The effect of plant hormones on phenolic production in Kauri trees

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Table of Contents

List of Tables

List of Figures

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.

Signed _____

Date _____

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I would like to gratefully thank Mark Duxbury for his guidance and supervision throughout the thesis and help whenever required. My gratitude and thanks to my wife Kristy Kwok who supported me throughout this thesis in so many ways. I would also like to thank Ms. Saeedeh Sadooghy-Saraby and Ms. Yan Wang for providing the glassware and chemicals required for the chemical testing.

Abstract

The aim of the research was to investigate if it is viable to use natural plant hormones to induce a defence response in the Kauri tree that might inhibit or stop infection via *Phytophthora.* Results showed that treatment with the hormone methyl jasmonate increased natural defensive phenolic compounds and lignin within the Kauri leaves. These phenolic compounds in turn were shown to inhibit but not stop the growth of *Phytophthora cinnamomi.* This was demonstrated in vivo and in vitro using detached leaf assays and inhibition of growth on V8 agar. Conversely treatment with the plant hormone salicylic acid was less effective at inducing phenolic production.

In conclusion it was found that plant hormone treatment alone was not enough to prevent *Phytophthora cinnamomi* from infecting Kauri. Although hormone treatment did increase phenolic production, this alone was not enough to eliminate the pathogen. In future it may be possible to combine plant hormone treatment with other methods to produce an effective means to eliminate or prevent further distribution of *Phytophthora.*

Chapter 1
Introduction

Life on Earth has existed for millions of years but perhaps the first complex multi-cell forms of life that existed were plants. Over this period of time plants have been able to develop sophisticated methods of defence to help ensure their survival (Walters 2010).

Although plants have gained many predators throughout the ages perhaps the most detrimental and hardest to resolve pests have been microbes. Due to the nature of microbes they can be hard to destroy and prevention can sometimes be economically and technically impossible.

Bacteria and fungi are capable of generating new strains with no way to predict when this could occur or what properties the new strain may acquire. These properties can include resistance to antibiotics, both natural and commercially produced, as well as other means of preventative measure such as anti-fungal elements which would be effective on the parental strain. Due to this constant genetic changing, microbial infections can be disastrous to agricultural crops as well as native forests alike.

As mentioned above vegetative infections are not particular in what plants are affected, that is to say any plant can be affected at any time. There are plants that have greater resistance to certain infections but no plant is immune to future infections which at this stage cannot be predicted.

Even though plants can be vulnerable there are certain defence mechanisms that they use to prevent and fight disease. These mechanisms are triggered by several conditions. They can include physical damage of the external layer of a plant, infection or environmental. Plants defence mechanisms can permanently be present on the plant (constitutive) or as previously mentioned can be activated by triggers (induced).

In reality the defence mechanisms of plants is quite complicated and requires a series of chemical pathways and changes to occur (Walters 2010). Pre mentioned triggers start a range of chemical reactions that can lead to cell sepsis or the production of chemicals that can be detrimental or fatal to invading pathogens or insects.

The aim of this thesis is to investigate if using the natural plant hormones methyl jasmonate and salicylic acid can induce a greater defence response within the kauri plant (*Agathis australis*). Furthermore to see if this response, if present, is possibly capable of inhibiting the spread of phytophthora in Kauri. The hypothesis to be tested is that phenolic compound production will increase within the plant following hormone treatment and this in turn will have a greater effect on inhibiting the growth of Phytophthora.

1.1 Agathis australis

One of the oldest and perhaps most well-known trees in New Zealand is the native *Agathis australis* or Kauri tree in the Maori language. Believed to originate and spreading globally during the Jurassic era this tree has become legend in the native folk lore. The largest tree in New Zealand is a Kauri named after the Maori forest god Tāne (Tane Mahuta), it is no surprise that this tree is seen as a national pride.

Although originally estimated to cover 1 – 1.5 million hectares in the New Zealand North Island, as of 2010 only 7500 hectares of old growth Kauri is now believed to survive. Originally logging for timber and furniture and fires were to blame for the great reduction in the number of trees, in modern times the decline of the Kauri is believed to be due to Kauri die back, a disease caused by a phytophthora species (Padamsee, Johansen et al. 2016).

1.2 Phytophthora

The genus *Phytophthora* is made up purely of plant damaging microorganisms. The name phytophthora literally means plant destroyer in the Greek language. *Phytophthora* are a type of Oomycetes which are eukaryotic organisms that appear similar to filamentous fungi (Kamoun, Furzer et al. 2015).

The first major outbreak of *Phytophthora*, and perhaps the most destructive in horticultural history, is the Irish Potato Famine between 1845 and 1846. The loss of a great number of potato crops due to *Phytophthora infestans* is believed to have lead to the death of up to a quarter of Ireland's population at the time, as many as 2 million people (Lamour 2013).

In New Zealand the species of Phytophthora causing major concern is that of *P. agathidicida*. This pathogen has been isolated from roots of dying and rotting *Agathis australis* (Kauri). Some symptoms of infection include the death of branches, lesions and the yellowing of the Kauri leaves. In some cases the infection can lead to the death of the tree. This disease is known as Kauri dieback and is regarded in modern times as the leading cause of declining numbers in the Kauri (Padamsee, Johansen et al. 2016). A second species of Phytophthora, *P. cinnamomi* , although less aggressive than *P. agathidicida* is also capable of killing Kauri under certain conditions and is widespread in the New Zealand environment (Newhook & Podger 1972, Johnstone et al 2009).

1.2.1 *Phytophthora agathidicida*

As mentioned above *P. agathidicida* has been found to be the pathogen responsible for Kauri dieback in New Zealand. Previously known as Phytophthora 'taxon Agathis' 'PTA', *P. agathidicida* was first identified as the pathogen causing Kauri dieback on mainland New Zealand as the connection was made between these Kauri and those found to be infected on Great Barrier Island (Beever, Coffey et al. 2007). Previously believed to be caused by *P. cocois* and *P. heveae*, research was done on the oospore width and results showed that although *P. cocois* and *P. heveae* did not have any significant difference in width, the oospores found in Kauri roots had a large width than any other *Phytophthora* species (Bellgard 2016).

