme catalyses the initial formation of $O_2^{\bullet -}$ which then dismutates to $H_2O_2$ (Almagro et al., 2009).

# Chapter 2. Experimental

## 2.0. Hormone Selection

The inducible defence mechanism of plants can be classified in to structural and biochemical mechanisms according to their function (Ales Lebeda 1999). These inducible defence mechanisms are mainly controlled by three important plant hormones, salicylate (SA), jasmonate (JA),and ethylene (ET) (Tina et al., 2011). These signalling pathways can synergistically interact or influence each other through a complex set of network interactions (Koornneef & Pieterse, 2008). From previous studies it has been observed that ethylene (ET) can act as a potential modulator for either salicylate (SA) or jasmonate (JA) mediated signalling pathways in plant defence mechanisms (Adie, Chico, Rubio-Somoza, & Solano, 2007), which in turn results in the synthesis of the pathogenesis related (PR) proteins. Among the proteins induced, because of the presence of the high number of isoenzymes and the capability of its participation in a wide range of plant physiological processes, the enzyme peroxidases are well known for the plant defence mechanisms (Almagro et al., 2009).

Based on the above observations, the kauri trees were initially treated with a synergetic combination of methyl jasmonate and ethephon, in order to observe the induction of the pathogenesis related proteins, especially the peroxidases.

## 2.1. Plant Material

The kauri trees were purchased from a local native plant nursery. The trees were grown in the AUT research laboratory under semi-controlled conditions. The trees were 1 to 2 m in height.

## 2.2. Methyl jasmonate and ethephon application

0.23 g of methyl jasmonate (Sigma) was dissolved in 200 µL of 99 % of ethanol, 0.5g of tween 20 and 25 mL of water. The tween 20 is a nonbiologically active detergent which helps to solubilize the methyl jasmonate and also acts as a wetting agent. The solution was sprayed on the kauri tree with the help of a chromatographic reagent atomizer in a distillation fume hood.

An ethephon solution containing 6.6 mg of ethephon (Sigma) in 10 mL of water, was also applied to the soil beneath the kauri at the same time. Ethephon spontaneously decomposes to release ethylene gas.

The leaf samples were collected on 0 (prior to the treatment), 3 and 6 day intervals (from a single branch at a time) and stored at -80 °C for further analysis.

As an initial analysis, to have a detectable amount of kauri proteins, the protein analysis was conducted as reported in Scalet et al (1995) for pine needles with minor modifications (Scalet, Federico, Guido, & Manes, 1995). The leaf tissue was homogenized in liquid nitrogen by mortar and pestle and transferred to a semi micro test tube, containing 5 volumes of 0.1M potassium phosphate buffer (pH 6.8). As Scalet et al (1995) suspected the presence of the endogenous phenolic compounds in the protein extract, the decision was made to add 1.5 % of polyvinylpyrrolidone (PVP) to the extracting solution as a phenolic compound scavanger. The powdered leaf tissue and the buffer solution were furthur homogenized with the help of an ultra-turrax and transferred to a 1.5 mL Eppendorf tube and centrifuged at 12000 g for 20 minutes at 4° C. The supernatant was used for protein confirmation analysis. On the basis of the protein analysis, the decision was taken to proceed with the hormone treatment and optimize the parameters for the enzyme extraction and enzyme assay with special reference to the peroxidase enzyme.

## 2.3. Method Development

### 2.3.1. Peroxidase assay

The peroxidase assay was performed as described by Maehly and Chance (1955) (B. Chance & Maehly, 1955)

The peroxidase activity was measured by the rate of formation of the oxidation product of guaiacol, the substrate, by peroxidases in presence of the oxidizing agent $H_2O_2$.The selection of guaiacol as a substrate for the enzymatic activity was because of its high reactivity with the majority of the isoforms of the peroxidase enzyme.

The chemical nature of the amber coloured reaction product had been reviewed and described as tetraguaiacol (B. Chance, and Maehly, A. C, 1964), 2,2'-dihydroxy-3,3'-dimethoxybiphenyl (Booth & Saunders, 1956), 4,4 dihydroxy-3,3'-dimethoxybiphenyl (Harauchi & Yoshizaki, 1982). Most recently the LC-APCI/MS analysis of the reaction mixture yielded a mass spectrum of consistent amount of 3,3'-dimethoxy-4,4'-dihydroxybiphenyl derivatives (Doerge, Divi, & Churchwell, 1997).

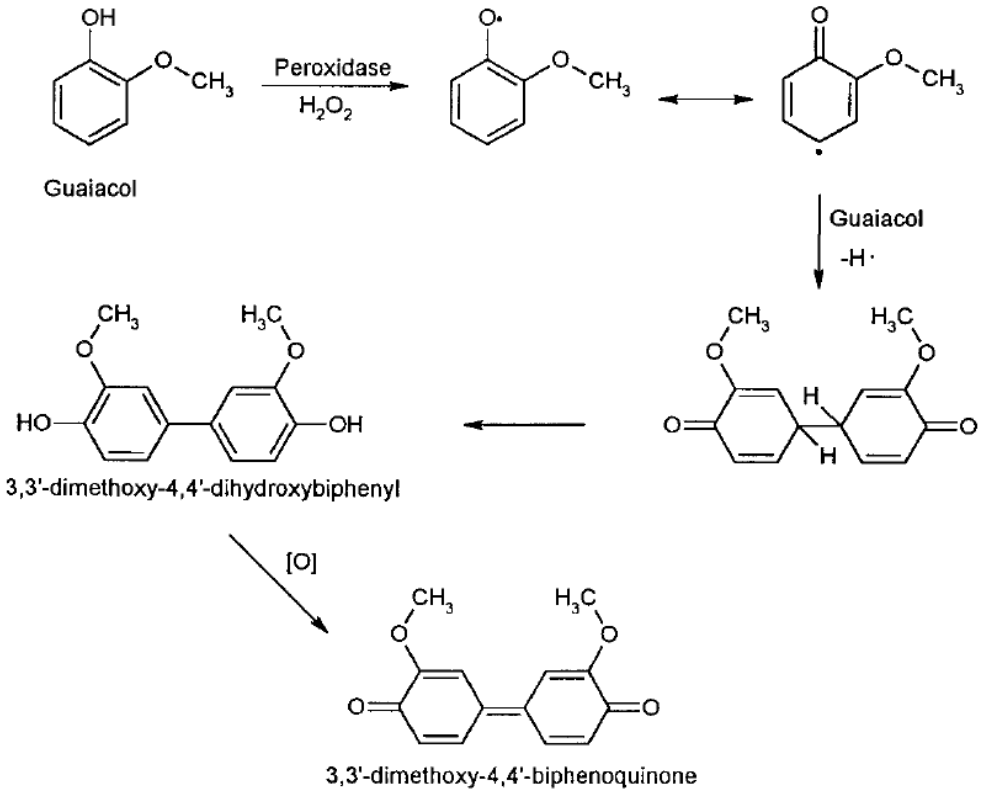



**Fig. 2.a Mechanism of action of the peroxidase assay**

(Doerge et al., 1997).

The colorimetric assay of the enzymatic activity is quantified by measuring the change in absorbance of the amber coloured 3,3'-dimethoxy-4,4'-dihydroxybiphenyl derivatives at 480 nm. The optimum parameters for the maximum quantitation of the peroxidase activity was further considered. Factors like time, temperature, pH, enzyme concentration/volume were considered during the enzyme assay optimization process.

At the same time, the possibility of the addition of phenolic compound scavengers such as PVP and PVPP (Herman & Shan, 2011) as well as the effect of metal chelators like EDTA were also examined during the enzyme extraction process.

### 2.3.2. Enzyme assay - Optimization of parameters

Since the composition of different plant extracts varies based on its spacial and geometrical entities, and also there was no published studies based on kauri peroxidase enzymes, it was necessary to optimize the parameters for the kauri peroxidase enzyme assay.

Since similar plant studies on peroxidase enzyme were conducted at the range of 25 °C – 30 °C, a series of assays were carried out at 25 °C and 30 °C, and the enzyme activities were observed at different time spans to optimize the time and temperature for the kauri peroxidase assay (Fig. 2.b.).

The results showed that the absorbance at 6 minutes comes well within the area of linear interpolation. Even though it was not significant, there was a slight increase in enzyme activity at 30 °C when compared to that of 25 °C. So the 30 °C and 6 minutes time span were selected as the optimum parameters for further analysis.

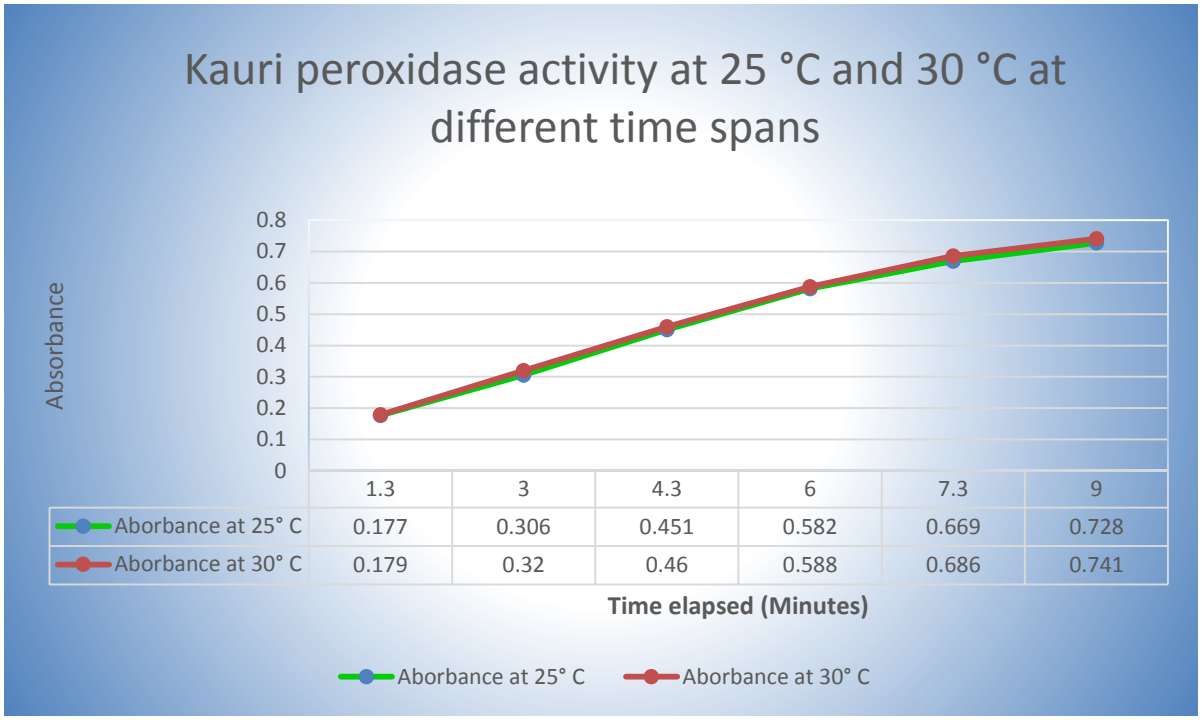



**Fig. 2.b. Peroxidase assay results of kauri leaf extract (without any treatment) at different time spans and at different temperatures.**

pH is an important factor that determines enzyme activity as it can alter the ionization state of amino acid chains or the ionization of the substrate (Pandit, 2012). The peroxidase enzyme is highly stable in the pH range of 5.0 to 9.0 (Schomburg, Salzmann, & Stephan, 1994). So the decision was taken to maintain pH 5.6 for the sodium acetate buffer for all of the further enzyme assay procedures.

Another set of assays were carried out to optimize the enzyme volume to obtain an absorbance value within the acceptable range of absorbance (<3) at the 6 minute period of time. The results obtained are given in the Table 2.I.

**Table 2.I. Peroxidase assay results with 0.1 mL and 0.05 mL of enzyme extract**

| **Kauri leaf assay (without any treatment)** | **Absorbance at 480 nm (at 6 minutes)** |
|---|---|
| Blank | 0 |
| Enzyme extract with 0.1 ml of enzyme extract | 0.846 |
| Enzyme extract with 0.1 ml of enzyme extract | 0.892 |
| Enzyme extract with 0.1 ml of enzyme extract | 0.876 |
| Enzyme extract with 0.05 ml of enzyme extract | 0.563 |
| Enzyme extract with 0.05 ml of enzyme extract | 0.572 |
| Enzyme extract with 0.05 ml of enzyme extract | 0.559 |

In general, absorbance values close to 0.4 give optimum spectrophotometric accuracy. An absorbance close to 0.4 also allows for an increase in absorbance due to subsequent hormone treatments. Based on the above observations it was decided to proceed with the 0.05 mL of enzyme extract for all of the further enzyme assays as 0.05 mL gives an absorbance closer to 0.4 than that of 0.1 mL enzyme extract.

Based on the above mentioned optimization parameters, the kauri peroxidase enzyme assay method was finalised as follows.

### 2.3.3. Method for the kauri peroxidase enzyme assay

The reaction mixture consisted of 0.05 mL of enzyme extract, 0.9 mL of 50 mM sodium acetate buffer (pH 5.6), 0.5 mL of 20 mM guaiacol and 0.5 mL of 60 mM $H_2O_2$. The linear increase in absorbance at 480 nm is monitored for 6 minutes at 30 °C (Baysal, Gürsoy, Örnek, & Duru, 2005).A volume of 0.05 mL of 50 mM sodium acetate buffer (pH 5.6) instead of the enzyme extract in the reaction mixture was treated as the reagent blank for the enzyme assay.

## 2.4. Enzyme extraction-Optimization of parameters

0.1 M potassium phosphate buffer (pH 6.8) was used as an extraction buffer. The choice of the phosphate buffer was based on the findings by Pandit, 2012 over the acetate and citrate buffer based on the maximum protein extraction as well as the stability and the activity of the peroxidase enzyme (Pandit, 2012). The decision for the selection of pH 6.8 for the phosphate buffer was based on previous similar studies (Kar & Mishra, 1976; Marcucci, Aleandri, Chilosi, & Magro, 2010).

Because of the possibility of the phosphate anions preventing the nucleophilic attack of the thiolate anion on the disulphide linkage within the enzyme, as the thiolate anion (rather than the thiol) is a reactive species in the thiol–disulfide interchange, an increase in the buffer concentration decreases the extent of thiol–disulphide interchange (Cecil & McPhee, 1959; Liu, 1977; Wu et al., 2000). So the strength of the phosphate buffer has been optimized to 0.1 M to increase the enzyme stability.

In order to maintain the enzyme stability all the extraction procedures were carried out < 4 °C. The leaflets were frozen in liquid nitrogen and ground to a fine powder for 2-3 minutes using a mortar and pestle in an ice bath. 0.1 g of the ground tissue was suspended in a semi micro test tube, and further homogenized with an ultra-turrax with 0.5 mL of 0.1 M potassium phosphate buffer (pH 6.8). The possibility that the plant polyphenols may inhibit the enzyme activity was also considered during parameter optimization stage. Since the polymers polyvinylpyrrolidone (PVP) and its cross-linked version polyvinylpolypyrrolidone (PVPP) are well known as effective polyphenol absorbents (by forming a strong hydrogen bonding complex with phenolic compounds), the possibility of addition of 1.5% w/v PVP and the same ratio of the combination of PVP and PVPP to the extraction solution was examined.The change in the enzyme activity due to addition of PVP and PVP+PVPP was assessed.

**Table 2.II. Peroxidase assay results without PVP, with PVP and the combination of PVP and PVPP**

| Kauri leaf assay (without any treatment) | Absorbance at 480 nm (at 6 minutes) |
|---|---|
| Blank | 0 |
| Enzyme extract alone | 0.461 |
| Enzyme extract alone | 0.483 |
| Enzyme extract with PVP alone | 0.582 |
| Enzyme extract with PVP alone | 0.591 |
| Enzyme extract with PVP and PVPP | 0.572 |
| Enzyme extract with PVP and PVPP | 0.558 |

The results (Table 2.II.) showed that the addition of PVP alone to the extracting solution improved the peroxidase activity, at the same time the combination of PVP and PVPP was not as effective as that of PVP alone. So addition of 1.5% w/v PVP alone to the extracting solution was confirmed for all of the furthur analysis.

Metal ions like $Fe^{2+}$, $Fe^{3+}$ and $Hg^{2+}$ exhibit a strong inhibitory effect on the peroxidase enzyme (Khatun et al., 2012; N Einollahi, S Abbasi, N Dashti, & F Vaezzadeh, 2006). According to Khatun et al (2012) metal ions like $Ni^{2+}$, $Pb^{2+}$, $Zn^{2+}$, $Al^{3+,}$ $Mg^{2+}$, $Cu^{2+}$, $Co^{2+}$ and $Cd^{2+}$ also posses a low inhibitory effect on the peroxidase enzyme. So the influence of EDTA as a metal chelator in the extracting solution, towards the enzyme activity was examined.

**Table 2.III. Peroxidase assay results with and without EDTA in the extracting solution (with PVP)**

| Kauri leaf assay (without any treatment) | Absorbance at 480 nm (at 6 minutes) |
|---|---|
| Blank | 0 |
| Enzyme extract alone | 0.603 |
| Enzyme extract alone | 0.591 |
| Enzyme extract with 0.5 mL 1mM EDTA | 0.551 |
| Enzyme extract with 0.5 mL 1mM EDTA | 0.568 |

The results showed that the addition of EDTA to the extracting solution slightly inhibited the enzyme activity.So the decision was taken to exclude EDTA from the extacting solution for all of the furthur analysis.

While considering all the optimization parameters, the peroxidase enzyme extraction method from the kauri leaf was finalised as follows.

### 2.4.1. Kauri Peroxidase extraction method from the leaf sample

The extraction was carried out on a single leaflet with five replicates. The leaflets were frozen in liquid nitrogen and ground to a fine powder for 2-3 minutes using a mortar and pestle in an ice bath. 0.1 g of the ground tissue was suspended in a semi micro test tube, and further homogenized with an ultra-turrax with 0.5 mL of 0.1 M Potassium phosphate buffer (pH 6.8) containing 1.5 % (w/w) polyvinylpyrrolidone (PVP), to remove the soluble phenolic compounds. The homogenate was mixed further with the help of a vortex and rapidly transferred to a 1.5 mL Eppendorf tube. The homogenates were centrifuged at 12,000g for 20 min at 4 ºC and the supernatants used for the further analysis.

# Chapter 3. Results and Discussion

## 3.1. Hormone treatment of kauri trees with methyl jasmonate and ethephon

Previous studies with other types of plants (e.g. tobacco) (Durrant & Dong, 2004; Sasaki et al., 2007) have shown that methyl jasmonate and ethephon may act synergistically to induce the enzyme activity. It was therefore decided to use this combination as the initial treatment.

0.23 g of methyl jasmonate (Sigma) was dissolved in 200 µL of 99 % of ethanol, 0.5g of tween 20 and 25 mL of water. The tween 20 is a nonbiologically active detergent which helps to solubilize the methyl jasmonate and also act as a wetting agent. The solution was sprayed on the kauri tree with the help of a chromatographic reagent atomizer in a distillation fume hood.

An ethephon solution containing 6.6 mg of Ethephon in 10 mL of water, was also applied to the soil at the same time to release ethylene.