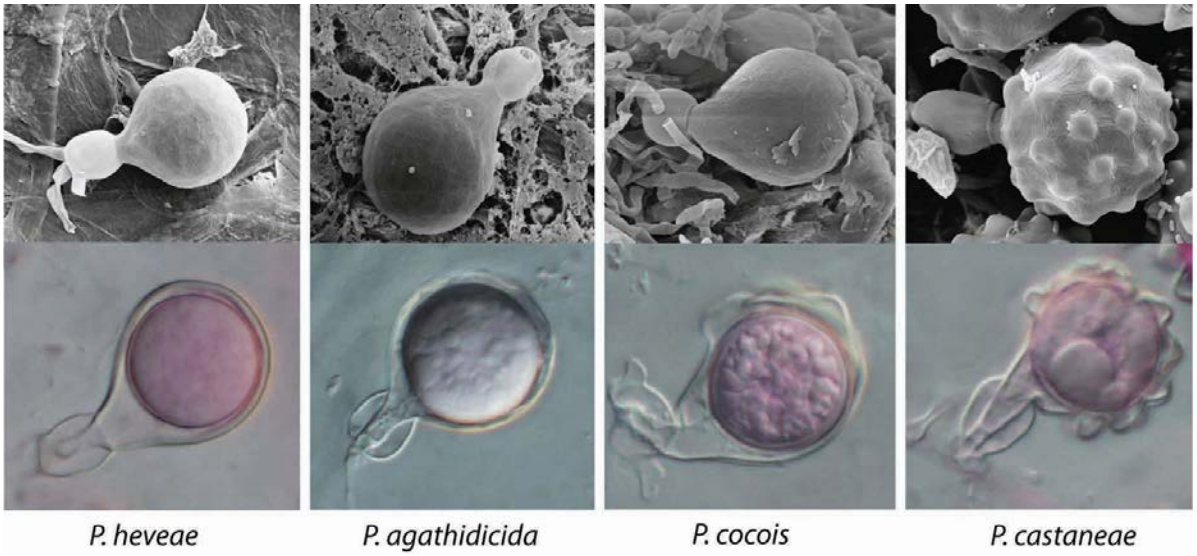


Figure 1 The above images show several morphological structures of various Phytophthora oogonium.

As with most *Phytophthora, P. agathidicida* infects the host plant, in this case Kauri, via the fine roots. From the fine roots the pathogen progresses further into the plant through the larger woody roots and from there infecting the cambium. Although very rare in some cases the xylem of the plant can be infected. Once the infection reaches the collar of the tree it forms what is known as a canker, sites at which tissue death occurs. Eventually the lesions caused can spread to form a ring entirely circling the trunk. The lesion ring causes vascular dysfunction which partially inhibits nutrients getting to the crown causing it to die off as well as chlorosis. This death usually occurs at the tips of the branches and slowly kills of the plant moving towards the trunk, hence the name "die back". (Beever, Coffey et al. 2007)

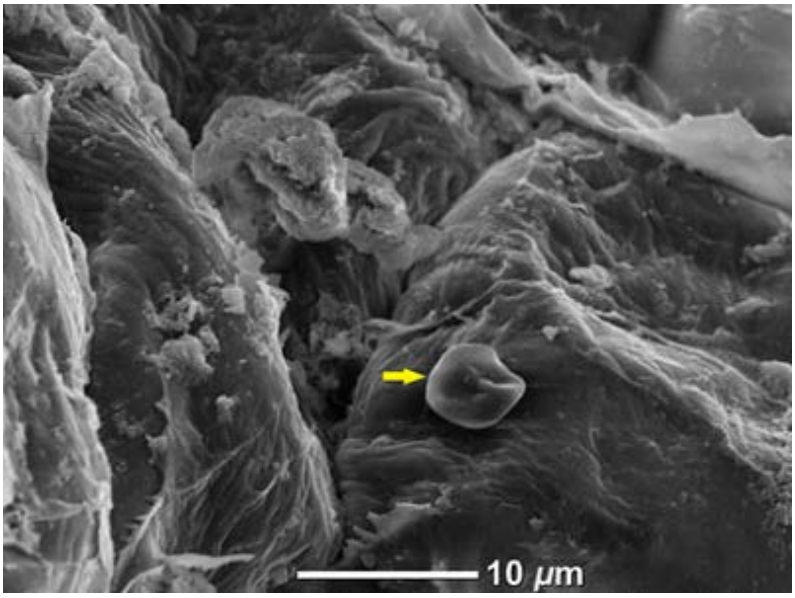


Figure 2 The image above shows a *Phytophthora agathidicida* cyst formed on a Kauri root (Beever, Coffey et al. 2007).

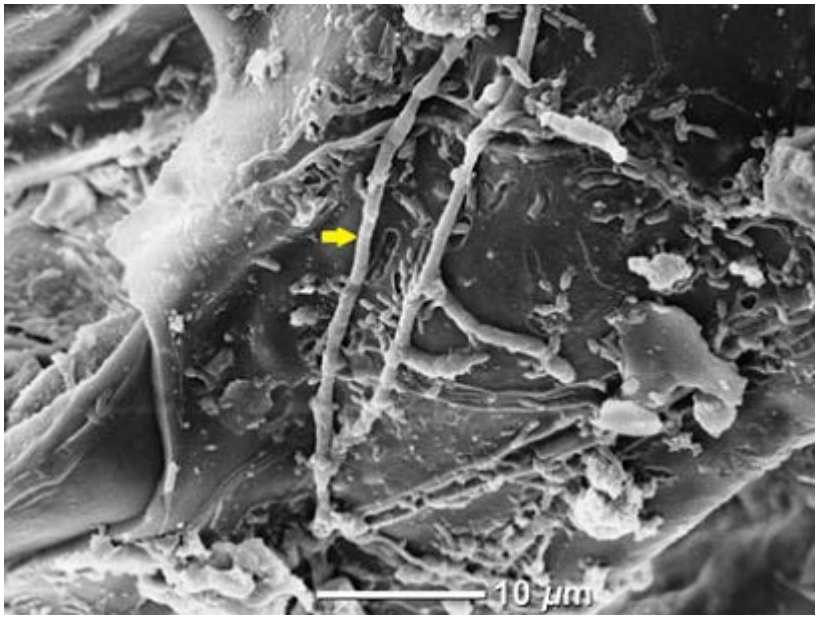


Figure 3 In the image above the ramifications of *Phytophthora agathidicida* hyphae can be seen.

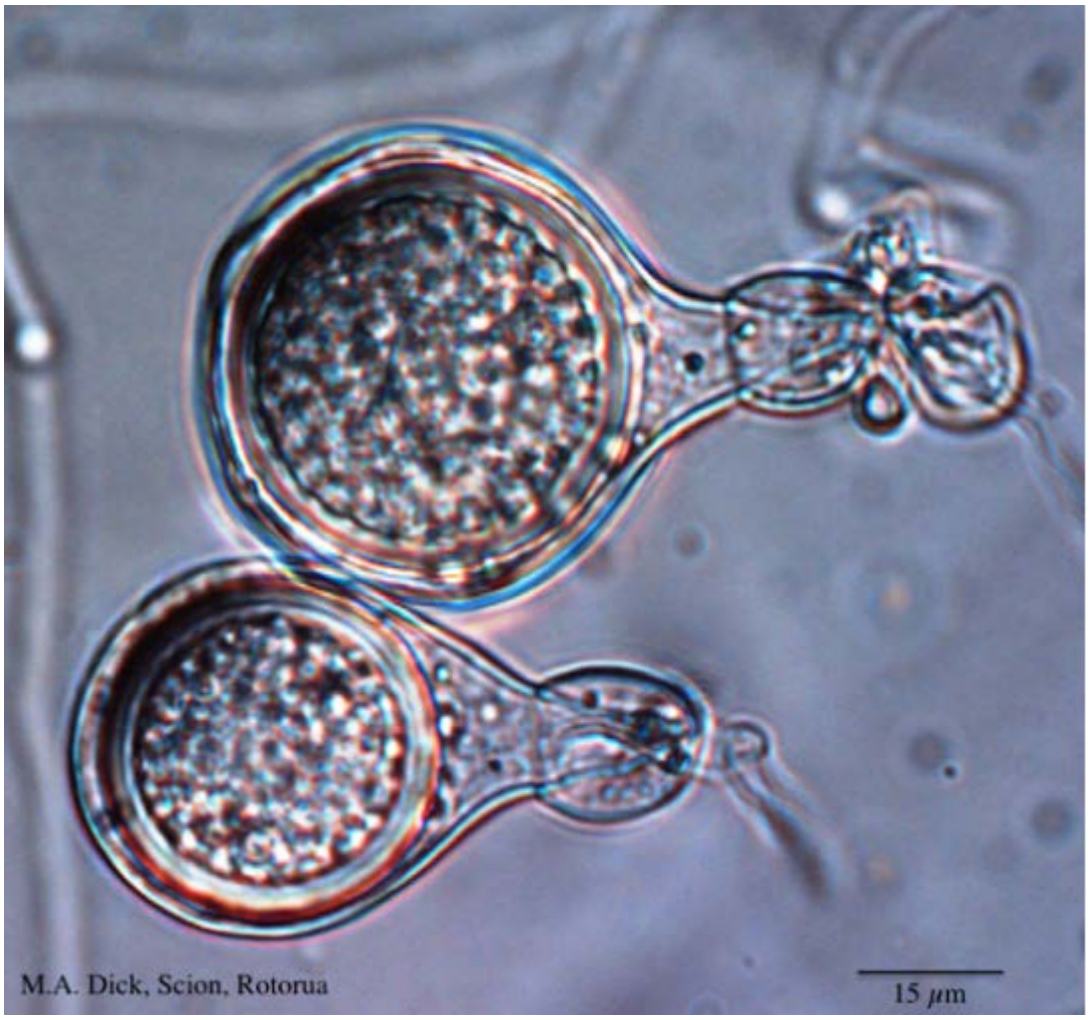


Figure 4 The above image is a light micrograph showing the oospores of *Phytophthora agathidicida* (Bellgard 2016).

1.2.2 *Phytophthora cinnamomi*

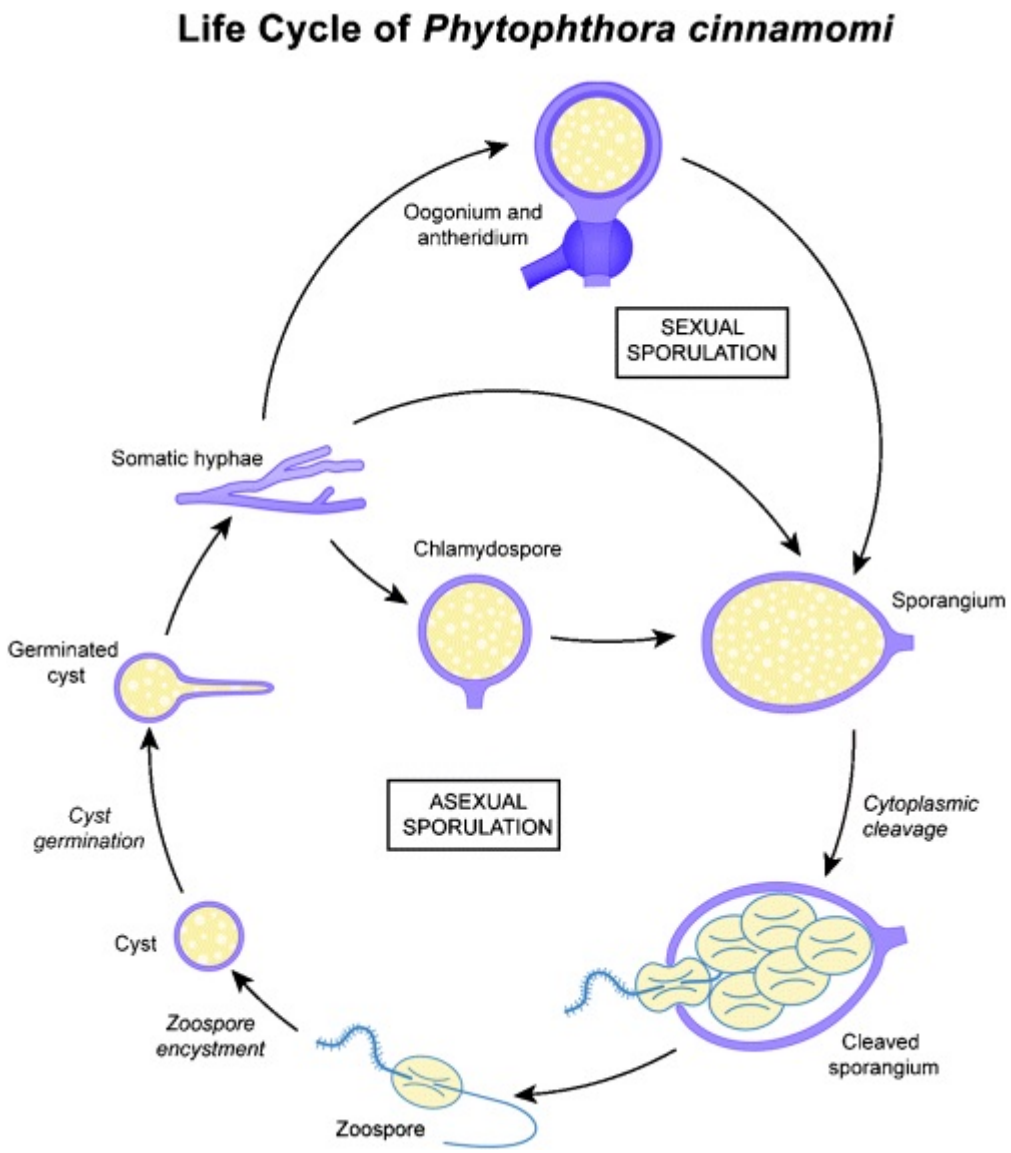
P. cinnamomi is one of the most destructive plant pathogens on a global scale, causing infections in a number of trees. The species name *cinnamomi* is derived from the plant from which it was first isolated, that being the cinnamon tree (Hardham 2005).An example of infection can be seen in the Mediterranean where it has been associated with the decline of *Quercus suber* also known as cork oak and in Brazil, it is known to cause root rot and crown root in *Araucaria angustifolia,* Brazilian pine. This plant pathogen also causes great economic loss through the infections of pineapples, cinnamon trees and avocado trees (Lamour 2013).