The leaf samples were collected on 0 (prior to the treatment), 3, 6, 9 and 12 day intervals (from a single branch at a time) and stored at -80 °C for the further enzyme assay analysis.

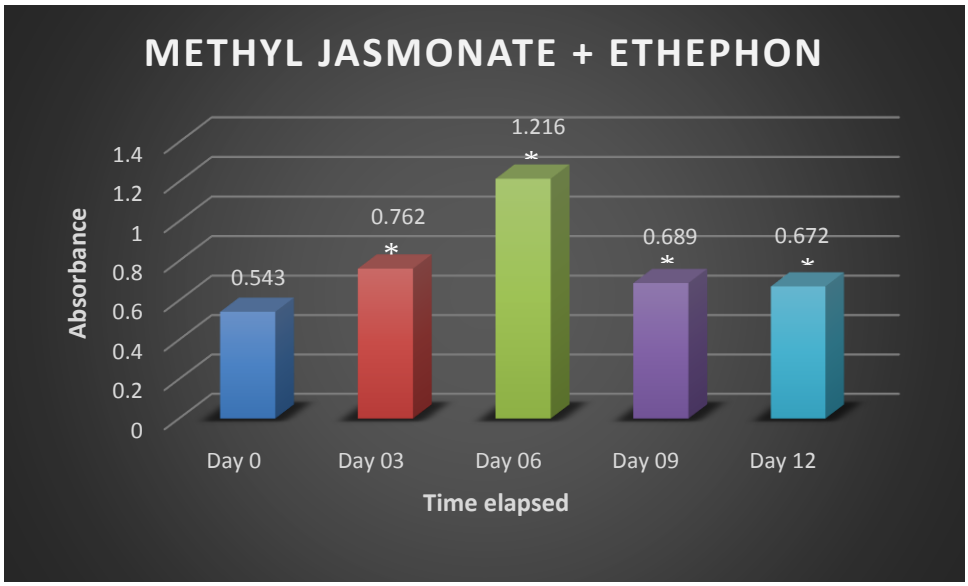
Five individual leaves from each day were individually subjected to peroxidase extraction and assay as described in section 2.4.1 & 2.3.3 respectively. The enzyme extractions and the assays were repeated in a similar manner for five replicates, on different days to avoid any possible environmental parameters that affects the enzyme activity.

The data obtained were statistically analysed by Dixon's Q test with 90 % confidence interval. The Dixon's Q test removes the outlier data points. The Q test were conducted between the replicate absorbance values of each days treatment and also between the average absorbance values conducted at different day intervals. The results are shown in table 3.I.

**Table 3.I. Peroxidase assay results showing enzyme activity at different day interval**

| **Kauri leaf assay** | **Absorbance at 480 nm (at 6 minutes) ( $\bar{x}$, n=5 )** |
|---|---|
| Blank | 0 |
| Day 0 | 0.543 |
| Day 3 | 0.762 |
| Day 6 | 1.216 |
| Day 9 | 0.689 |
| Day 12 | 0.672 |

**Fig. 3.a. Comparison of peroxidase activity by absorbance at different day interval on methyl jasmonate and ethephon application**




* $p < 0.05$, ** $p < 0.01$ Compared to control (day 0)

The absorbance values show a large increase up to day 6, with the observed maximum activity at day 6 being a 123.9% increase (when compared to day 0), and then decreased as days went on.

The data obtained were further analysed by a two tailed *t* test to compute any statistical significance between the absorbance levels obtained at different day intervals against the absorbance value obtained at day 0 (prior to the treatment). A *p*-value of 0.05 or less were considered as rejecting the null hypothesis and considered as statistically significant. A *p*-value

of 0.01 or less were considered as rejecting the null hypothesis and considered as highly statistically significant. The data obtained are given in table 3.II.

**Table 3.II. Two tailed *t* test *p*-value comparison of peroxidase activity by absorbance against day 0 (absorbance prior to the treatment)**

| **Days** | ***p*- value** |
|---|---|
| Day 0 vs day 3 | $1.7893\times 10^{-10}$ |
| Day 0 vs day 6 | $6.5418\times 10^{-15}$ |
| Day 0 vs day 9 | $9.0668\times 10^{-13}$ |
| Day 0 vs day 12 | $8.5824\times 10^{-12}$ |

The *p*-value data revealed a significant impact of methyl jasmonate and ethephon on the kauri peroxidase activity at different day intervals.

As the current dosage of synergetic combination of methyl jasmonate and ethephon had a significant impact on the kauri peroxidase activity, the decision was taken to do a hormone treatment by methyl jasmonate alone to observe its influence on the peroxidase activity.

## 3.2. Hormone treatment with methyl jasmonate alone

0.23 g of methyl jasmonate (Sigma) was dissolved in 200 µL of 99 % of ethanol, 0.5g of tween 20 and 25 mL of water. The solution was sprayed on the kauri tree with the help of a chromatographic reagent atomizer in a distillation fume hood. The leaves were collected from a single branch at 0 (prior to the treatment), 3, 6, 9 and 12 day intervals and stored at -80 °C for the further analysis.

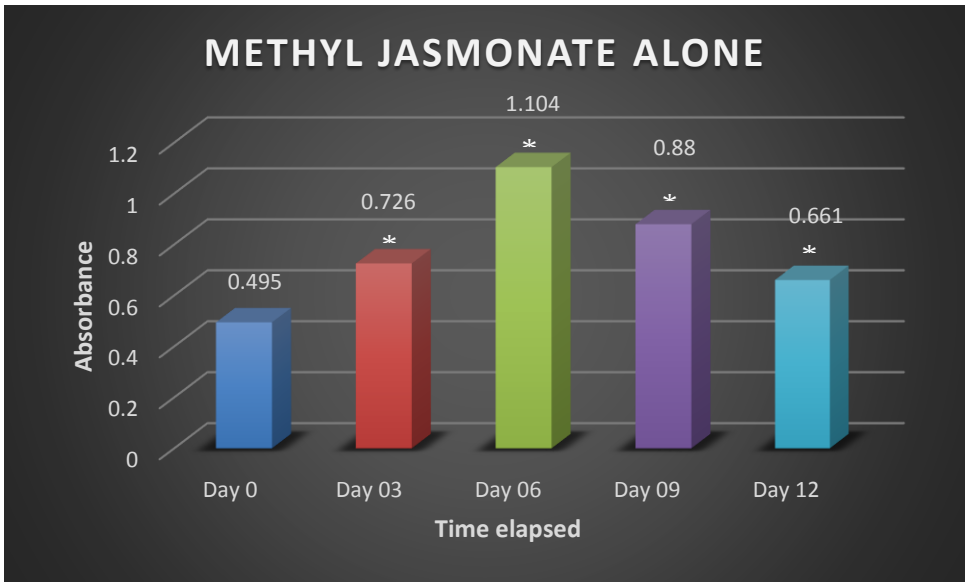
The peroxidase extraction, assay and the statistical analysis were conducted as described in sections 2.4.1, 2.3.3 & 3.1.

The assay results were statistically analysed by Dixon's Q test with 90 % confidence interval as described in section 3.1. The results are given below in the table,

**Table 3.III. Peroxidase assay results showing enzyme activity at different day interval**

| **Kauri leaf assay** | **Absorbance at 480 nm (at 6 minutes) ( $\bar{x}$, n=5 )** |
|---|---|
| Blank | 0 |
| Day 0 | 0.495 |
| Day 3 | 0.726 |
| Day 6 | 1.104 |
| Day 9 | 0.88 |
| Day 12 | 0.661 |

**Fig. 3.b. Comparison of peroxidase activity by absorbance at different day interval on methyl jasmonate application**




* $p < 0.05$, ** $p < 0.01$ Compared to control (day 0)

The data obtained show a large increase in peroxidase activity up to day 6 and then it tends to decrease gradually. The peroxidase activity at day 6 shown a 123.03% fold increase in activity when compared to day 0 (before treatment).

The statistical significance of the enzyme activities at different days intervals against day 0 (activity prior to the treatment) has been analysed by a two tailed *t* test. The observations are given in table below,

**Table 3.IV. Two tailed *t* test *p*-value comparison of peroxidase activity by absorbance against day 0 (absorbance prior to the treatment)**

| **Days** | ***p*- value** |
|---|---|
| Day 0 vs day 3 | $3.7635\times 10^{-10}$ |
| Day 0 vs day 6 | $3.8537\times 10^{-10}$ |
| Day 0 vs day 9 | $8.9170\times 10^{-10}$ |
| Day 0 vs day 12 | $1.8149\times 10^{-09}$ |

The data obtained revealed a highly significant impact of foliar application of methyl jasmonate on the kauri peroxidase activity at different day intervals.

Since both the hormone treatments, the synergetic combination of methyl jasmonate and ethephon and the methyl jasmonate alone, exhibit a substantial statistical significance towards the kauri peroxidase activity within their groups, and also because of the limitations of the time constrains to proceed with multiple treatments on different trees to compare the statistical significance between the groups, it was decided to proceed with a further double dose hormone treatment of methyl jasmonate alone to observe its impact on the enzyme activity.

## 3.3. Hormone treatment with methyl jasmonate alone – Double dose treatment

A kauri tree was treated with 0.50 g of methyl jasmonate (Sigma) in 200 µl of 99 % of ethanol, 0.5g of tween 20 and 25 mL of water as described in section 3.1. The leaves were collected from a single branch at 0 (prior to the treatment), 3, 6, 9 and 12 day intervals and stored at -80 °C for the further analysis.

The enzyme extraction, assay and the statistical analysis were conducted as described in sections 2.4.1, 2.3.3 & 3.1.