Country \ State	**Origin**	**Invasive**
Australia		
- Australian Northern Territory	Introduced	
- New South Wales	Introduced	
- Queensland	Introduced	Invasive
- South Australia	Introduced	Invasive
- Tasmania	Introduced	Invasive
- Victoria	Introduced	Invasive
- Western Australia	Introduced	Invasive
- Cook Islands		
- Fiji		
- Federated States of Micronesia		
New Zealand	Introduced	Invasive
Papua New Guinea		
Samoa		

(International 2016)

Table 1 information obtained from a datasheet produced by The Centre for Agriculture and Bioscience International. It shows the areas in Oceania in which *Phytophthora cinnamomi* is established.

It is believed that *Phytophthora cinnamomi* originated north of Australia in Papa New Guinea and was originally isolated in 1922 but as can be seen in the table, it has spread throughout Oceania (Hardham 2005). The main mode of infection is via roots where it can cause root rot, however it can also cause infection via stems causing stem cankers (Reitmann, Berger et al. 2016).



(Hardham 2005)

Figure 5 The above image shows the life cycle of *Phytophthora cinnamomi* including both asexual and sexual reproduction cycles.

The life cycle of *Phytophthora cinnamomi* begins as a zoospore which are produced within a sporangium. Once released from the sporangium the zoospore moves through any water contained with the soil until it comes into contact with a feeder root from a host plant. The zoospores form a cyst with the plant root tissue and will wait for favourable conductive

conditions before undertaking asexual sporulation. During this sporulation stage hyphae are produced which in turn develop new sporangium thus completing the life cycle (Ríos, Obregón et al. 2016).

Although *Phytophthora cinnamomi* can also reproduce sexually it is of rare concern as the requirements for oospore formation requires two mating types of *P. cinnamomi.* The two mating types, A1 and A2, are not distributed evenly in the environment. A1 is rare in comparison to A2, this is believed to be due to the ability of the A2 type to self produce by forming self oospores(Crone, McComb et al. 2013). In the case where sexual mating does occur between A1 and A2 types oospores are formed within an oogonium. These oospores are very hardy and consist of very thick cells walls with a chemical composition which allows them to survive conditions that would usually destroy zoospores, such a droughts or attack from other microbes (Jung, Colquhoun et al. 2013).

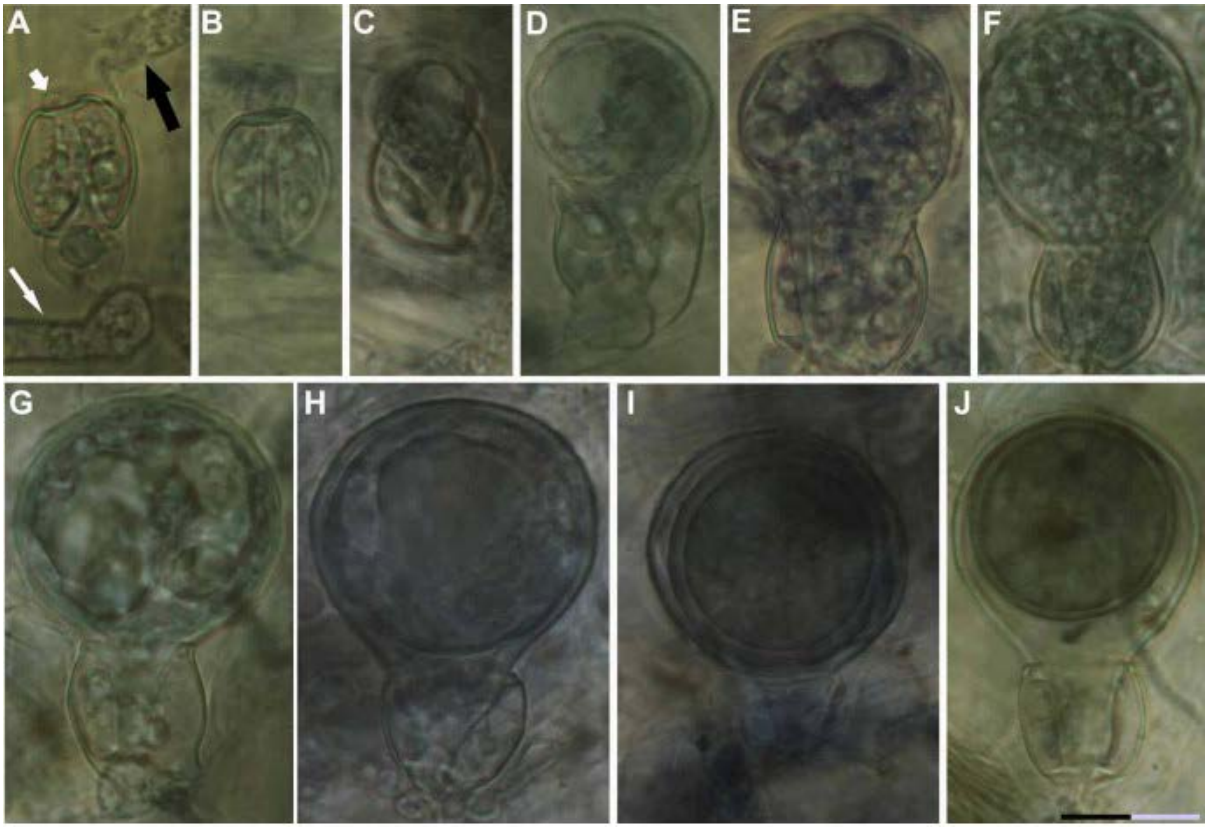


Figure 6 The above images show oospores from *Phytophthora cinnamomi*. In image A the short white arrow can be seen indicating the distal end of the oogonium while the long white arrow indicates the hyphae from which the oogonium was formed. The black arrow is pointing to the hyphae which is providing the antheridium. The images from A - I show the formation of a oospore at varies stages and J showing an aplerotic oospore (Crone, McComb et al. 2013).

P. cinnamomi zoospores provide the greatest danger to trees as they infect via soil. The zoospore eukaryotic cell is bi-flagellated and is therefore motile in any water contained in the soil. It uses this medium to spread through the soil making it hard to prevent and contain the pathogen. During this phase the zoospores find living plant material using electro and chemostatic signals (Turrà and Di Pietro 2015). The pathogen has also been found to survive up to 34 months after the death of the host plant creating a vector for contamination even after the tree is no longer living or of economical use (Collins, McComb et al. 2012). For this reason it is important in agricultural farming for infected trees to be disposed of correctly to help reduce spread to further plants.

In nurseries phenylamides have been used to control root rot caused by *Phytophthora* species. These phenylamides include metalaxyl which has also been shown to be effective in treating conifers. Another compound used is salts of phosphorus acid, this too has shown to be effective in several plant species in the defence against *Phytophthora.* When treated with the compounds plants have been shown to activate their own defences against the pathogen as well as the compound acting directly against it. In terms of industrial crops, potassium phosphonate has been used successfully in the reduction of root rot in radiata pine caused by *P. cinnamomi* (Reglinski, Spiers et al. 2009).

In Australia *P. cinnamomi* has become more a major concern in the Western Australia State. Jarrah dieback, the name given to the sudden dieback of jarrah trees in large numbers in the 1940's is caused by *P. cinnamomi*. The large forests made up of jarrah (*Eucalyptus marginata*) have largely been affected by this pathogen which was discovered to be the cause in 1964. Areas in which this disease was commonly occurring were described as waterlogged during the winter season. The conditions found during the summer months are known as hot and dry indicating that *P. cinnamomi* most likely survives during this in spore form, either as a chlamydospore or a oospore (Davison 2015).

Wet conditions have been found to be a link between severe outbreaks of dieback in the jarrah forest. It has been found that mass deaths occur in areas where a concreted lateritic layer is found to be within 1m of the soil surface. This layer causes water logging during the wet season which allows zoo spores to spread through the soil spreading the disease amongst suitable areas.

In 2007 the resistance of jarrah to *P. cinnamomi* has been discovered at a genetic level in jarrah in several plant trials. A study undertaken over 13 years found clones of genetically resistant jarrah grew with greater average heights and with decreased mortality than those without resistance (Stukely, Crane et al. 2007). These resistant plants could be valuable in

the rehabilitation of areas greatly affected by the dieback. Further investigation into the genes of these plants could also provide information for future treatments or prevention of *Phytophthora* infestations. This possible knowledge combined with current treatments, such as treatments with phosphorus acid based products, show promising signs in fighting dieback.

In terms of environment similar conditions can be seen between common areas in New Zealand and Western Australia were *phytophthora* species are present. Forests were Kauri are found in New Zealand tend to have high rain fall, much like those in the jarrah forest. The difference between the two environments being the long hot and dry summers found in the jarrah forests.

With respect to *P. cinnamomi* in New Zealand, Johnston et al made the following comment, "*Phytophthora cinnamomi* is not native to New Zealand, but perhaps it was introduced by Polynesians, as Phytophthora-like symptoms were observed in the early 1800's. The fungus is widespread in native forest soils. It can cause noticeable, localised damage during periods of weather suited to inoculum build-up (warm, wet winters), followed by periods of stress on the plants (unusually dry summers). However, the moderate New Zealand climate means that in native forests damage is rarely visible in above-ground parts of plants. Some roots are killed, but in New Zealand plants generally require only a proportion of their roots for normal growth. Despite major disease episodes being rare, P. cinnamomi could have subtle, longer term, effects on New Zealand's vegetation. Variation in plant susceptibility alone will cause changes to plant community structure over time. Seedlings of some major forest trees are highly susceptible. Although kauri (Agathis australis) forests appear to be re-establishing vigorously in many parts of northern New Zealand, at some sites seedlings survive only when protected against *Phytophthora* by fungicides" (Johnston et al 2009).

P. cinnamomi in New Zealand is wide spread in the native soil. It causes damage in low levels to native and introduced plant species in water logged conditions and can effect, to a small extent via seedling infections, commercial crops such as *pinus radiata*.

1.3 Types of defence

Plants are effectively the bottom of the food chain in that almost all living organisms can feed off and derive nutrients from plants in one way or another. There are several major organisms that attack and feed off plants, these are, microorganisms, insects, nematodes, other plants and animals. To compensate for this large range of predators plants have developed many methods of defence that range from simple passive structures to complicated hormone induced chemical defences (Walters 2010).

There are two categories of chemical defence in which a plant can defend itself. The two categories are phytoalexins, produced by the plants in response to attack, and phytoanticipins, produced by the plant during normal growth conditions.

When a plant is attacked by a microbial pathogen it produces phytoalexins *de novo.* This is considered a responsive defence as the phytoalexin chemicals are only synthesised in response to attack. Phytoanticipins are classified as compounds that are produced from chemicals already present in the plant after attack or chemicals that are already present in the plant itself before attack (Sanchez Maldonado, Schieber et al. 2015).

Within plants substrates and enzymes are separated from each via cellular compartments. The reason for this separation is to avoid the premature reactions that would take place when the substrates and enzymes mix producing biocidal compounds. When the plant is damaged either by microorganism or physically the cellular compound walls are destroyed. This causes the enzymes and substrates to chemically react and produce biocidal compounds which in turn can help defend the plant (Sanchez Maldonado, Schieber et al. 2015).

1.3.1 Structural defences

The most basic, but by no means insignificant, form of defence used by plants is the physical make up of the external plant. Perhaps one of the most common and well known examples is the use of thorns by a rose bush. These thorns help protect the plant from herbivores through the threat of pain and injury to any animal trying to physically make contact with the plant.

Another form of structural defence is the outer most layer of the plant. This outer layer is known as the cuticle and forms a protective barrier between the inner, nutrient filled, section of the plant and the outside environment.

1.3.2 Chemical defences

Regulation

As with animals, plants use hormones to control and regulate internal responses to certain situations. By situations I imply response to attack, change in environment and reproduction to name a few. Due to the specific nature of this study the hormones that control defence responses will be the key targets.

When a plant is harmed or in danger from an attacker one way in which it can defend itself is by producing chemicals via a complex pathway. The signal molecules used in plants are commonly known as jasmonates (Turner, Ellis et al. 2002). One of the most common jasmonates used by plants is a hormone known as methyl jasmonate.

When physically damaged plants produce the lipid linolenic acid. The build up of linolenic acid therefore often indicates that the plant is in some sort of distress. It makes sense then that linolenic acid is a precursor for jasmonic acid, which as mentioned previously, is used as a primary defence chemical by the plant. The degradation of precursors to form the required defence chemicals requires many different chemicals but mainly relies on enzymes which catalyse the degradation and formation of simpler chemicals.