The absorbance data obtained were statistically analysed by Dixon's Q test with 90 % confidence interval as described in section 3.1. The results are given below in the table,

**Table 3.V. Peroxidase assay results showing enzyme activity at different day interval**

| **Kauri leaf assay** | **Absorbance at 480 nm (at 6 minutes) ( $\bar{x}$, n=5 )** |
|---|---|
| Blank | 0 |
| Day 0 | 0.534 |
| Day 3 | 1.78 |
| Day 6 | >3 * |
| Day 9 | 2.816 * |
| Day 12 | 1.557 * |

*The data is unreliable since the leaves started senesceing and became brown, which would have affected the enzyme activity.

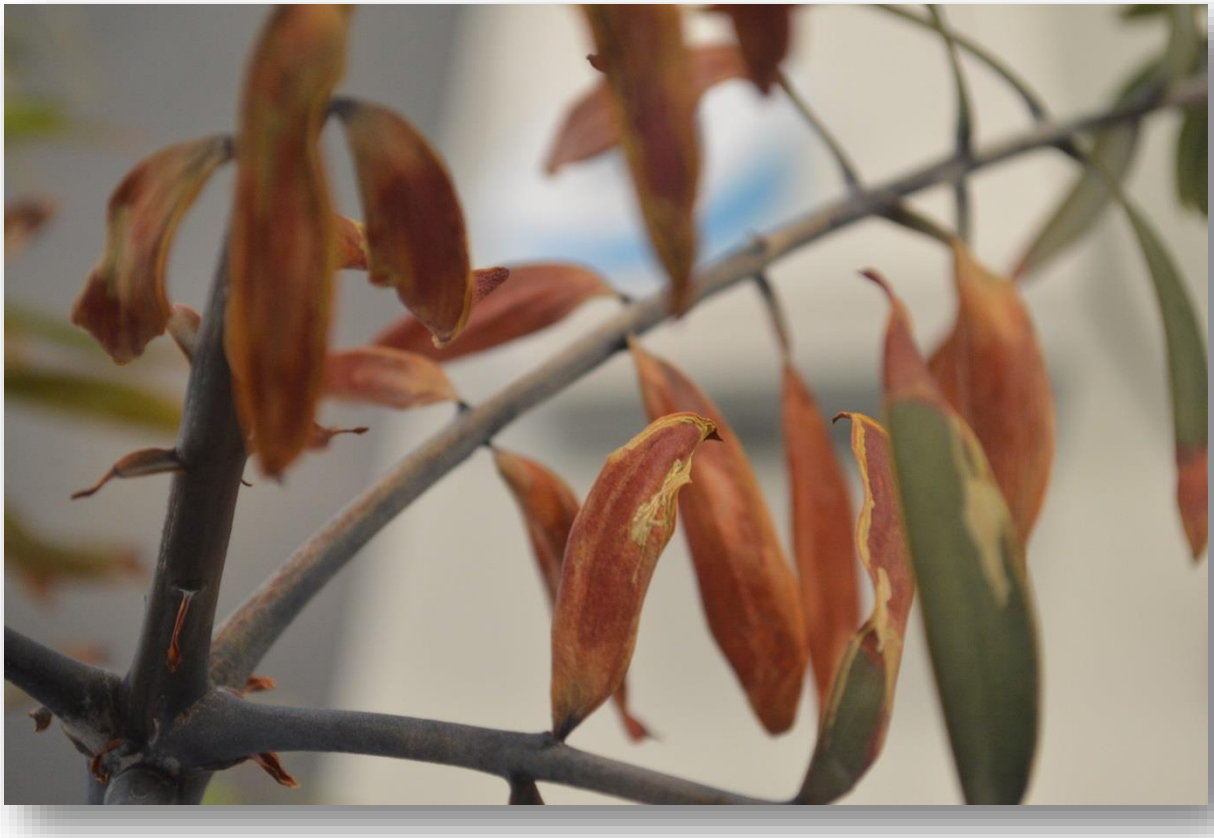


**Fig. 3.c. Leaf senesceing due to methyl jasmonate double dose treatment**

Because of the higher possibilities of random errors and internal quenching the absorbance data above 3 was considered as invalid. Even though there was a possibility of further dilution, the unreliability of the enzyme levels due to the leaf senescening caused a decision to be made to proceed with the single dose treatments for the further experiments.

Since both the hormone treatments, the synergetic combination of methyl jasmonate and ethephon and the methyl jasmonate alone (single dose), show the highest increase in peroxidase activity at day 6, the decision was taken to do a further hormone retreatment at day 6 and extend the observations until day 18 at three day intervals. This was to determine if the enzyme activity could be increased even higher than day 6.

## 3.4. Hormone treatment with methyl jasmonate and ethephon with day 6 retreatment

0.23 g of methyl jasmonate (Sigma) was dissolved in 200 µL of 99 % of ethanol, 0.5g of tween 20 and 25 mL of water. The tween 20 is a nonbiologically active detergent which helps to solubilize the methyl jasmonate and also act as a wetting agent. The solution was sprayed on the kauri tree with the help of a chromatographic reagent atomizer in a distillation fume hood.

An ethephon solution containing 6.6 mg of ethephon in 10 mL of water, was also applied to the soil at the same time.

The tree was retreated at day 6 in an exactly similar manner as described above.

The leaf samples were collected on 0 (prior to the treatment), 3, 6 (prior to the second treatment), 9, 12, 15 and 18 day intervals (from a single branch at a time) and stored at -80 °C for the further enzyme assay analysis.

Five replicates of individual leaves from each day were individually subjected to peroxidase extraction and assay as described in section 2.4.1 & 2.3.3 respectively. The enzyme extractions and the assays were repeated in a similar manner five times on different days to avoid any possible environmental parameters that affects the enzyme activity.

Apart from the initial analysis, the factor of leaf sample weight was also considered during the statistical summarization to improve the accuracy of the enzyme activity analysis.

The data obtained were statistically analysed by Dixon's Q test with 90 % confidence interval (Shoemaker, Garland, & Steinfeld, 1974). The Dixon’s Q test were conducted between the

replicate absorbance values of each day treatment and also between the average absorbance/leaf sample weight values of each days conducted at different day intervals.

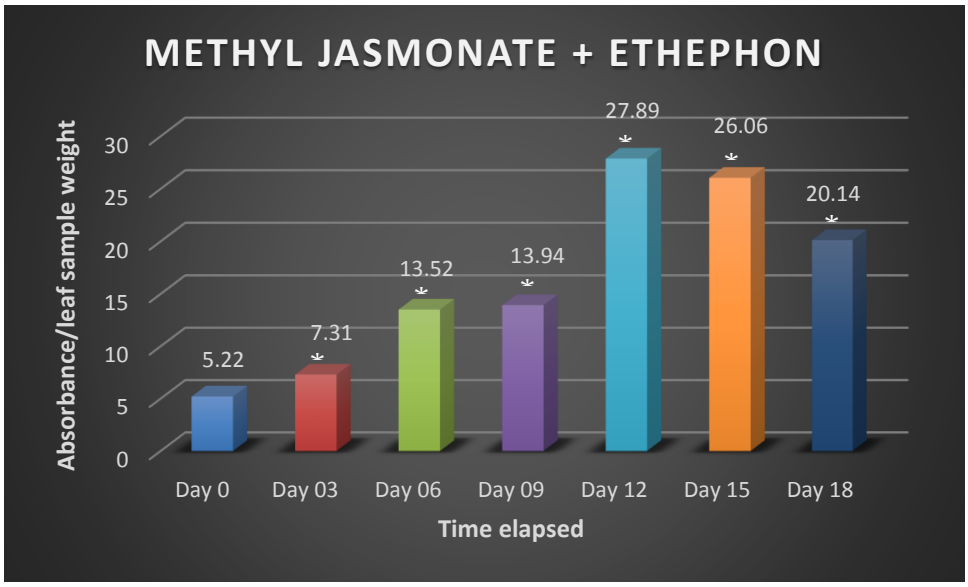
See appendix for the detailed statistical analysis for the peroxidase activity by methyl jasmonate and ethephon hormone treatment with day 6 retreatment.

The Q test results are summarized in the table below,

**Table 3.VI. Peroxidase assay results showing enzyme activity at different day interval**

| **Kauri leaf assay** | **Absorbance at 480 nm/Leaf sample weight (g) (at 6 minutes) ( $\bar{x}$, n=5 )** |
|---|---|
| Blank | 0 |
| Day 0 (Initial treatment) | 5.22 |
| Day 3 | 7.31 |
| Day 6 (Retreatment) | 13.52 |
| Day 9 | 13.94 |
| Day 12 | 27.89 |
| Day 15 | 26.06 |
| Day 18 | 20.14 |

**Fig. 3.d. Comparison of peroxidase activity by absorbance/Leaf sample weight at different day interval on methyl jasmonate and ethephon application with day 6 retreatment**




* $p < 0.05$, ** $p < 0.01$ Compared to control (day 0)

The data obtained revealed a substantial influence of foliar application of synergetic combination of methyl jasmonate and ethephon on the kauri peroxidase activity. The enzyme activity increased gradually until day 9, and it increased drastically to its maximum at day 12, then it was observed to decrease gradually as days went on. The peak enzyme activity at day 12 had shown a 434.29 % increase when compared to that of day 0 (the enzyme activity prior to the treatment).

The statistical significance of the increase in enzyme activity observed at different day intervals against the activity value obtained at day 0 (prior to the treatment) has been tabulated by conducting a two tailed *t* test within the group. A *p*-value of 0.05 or less were considered as rejecting the null hypothesis and considered as statistically significant. The data obtained are given in table 3.VII.