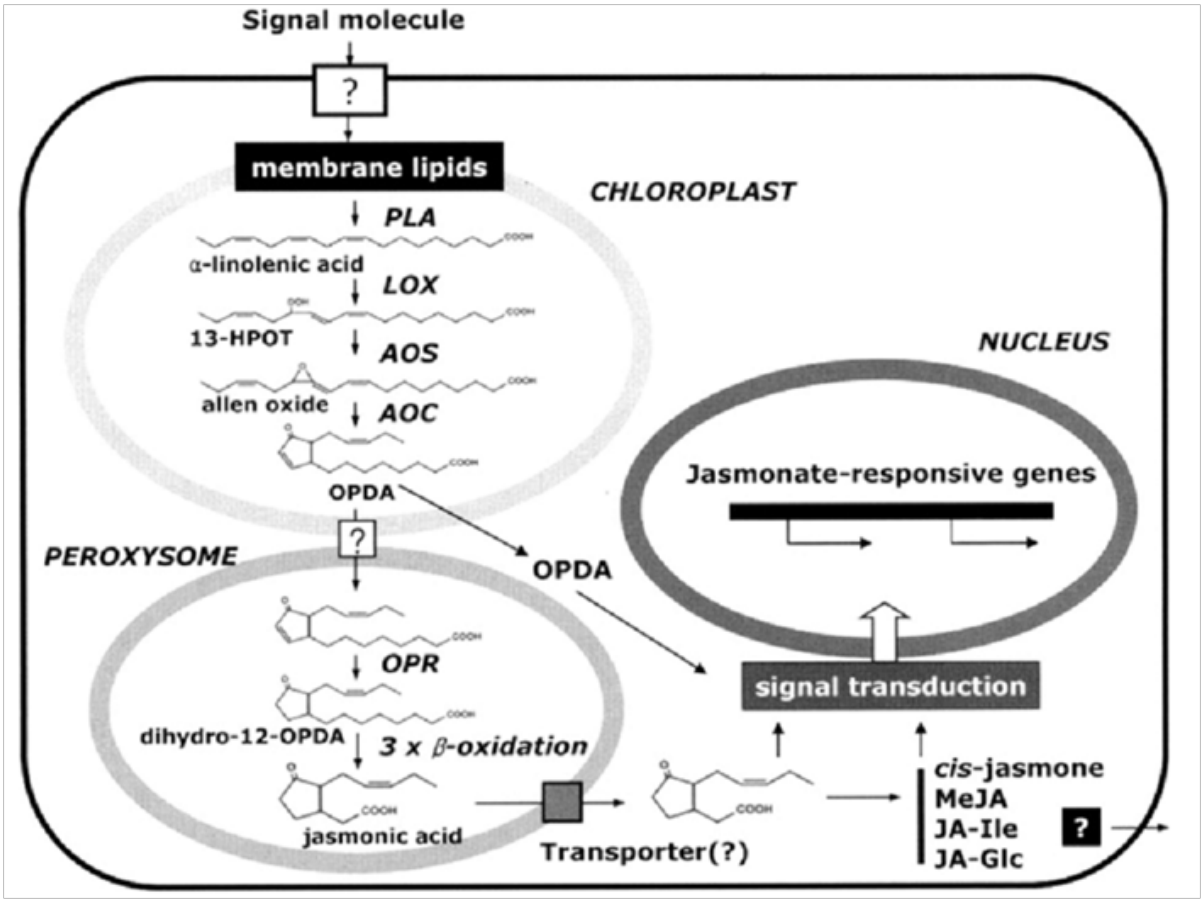


Figure 7 This figure shows the octadecanoic pathway by which jasmonic acid is formed with a plant (Cheong and Choi 2007).

The chemical pathway by which linolenic acid is converted to jasmonic acid is known as the octadecanoic pathway. This biochemical pathway begins with linolenic acid being released by lipases from the chloroplasts present in the plant. Within the chloroplasts two biosynthesis steps take place that are catalysed by the enzymes 13-LOX, 13-AOS and AOC. The end product of this synthesis within the chloroplasts is cis-(+)-12-oxophytodienoic acid. Once produced the oxophytodienoic acid is then transferred from the chloroplast to the peroxisomes via the cell cytosol(Svyatyna and Riemann 2012).

OPDA, now present in the peroxisomes, is reduced by the enzyme 12-Oxophytodienoate reductase 3, also known as OPR3, via three beta oxidation steps to form (3R,7R)-(-)-Jasmonic acid (JA) (Svyatyna and Riemann 2012).

One of the key groups chemicals in the defence of a plants are reactive oxygen species (ROS). This group of chemicals, which includes hydrogen peroxide H_2O_2, are involved after the infection of a plant by pathogens. ROS are key chemicals that act as a trigger and also directly in the formation of reactions that create protective agents(Yarullina, Kasimova et al. 2015).

1.3.3 Jasmonic acid

Jasmonic acid's role in the defence of plants is known to be as one of the chemicals responsible for the induction in plant tissue of ROS and proteins responsible for challenges to the plant from pathogens. As mentioned above jasmonic acid is a product of the octadecanoid pathway and in turn is a signal chemical used to trigger the production of proteins and proteinase inhibitors (Yang, Tang et al. 2011). During this thesis methyl jasmonate was used as a treatment hormone. Methyl jasmonate is an esterified volatile form of jasmonic acid which is commonly used in experiments where hormone treatments are used.

In plants growing in favourable conditions with no current or recent attack or infection, very low levels of jasmonic acid will be present. Jasmonic acid will accumulate however in plants when infection or physical damage has occurred.

Plants treated with jasmonic acid have been shown to have an increased production of wound induced proteins. An example of this is an increase in the enzyme Phenylalanine Ammonia Lyase (PAL) which controls conversion of the amino acid phenylalanine into a series of phenolic compounds and lignin via the phenylpropanoid pathway. Jasmonic acid also induces proteinase inhibitors. Considered a less specific signal, as any wounding of the plant will cause production, jasmonic acid is seen to be more effective in defending plants against necrothrophic pathogens and phytophages (Ahmad, Hayat et al. 2013).

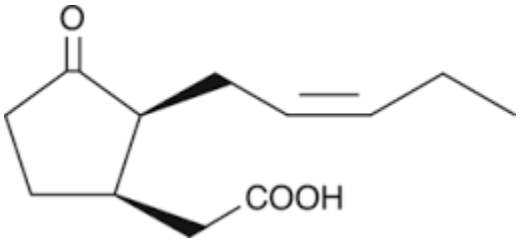


Figure 8 The above image is the chemical structure of jasmonic acid

1.3.4 Salicylic acid

2-hydroxy benzoic acid, commonly known as salicylic acid, as a plant hormone has many functions in plants. The roles vary from influencing seed germination and stomatal closure to respiration. Although these functions are affected by salicylic acid, the effects may not be direct. This is due to salicylic acid modulating the signalling of other hormones within the plant such as jasmonic acid (Ahmad, Hayat et al. 2013).

Salicylic acid is involved in the promoted production of H_2O_2. SA inhibits the activity of catalase, which breaks down H_2O_2 into water and oxygen, causing an increase of intracellular H_2O_2, thus increasing radicals via self-transformation causing lipid peroxidation. The radicals can help create H_2O_2 by acting on nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase within the plasma membrane of the cell producing H_2O_2 (Li, Liu et al. 2013).

Although salicylic acid is essential in the defence of plants it can also inhibit the effect of other defensive chemicals. Research has shown that plants treated with salicylic acid have shown a decrease in activity associated with jasmonic acid. This in turn can have a detrimental effect on the defence of a plant. A synergism effect however was found to exist between jasmonic acid and salicylic acid with plants. This effect was in the metabolism of phenolics in relation to Phenylalanine ammonia lyase (PAL), which has an effect on defensive structures such as lignin (Yang, Tang et al. 2011).

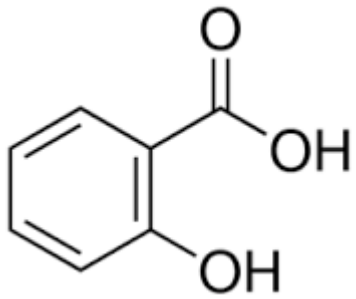


Figure 9 The above image is the chemical structure of salicylic acid

1.4 Phenolic compounds

Phenolic compounds are found naturally within higher plant species with many of them shown to have anti-microbial effects. These compounds can be grouped into soluble and insoluble compounds depending on where they are found within the plant. Soluble phenolic compounds can be easily extracted using organic solvents however insoluble phenolic compounds are more difficult to extract due to the nature of their location. Insoluble phenolic compounds are found bound to proteins and polysaccharides in the cell wall.