**Table 3.VII. Two tailed *t* test *p*-value comparison of peroxidase activity against day 0 (Enzyme activity prior to the treatment)**

| **Days** | ***p*- value** |
|---|---|
| Day 0 vs day 3 | $2.7232\times 10^{-6}$ |
| Day 0 vs day 6 | $5.4166\times 10^{-7}$ |
| Day 0 vs day 9 | $2.0779\times 10^{-9}$ |
| Day 0 vs day 12 | $1.3226\times 10^{-9}$ |
| Day 0 vs day 15 | $1.3718\times 10^{-7}$ |
| Day 0 vs day 18 | $5.0786\times 10^{-11}$ |

The *t* test *p* value results revealed that the foliar application of synergetic combination of methyl jasmonate and ethephon made a highly significant impact on kauri peroxidase activity on each observed day interval.

Since the day 6 hormone retreatment of synergetic combination of single dose methyl jasmonate and ethephon was observed to be highly significant on the kauri peroxidase activity and also the foliar application of methyl jasmonate alone (single dose day 0 treatment) had shown a significant impact on the peroxidase activity during the preliminary analysis, it was decided to conduct a day 6 hormone retreatment of single dose methyl jasmonate alone to observe its impact on the peroxidase activity until day 18 at three days intervals.

## 3.5. Hormone treatment with methyl jasmonate alone with day 6 retreatment

The kauri tree was sprayed with 0.23 g of methyl jasmonate (Sigma) dissolved in 200 µL of 99 % of ethanol, 0.5g of tween 20 and 25 mL of water in a distillation fume hood.

The tree was retreated at day 6 in an exactly similar manner as described above.

The leaf samples were collected on 0 (prior to the treatment), 3, 6 (prior to the second treatment), 9, 12, 15 and 18 day intervals (from a single branch at a time) and stored at -80 °C for the further enzyme assay analysis.

The enzyme extraction, assay and the statistical analysis were conducted as per the sections 2.4.1, 2.3.3 & 3.1. respectively.

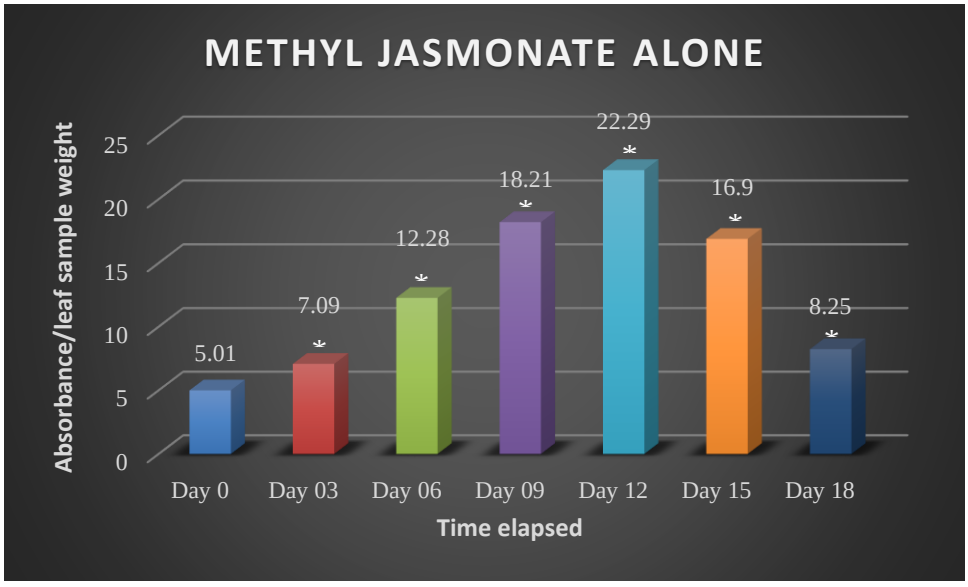
See appendix for the detailed statistical analysis for the peroxidase activity by hormone treatment of methyl jasmonate alone with day 6 retreatment.

The Q test results comparing the peroxidase activity at different day interval are summarized in the table below,

**Table 3.VIII. Peroxidase assay results showing enzyme activity at different day interval**

| **Kauri leaf assay** | **Absorbance at 480 nm/Leaf sample weight (g) (at 6 minutes) ( $\bar{x}$, n=5 )** |
|---|---|
| Blank | 0 |
| Day 0 (Initial treatment) | 5.01 |
| Day 3 | 7.09 |
| Day 6 (Retreatment) | 12.28 |
| Day 9 | 18.21 |
| Day 12 | 22.29 |
| Day 15 | 16.9 |
| Day 18 | 8.25 |

**Fig. 3.e. Comparison of peroxidase activity by absorbance/Leaf sample weight at different day interval on methyl jasmonate application with day 6 retreatment**




* $p < 0.05$, ** $p < 0.01$ Compared to control (day 0)

The data obtained revealed an increase in peroxidase activity until day 12 by methyl jasmonate application. The enzyme activity was observed at its maximum at day 12 and it tends to decrease gradually towards the end of the observations. The peroxidase activity at day 12 had shown 344.91% fold increase when compared to day 0 (Activity prior to the treatment). This was slightly less than the synergistic combination.

The statistical significance of the enzyme activities at different days intervals against day 0 (activity prior to the treatment) has been analysed by a two tailed *t* test. The observations are given in table below,

**Table 3.IX. Two tailed *t* test *p*-value comparison of peroxidase activity by absorbance against day 0 (absorbance prior to the treatment)**

| **Days** | ***p*- value** |
|---|---|
| Day 0 vs day 3 | $4.6328\times 10^{-07}$ |
| Day 0 vs day 6 | $4.9990\times 10^{-09}$ |
| Day 0 vs day 9 | $7.6386\times 10^{-06}$ |
| Day 0 vs day 12 | $6.7454\times 10^{-12}$ |
| Day 0 vs day 15 | $8.3118\times 10^{-08}$ |
| Day 0 vs day 18 | $8.3212\times 10^{-08}$ |

The *t* test *p* value data revealed that the foliar application of methyl jasmonate alone made a highly significant impact on the kauri peroxidase activity on each observed days when compared to the day 0 activity.

Since the foliar application of synergistic combination of methyl jasmonate and ethephon and the methyl jasmonate alone were observed to be highly significant in the induction of peroxidase activity, it was decided to conduct a single dose hormone treatment of ethephon alone with day 6 retreatment to compare the effectiveness of the individual hormone applications.

## 3.6. Hormone treatment with ethephon alone with day 6 retreatment

A kauri tree was treated with 6.6 mg of ethephon dissolved in 10 mL of water, in the soil around the pot, in a distillation fume hood.

The treatment was repeated at day 6 in an exactly similar way as mentioned above.

The leaf samples were collected on 0 (prior to the treatment), 3, 6 (prior to the retreatment), 9, 12, 15 and 18 day intervals (from a single branch at a time) and stored at -80 °C for the further enzyme assay analysis.

The enzyme extraction, assay and data analysis were conducted as per the respective sections 2.4.1, 2.3.3 & 3.1.

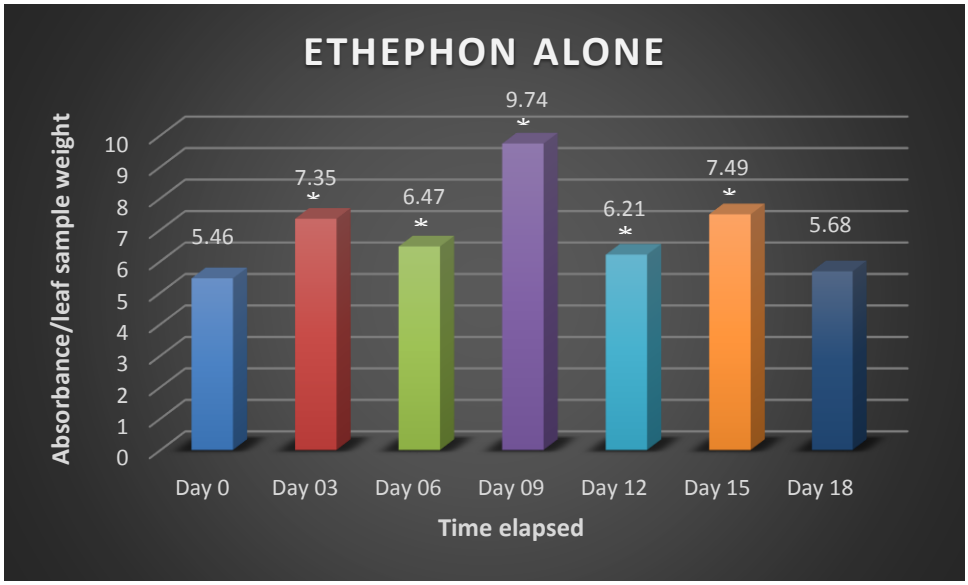
See appendix for the detailed statistical analysis for the peroxidase activity by hormone treatment of ethephon alone with day 6 retreatment.

The results after the application of the Q test summarizing the peroxidase activity at different day interval are given below,

**Table 3.X. Peroxidase assay results showing enzyme activity at different day interval**

| **Kauri leaf assay** | **Absorbance at 480 nm/Leaf sample weight (g) (at 6 minutes) ( $\bar{x}$, n=5 )** |
|---|---|
| Blank | 0 |
| Day 0 (Initial treatment) | 5.46 |
| Day 3 | 7.35 |
| Day 6 (Retreatment) | 6.47 |
| Day 9 | 9.74 |
| Day 12 | 6.21 |
| Day 15 | 7.49 |
| Day 18 | 5.68 |

**Fig. 3.f. Comparison of peroxidase activity by absorbance/Leaf sample weight at different day interval on ethephon application with day 6 retreatment**




* $p < 0.05$, ** $p < 0.01$ Compared to control (day 0)

The peroxidase activity was observed to increase during the course of the treatment. However, there is no consistency observed in the variation in enzyme activity. The enzyme activity reached its maximum at day 9 and it showed a 78.39% increase when compared to day 0 (Activity prior to the treatment).