Although insoluble phenolic compounds cannot be extracted together with the soluble phenolic compounds, it is important to measure both to get an effective measure of the total content within a plant (Wang, Huang et al. 2016). Another factor taken into account is number of different phenolic compounds, which can number into the thousands (Dias, Sousa et al. 2016). By carrying out tests on the total group of compounds much time can be saved by avoiding complex separation techniques.

One known property of phenolic compounds is their fungistatic effect, helping to prevent the spread of a pathogenic fungal infection (Cvikrová, Malá et al. 2006). As a triggered response the plant produces phenolic compounds to try and halt any advancement of the attacker. This could be biotic or abiotic.

There are many chemicals grouped under phenolic compounds however this study will focus on a few chosen due to their known characteristics in plant defence.

1.4.1 Tannins

Tannins are found throughout plants including bark, leaves and fruit. The molecular weight is relatively small in the range of 500 to 3000. The use of tannins within a plant consists of microorganism deterrents as well as defence against external predators (Sanchez Maldonado, Schieber et al. 2015). There are two types of tannins present naturally in plants, they are grouped as hydrolysable tannins and condensed tannins. The basic structure of condensed tannins consists of carbon to carbon linked flavan-3-ol monomeric units. Hydrolysable tannins on the other hand consist of polyphenols containing a polyol central core. Gymnosperms are known to produce only condensed tannins whilst dicotyledons

produce a combination of both hydrolysable and condensed tannins (Selvakumar, Saha et al. 2007).

The four most common monomers involved in the synthesising of tannins are catechin, gallocatechin, fisetinidol and robinetinidol. These four monomers are the predominant structural building blocks for the following oligomers, procyanidin, prodelphinidin, profisetinidin and prorobinetinidin respectively.

Buds from pinus spp. were analysed to find the composition of flavan-3-ols within. The research found that the majority of flavan-3-ols found within the buds were epigallocatechin benzyl mercaptan adduct followed closely by epicatechin benzyl mercaptan adduct. Smaller quantities of catechin benzyl mercaptan adduct and catechin were also found within the extraction (Ropiak, Ramsay et al. 2016).

In terms of bacterial protection it has been found that extracts from Pinus spp. needles can have an inhibitory effect against common agriculture pathogens e.g. Azotobacter sp., Rhizobium sp. and Bacillus halodurans. Interestingly it was also discovered that the extraction solution impacted on the inhibition. When tannins were extracted using a solution of just water inhibition was found to be greater compared to tannins extracted with 70% acetone solution. The amount of tannins extracted, however, in terms of tannic acids, was greater with the acetone solution compared with water extraction (Selvakumar, Saha et al. 2007).

Fungal cultures were found to be more resistant to tannins extracted from pine needles than bacteria. The tannin extracts showed no inhibition in the growth of *Pleurotus djamor* however inhibition was seen in higher concentration of extract with *Trichoderma virescens* and *Trichoderma reesii*. Tannin concentration of 3000ppm were required before inhibition could be seen in the fungal cultures where as the bacterial cultures were inhibited with as low as 50ppm (Selvakumar, Saha et al. 2007).

Some fungal cultures have been found to produce tannases, this is most likely the reason that fungal cultures are more immune to the inhibition of tannins that bacteria (Purohit, Dutta et al. 2006).

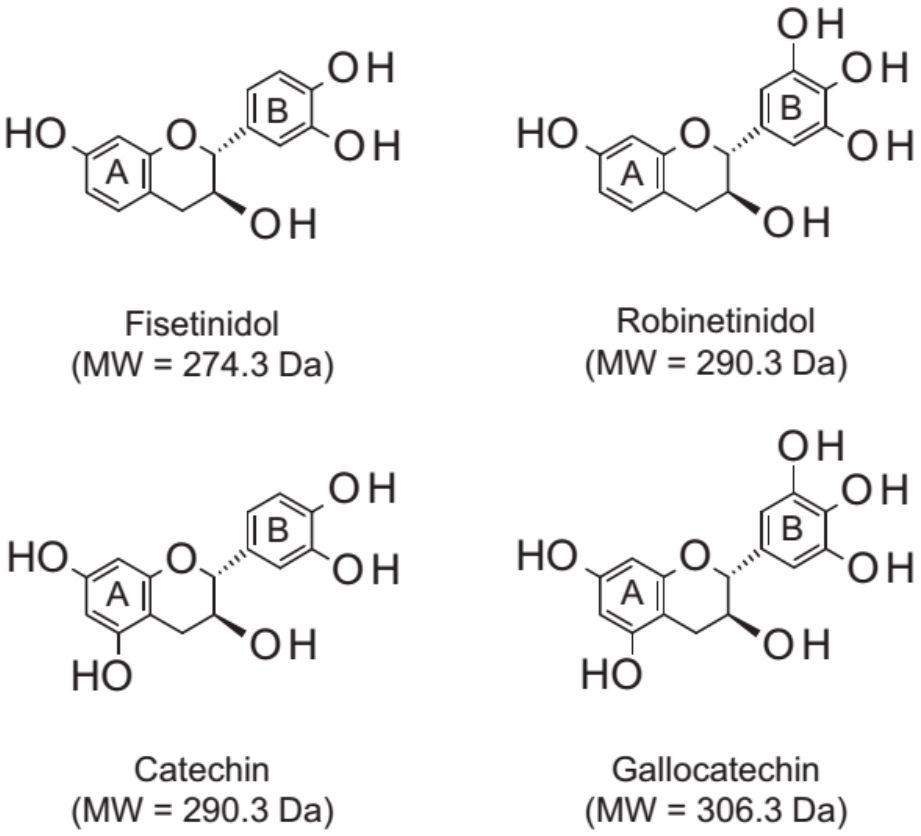


Figure 10 The above image shows the four most common flavan-3-ol units found in condensed tannins and their structure (Bianchi, Gloess et al. 2014).

Tannins have been investigated for the biocidal effects that they can have on pathogenic parasites and other microorganisms. Research has been done to show the effects of tannins, in particular condensed tannins, in vitro and vivo. An example is the use of tannins extracted from Pinus radiata to inhibit the larval development and viability of two livestock nematodes (Teladorsagia circumcincta and Trichostrongylus colubriformis). It was found that by introducing condensed tannins into the diets of ruminant animals, in amounts found normally in their foliage, the nematodes development was inhibited and therefore showing that the condensed tannins are directly effecting the viability of the parasites (Molan 2014). Other research has also shown that condensed tannins extracted from Pinus ssp. can have an excystation effect on Hymenolepis diminuta (Dhakal, Meyling et al. 2015).