The table 3.XI compares the statistical significance of the variation in peroxidase activity at different day intervals by computing the *t* test *p* value data when compared to the enzyme activity prior to the treatment.

**Table 3.XI. Two tailed *t* test *p*-value comparison of peroxidase activity by absorbance against day 0 (absorbance prior to the treatment)**

| **Days** | ***p*- value** |
|---|---|
| Day 0 vs day 3 | $4.3637\times 10^{-07}$ |
| Day 0 vs day 6 | $4.3048\times 10^{-05}$ |
| Day 0 vs day 9 | $8.6807\times 10^{-10}$ |
| Day 0 vs day 12 | 0.001153458 |
| Day 0 vs day 15 | $1.0921\times 10^{-06}$ |
| Day 0 vs day 18 | 0.071285527 |

The *t* test *p* value result shows that the ethephon hormone treatment alone could make a highly statistically different increase in activity of peroxidase enzymes on kauri plants at different day intervals. The enzyme activity at day 9 has been observed to have the highest absorbance when compared to the other observations with respect to the enzyme activity at day 0.The activity reduced back to control level at day 18 with no significant difference.

## 3.7. β-amino-butyric acid (BABA)

*β*-amino-butyric acid (BABA) is a non-protein amino acid. It has been used as a potential plant resistance inducer against a wide range of pathogens in several plant species. The experimental observations by Ton et al. (2005) revealed that Arabidopsis plants treated with BABA developed an enhanced capacity to resist biotic and abiotic stresses (Ton et al., 2005). The foliar application of BABA resulted in local and systemic induction of plant defence mechanisms in tomato plants as an accelerator of the SA response pathway (Oka, Cohen, & Spiegel, 1999). Based on the similar studies in the field with *β*-amino-butyric acid (BABA) (Cao et al., 2014; Cohen, Niderman, Mosinger, & Fluhr, 1994; Jakab et al., 2001; Zhong et al., 2014) in different plant species it was decided to conduct a hormone treatment with BABA to observe its activity on the kauri peroxidase enzyme and compare its effectiveness with the other observed hormone applications.

### 3.7.1. Hormone treatment with *β*-amino-butyric acid (BABA) with day 6 retreatment

A kauri tree was sprayed with 0.0250 g of *β*-amino-butyric acid (BABA) dissolved in 25 mL of water (1000 µg/mL) with the help of a chromatographic reagent atomizer in a distillation fume hood.

The treatment was repeated at day 6 as described above.

The leaves were collected at 0 (prior to the initial treatment), 3, 6 (prior to the second treatment), 9, 12, 15 and 18 day intervals and stored at -80 °C for the further analysis.

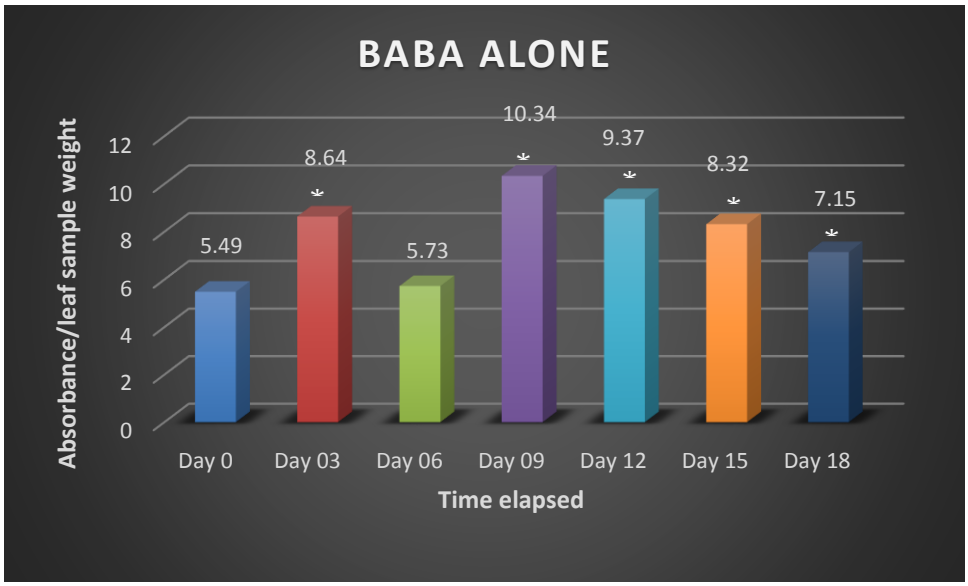
The enzyme extraction, assay and the statistical analysis were conducted as described in section 2.4.1, 2.3.3 & 3.1. respectively.

The absorbance data obtained were statistically summarized by Dixon's Q test with 90 % confidence interval as described in section 3.1. The results are given below in the table,

**Table 3.XII. Peroxidase assay results showing enzyme activity at different day interval**

| **Kauri leaf assay** | **Absorbance at 480 nm/Leaf sample weight (g) (at 6 minutes) ( $\bar{x}$, n=5 )** |
|---|---|
| Blank | 0 |
| Day 0 (Initial treatment) | 5.49 |
| Day 3 | 8.64 |
| Day 6 (Retreatment) | 5.73 |
| Day 9 | 10.34 |
| Day 12 | 9.37 |
| Day 15 | 8.32 |
| Day 18 | 7.15 |

**Fig. 3.g. Comparison of peroxidase activity by absorbance/Leaf sample weight at different day interval on *β*-amino-butyric acid (BABA) application with day 6 retreatment**




* $p < 0.05$, ** $p < 0.01$ Compared to control (day 0)

The data obtained revealed that the hormone treatment with *β*-amino-butyric acid (BABA) could make a positive impact on the kauri peroxidase activity as the activities were observed to increase at all the observations (relative to the control). Similar to the ethephon application, there was no consistency observed in the variation of enzyme activity during the course of the

treatment. The enzyme activity reached its maximum at day 9, and it showed a 88.34% increase when compared to day 0 (the enzyme activity prior to the treatment).

The table below 3.XIII shows the *t* test *p* values of enzyme activity at different day intervals against the activity at day 0.

**Table 3.XIII. Two tailed *t* test *p*-value comparison of peroxidase activity by absorbance against day 0 (absorbance prior to the treatment)**

| **Days** | ***p*- value** |
|---|---|
| Day 0 vs day 3 | $4.1105\times 10^{-06}$ |
| Day 0 vs day 6 | 0.067336826 |
| Day 0 vs day 9 | $6.6569\times 10^{-08}$ |
| Day 0 vs day 12 | $2.5637\times 10^{-07}$ |
| Day 0 vs day 15 | $4.3820\times 10^{-05}$ |
| Day 0 vs day 18 | 0.005769481 |

The peroxidase activities at most observed days showed highly statistically significant results upon BABA application when compared to the enzyme activity prior to the treatment with the exception of day 6.

## 3.8. Salicylic acid

As discussed earlier in the report the plant hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are the most important components of the different signalling pathways of the plant defence mechanism. As it has been observed that the $\beta$-amino-butyric acid (BABA), as an accelerator for the SA response pathway, could make a significant impact on the kauri peroxidase activity, it was decided to proceed with a salicylic acid hormone treatment on kauri plants to observe its impact on the peroxidase activity and compare its effectiveness with the other observed hormone treatments.

### 3.8.1. Hormone treatment with salicylic acid with day 6 retreatment

0.0345 g of salicylic acid dissolved in 2 mL of methanol, because of its poor solubility in water at room temperature, the solution was further diluted with 23 mL of deionized water. The solution was sprayed on the kauri tree with the help of a chromatographic reagent atomizer in a distillation fume hood.

The treatment was repeated on day 6 in a similar manner.

The leaf samples were collected on day 0 (prior to the initial treatment), 3, 6 (prior to the second treatment), 9, 12, 15 and 18 day intervals and stored at -80 °C for the further analysis.

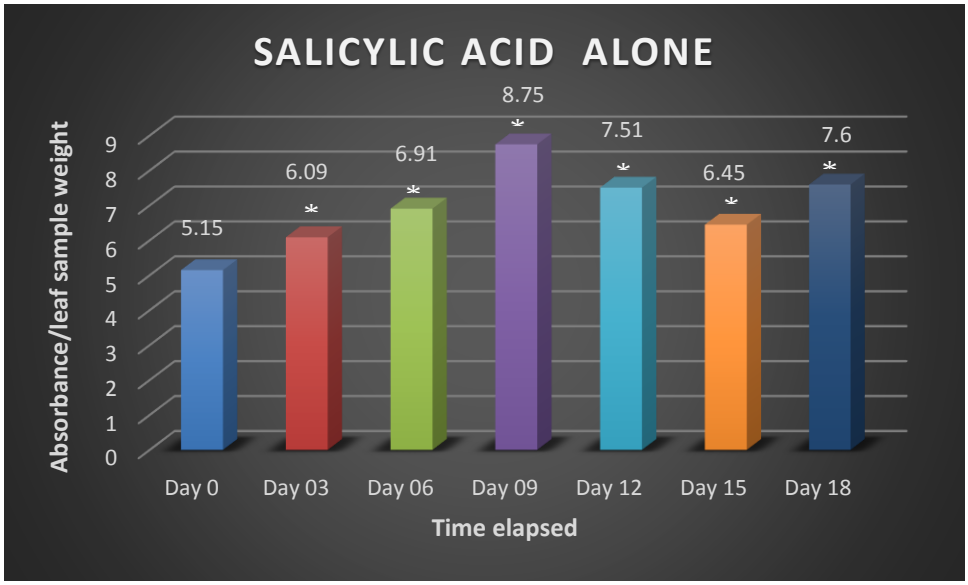
The enzyme extraction, enzyme assay and the statistical summarizations were conducted as described in sections 2.4.1, 2.3.3 & 3.1. respectively.

The Q test results of the enzyme activity at different day intervals are given below,

**Table 3.XIV. Peroxidase assay results showing enzyme activity at different day interval**

| **Kauri leaf assay** | **Absorbance at 480 nm/Leaf sample weight (g) (at 6 minutes) ( $\overline{x}$, n=5 )** |
|---|---|
| Blank | 0 |
| Day 0 (Initial treatment) | 5.15 |
| Day 3 | 6.09 |
| Day 6 (Retreatment) | 6.91 |
| Day 9 | 8.75 |
| Day 12 | 7.51 |
| Day 15 | 6.45 |
| Day 18 | 7.60 |

**Fig. 3.h. Comparison of peroxidase activity by absorbance/Leaf sample weight at different day interval on salicylic acid application with day 6 retreatment**




* $p < 0.05$, ** $p < 0.01$ Compared to control (day 0)

A gradual increase in peroxidase activity was observed until day 9 upon the salicylic acid treatment. The enzyme activity observed at its maximum at day 9 (69.9% increase in activity when compared to day 0 (the activity prior to the treatment)) and it tends to decrease when days went on.

The *t* test *p* values of the enzyme activity at different day intervals against the activity prior to the treatment are given in table 3.XV.

**Table 3.XV. Two tailed *t* test *p*-value comparison of peroxidase activity by absorbance against day 0 (absorbance prior to the treatment)**

| **Days** | ***p*- value** |
|---|---|
| Day 0 vs day 3 | 0.000469493 |
| Day 0 vs day 6 | $8.8194\times 10^{-05}$ |
| Day 0 vs day 9 | $2.9199\times 10^{-08}$ |
| Day 0 vs day 12 | $2.4260\times 10^{-05}$ |
| Day 0 vs day 15 | $9.7096\times 10^{-05}$ |
| Day 0 vs day 18 | $1.0136\times 10^{-05}$ |

The *t* test *p* value result reveals that the Salicylic acid treatment could make a highly significant impact on the peroxidase activity in all of the observed day intervals with respect to the activity prior to the treatment.

Because of the limitations of the time constrain and the restrain to avoid destroying too many plants for the current research, the *t* test comparison were only possible 'within' the group of the population (i.e. each plant acted as its own control). At this stage, in order to compare the variations in enzyme activities at different day intervals, it was necessary to conduct a blank analysis on a control plant at different specified day intervals. In order to improve the reliability on the results, two other important factors also were taken in account.

The mechanical wounding due to the leaf harvesting could also make an influence on the Peroxidase expression in plant species (Birecka & Miller, 1974; Svalheim & Robertsen, 1990). According to Everse & Grisham (1990) the mechanical wounding can promote the expression of cationic isoperoxidases in plant leaves (Everse & Grisham, 1990). The enzyme analysis on the blank treated control plant will be able to help to analyse and compare the peroxidase activities at different day intervals due to the mechanical wounding by leaf harvesting.

Since the hormone treatment with methyl jasmonate and ethephon was observed to be the most efficient combination to induce the kauri peroxidase activity (434.29 % increase in activity at day 12 when compared to the peroxidase level prior to the hormone treatment), it was decided to conduct the blank analysis with 200 µL of 99 % of ethanol, 0.5g of tween 20 in 25 mL of water in order to nullify any contributory effect by these reagents towards the enzyme activity.

## 3.9. Control blank treatment with day 6 retreatment

A Kauri tree was sprayed with a mixture containing 200 µL of 99 % of ethanol, 0.5g of tween 20 and 25 mL of water in a distillation fume hood.

A solution of 10 mL of deionized water was also applied to the soil at the same time.

The leaf samples were collected on 0 (prior to the initial treatment), 3, 6 (prior to the second treatment), 9, 12, 15 and 18 day intervals (from a single branch at a time) and stored at -80 °C for the further enzyme assay analysis.

The treatment repeated exactly in a similar manner at day 6.

The enzyme extraction, assay and the statistical summarizations were conducted as per the sections 2.4.1, 2.3.3 & 3.1. respectively.

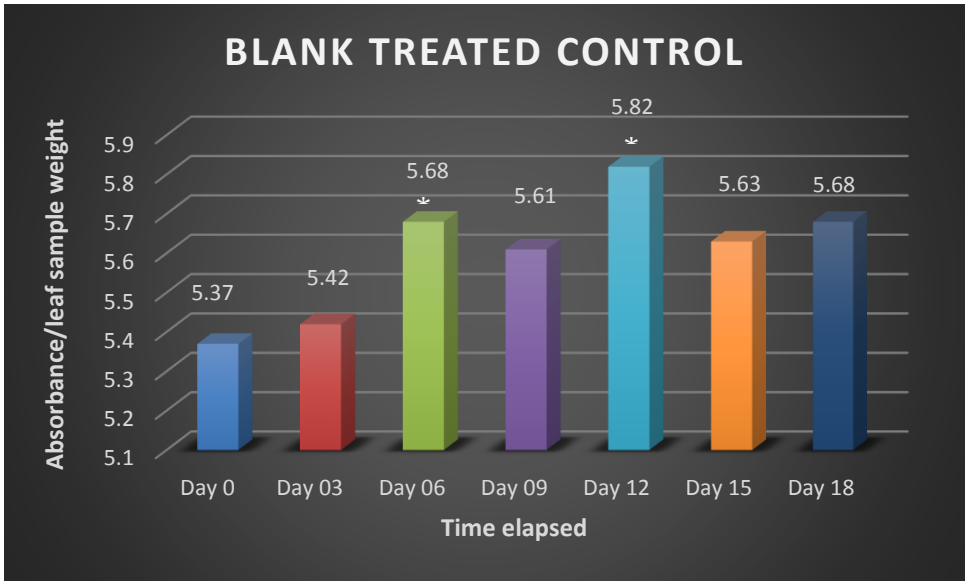
See appendix for the detailed statistical analysis for the peroxidase activity on blank treated control plant with day 6 retreatment.

The results comparing the peroxidase activity after the application of the Q test at different day intervals are given below,

**Table 3.XVI. Peroxidase assay results showing enzyme activity at different day interval**

| **Kauri leaf assay** | **Absorbance at 480 nm/Leaf sample weight (g) (at 6 minutes) ( $\bar{x}$, n=5 )** |
|---|---|
| Blank | 0 |
| Day 0 (Initial treatment) | 5.37 |
| Day 3 | 5.42 |
| Day 6 (Retreatment) | 5.68 |
| Day 9 | 5.61 |
| Day 12 | 5.82 |
| Day 15 | 5.63 |
| Day 18 | 5.68 |

**Fig. 3.i. Comparison of peroxidase activity by absorbance/Leaf sample weight at different day interval on blank treated control plant with day 6 retreatment**




* $p < 0.05$, ** $p < 0.01$ Compared to control (day 0)

The peroxidase activity showed a very slight increase in activity at different day intervals. As mentioned earlier in the report, it could be because of the mechanical wounding by the leaf harvesting or the contributory effect by the additional reagents used in the hormone treatment or could be the effect of both. However the percentage increase (8.3%) was very weak compared to the other treatments.

**Table 3.XVII. Two tailed *t* test *p*-value comparison of peroxidase activity by absorbance against day 0 (absorbance prior to the treatment)**

| **Days** | ***p*- value** |
|---|---|
| Day 0 vs day 3 | 0.28505955 |
| Day 0 vs day 6 | $5.5729\times 10^{-05}$ |
| Day 0 vs day 9 | 0.000319869 |
| Day 0 vs day 12 | $2.1826\times 10^{-05}$ |
| Day 0 vs day 15 | 0.000168502 |
| Day 0 vs day 18 | 0.0004828211 |

## 3.10. Data comparison for all treatments and blank treated control

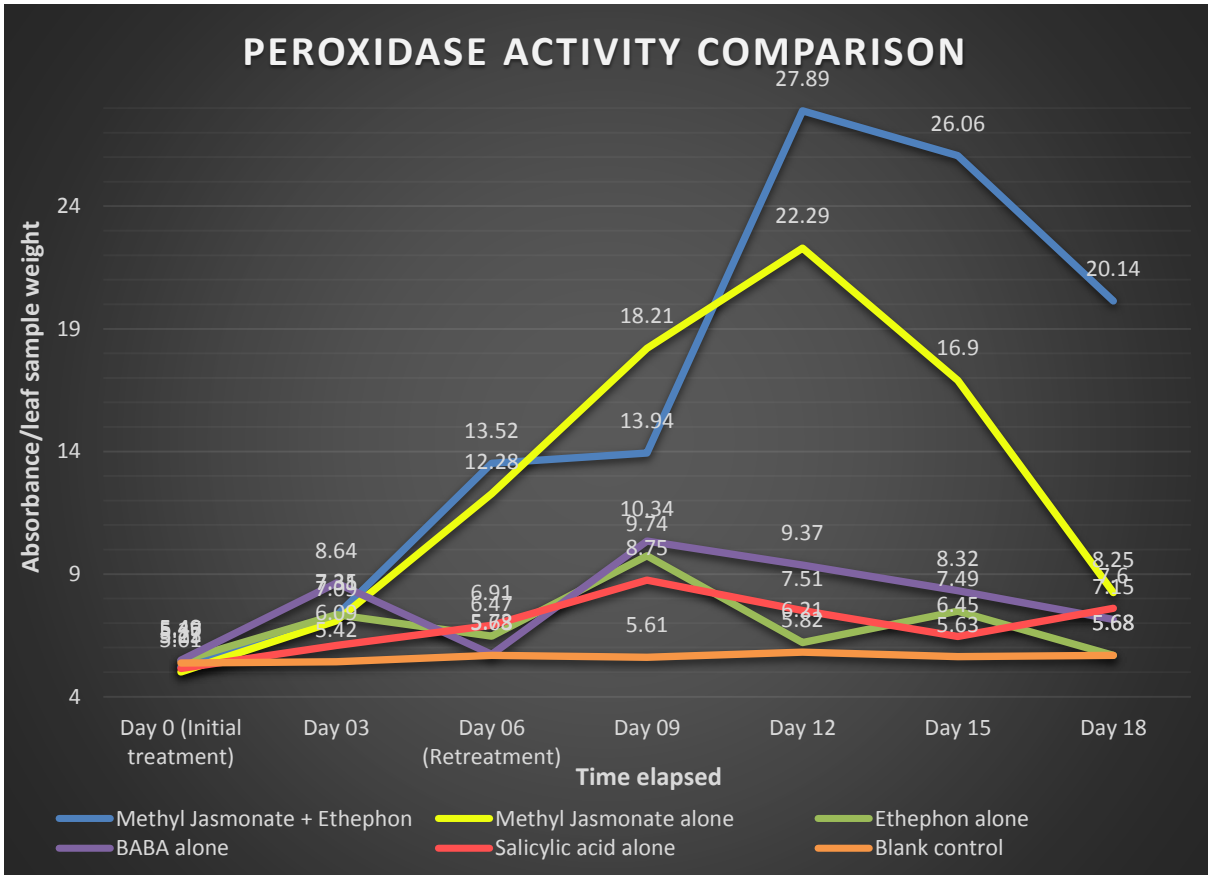



**Fig. 3.j. Data comparison for all treatments and blank treated control**

The synergetic combination of methyl jasmonate and ethephon is observed to be the most effective hormone treatment option to induce the peroxidase activity level in the kauri tree. The hormone combination made a 379.21% increase in enzyme activity at day 12 when compared to the activity level at day 12 with the blank treated control plant. The hormone treatments with *β*-amino-butyric acid (BABA) (84.31 % increase) and salicylic acid (55.97% increase) also noticeably improved the enzyme activity in the kauri tree. Even though the individual hormone applications, methyl jasmonate (282.99% increase at day 12) and ethephon (73.62 % increase at day 9) alone, could make a significant impact on the peroxidase activity, the combination is observed to be the most effective treatment option.

## 3.11. Discussion

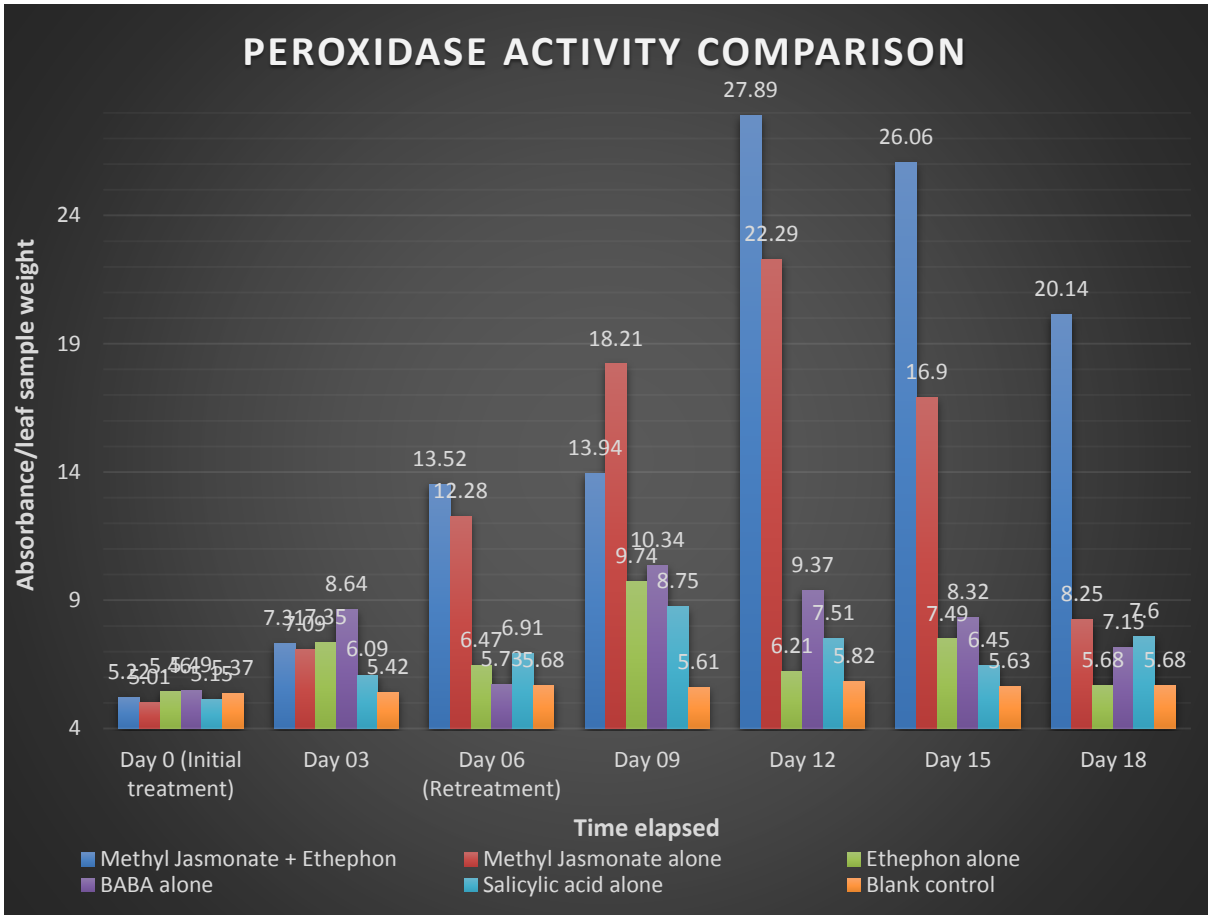



**Fig. 3.k. Data comparison for all treatments and blank treated control**

Two possible options were considered at the time of the commencement of the current research. Either treat one hormone on multiple trees or treat multiple hormones on individual trees. The first option gives better replication but less information whereas the second option gives more information but has less scientific certainty that the results are reproducible. While considering different factors, such as, limited time frame, length of the experimental procedures, uncertain nature of the enzyme analysis, restrain to avoid destroying too many plants for the experimental purposes, the second option was chosen to produce an efficient and meaningful result within the limited period of time.

In terms of kauri dieback, it is very hopeful to notice on the response of the kauri trees by different plant hormones towards the peroxidase activity. The peroxidase activity observed to be considerably significant and consistent with all the available hormone treatments within the 9-15 day intervals. Among them the synergetic combination of methyl jasmonate and ethephon

is observed to be the most effective and successful hormone treatment option in terms of the induction of peroxidase activity in the kauri trees.

In order to get the maximum reliability to the result, special considerations were given from the selection of the plant materials to the individual stages of enzyme extraction and assay procedures.

Since there were no known methods available specifically for the kauri peroxidase extraction and assay, the current research has given a large importance to the method development, especially in optimization of the experimental parameters for the maximum quantization of the peroxidase activity.

Similar aged (7-8 years) plant materials were purchased from a single local native plant nursery.

Even though it was time consuming the pilot studies were very useful and added a lot of value to the current research. Interestingly, the enzyme activities of the leaves from the different branches of the same plant showed a slight variation in its activity. In order to standardize the observations, the leaf harvestings were limited from a single branch at a time. Similarly the enzyme activity at the apical leaves were observed to be lower when compared to the activity levels of the mature leaves. So all the leaf samples was collected around the middle area of the branch at a time. Inclusion of the dried latex with the leaf samples was also observed to increase the enzyme activity, so, careful attention were taken not to include any kind of plant secretion with the leaf samples during the preservation or the analysis stages. And also it could be an interesting observation for the future studies in this area.

# Chapter 4. Conclusion

## 4.0. Conclusion

A potential new hormone treatment option for the kauri dieback disease has been developed. Foliar application of the synergistic combination of methyl jasmonate and ethephon was found to be an effective hormone treatment option for the induction of the defensive enzyme, peroxidase, in kauri trees. The optimum quantization of the peroxidase enzyme from the kauri leaf samples was achieved by the development of new enzyme extraction and assay methods with special reference to the kauri leaf tissue. Currently there is no treatment options available for an effective management of PTA in kauri trees, at the same time the current method is the first known hormone treatment option for the kauri dieback disease.

As the PTA is an exotic species, either the pathogenic attack bypasses the entire kauri natural defense system or the plants natural defense system is not capable of preventing the pathogenic attack. Either way the current findings are very relevant in terms of enhancement of the plants immunity. Because of the time constrain and the limitations of the resources it was not possible to analyze the effectiveness of the current hormone combination against the PTA infected plants. It is recommended to conduct further research in this area to measure the effectiveness of the hormone combination against the PTA infections on the Kauri trees.

At the same time the current research gives an insight for the many other options for future research opportunities. It will be worthwhile to analyze the peroxidase activity level with an extension of observations until day 24 or more with the same enzyme combination, methyl jasmonate and ethephon, with day 12 retreatment. Since ethephon and BABA show the same pattern of peroxidase activity levels with individual hormone treatments (observations of maximum activity at day 9 and comparatively less activity at day 6) and the combination of methyl jasmonate and ethephon were observed to be highly significant in terms of the induction of the peroxidase activity, it will be valuable to conduct further research into a synergistic combination of Methyl Jasmonate and BABA against the kauri peroxidase enzyme. A day 3 individual retreatment option for ethephon and BABA is also recommended for further research.

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# Appendix