1.4.2 Lignin

Lignin is one of the most abundant phenolic polymer compounds found in a plant and is used to create rigidity within the plant as well as exterior strength. Vascular plants have secondary cells walls that are comprised with a large percentage of lignin or lignin like compounds. Within cells the amount of lignin varies, this includes the taxa, types and can even vary within the same cell (Bagniewska-Zadworna, Barakat et al. 2014).

Many plants produce lignin as a form of defence during infection, invasion or through physical damage. The process of lignification involves the formation and deposition of phenolic polymers, with the majority being lignin's. This process strengthens the vascular body of the plant and can help prevent further damage to the plant (Barceló 1997).

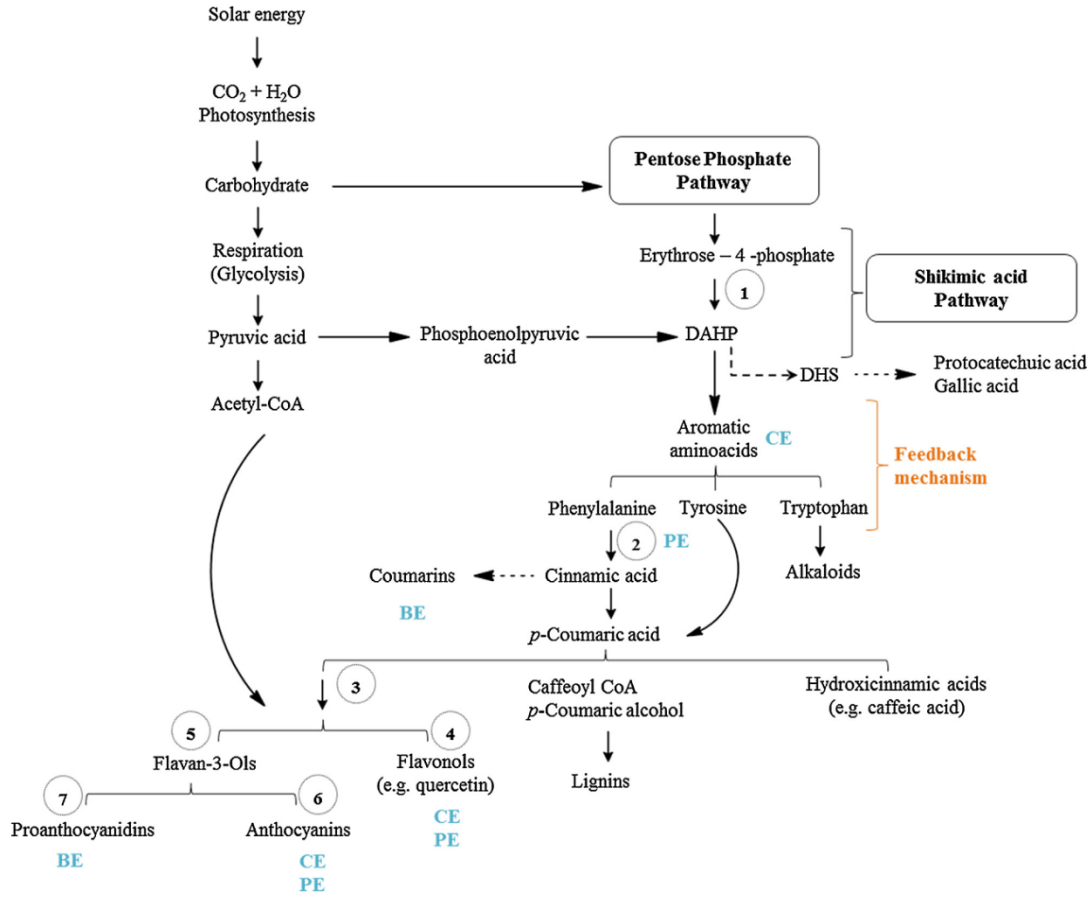


Figure 11 The above is a chemical pathway showing the synthesis of some of the more common phenolic compounds including Lignins, Flavonols, Gallic acid and Anthocyanins (Dias, Sousa et al. 2016).

The basic lignin structure consists of three primary monolignols. These consist of *p*-coumaryl, coniferyl, and sinapyl alcohol, which are coupled through an oxidation reaction. When the three monolignols produce lignin they create three different lignin units. *p*-coumaryl,

coniferyl, and sinapyl alcohol produce the lignin units H (*p*-hydroxyphenyl), G (guaiacyl), and S (syringyl), respectively (del Río, Lino et al. 2015). The three primary monolignols are bound in various orders through carbon-carbon bonds and ether bonds. The above units are bonded together randomly through carbon to carbon bonds and ester bonds. Lignin can then finally be grouped on three separate grounds depending on the units it consists of, these groups are G, SG, HSG. As the name suggest these lignin's contain either guaiacyl units, guaiacyl and syringyl units or a combination of all three of the groups (Gong, Xiang et al. 2016). For this reason the lignin composition contained in different taxa of plants and in fact even within different regions of the same plant can be different from one another.

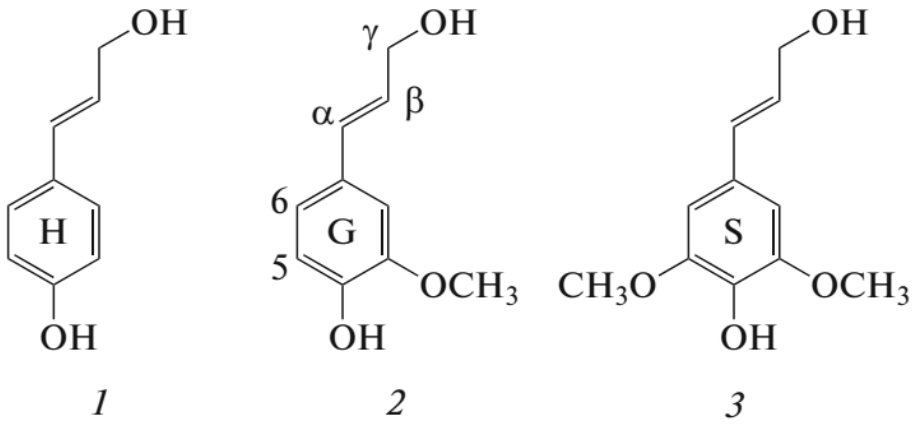


Figure 12 The above image shows the three monolignols that create the precursor units of lignin. (1) *p*-coumaryl, (2) coniferyl and (3) sinapyl (Feofilova and Mysyakina 2016)

Although it is not uncommon to find a mixture of lignin within a plant in general the lignin unit groups can be quantified within plant groups. For example lignin found in the wood of conifer trees, which include Kauri, consist mainly of G lignin groups and only a small amount of H groups with a trace amount of S groups. This can be compared to deciduous trees which contain lignin consisting of an almost equal amount of S group and G group lignin units with only a trace amount of H group units. Along with composition the amount of lignin present within a plant groups varies, coniferous wood for example contains on average 23-38% lignin whilst deciduous trees contain only 14-25%. Visually this can be seen with coniferous trees commonly having a thicker bark layer when compared to deciduous trees (Feofilova and Mysyakina 2016).

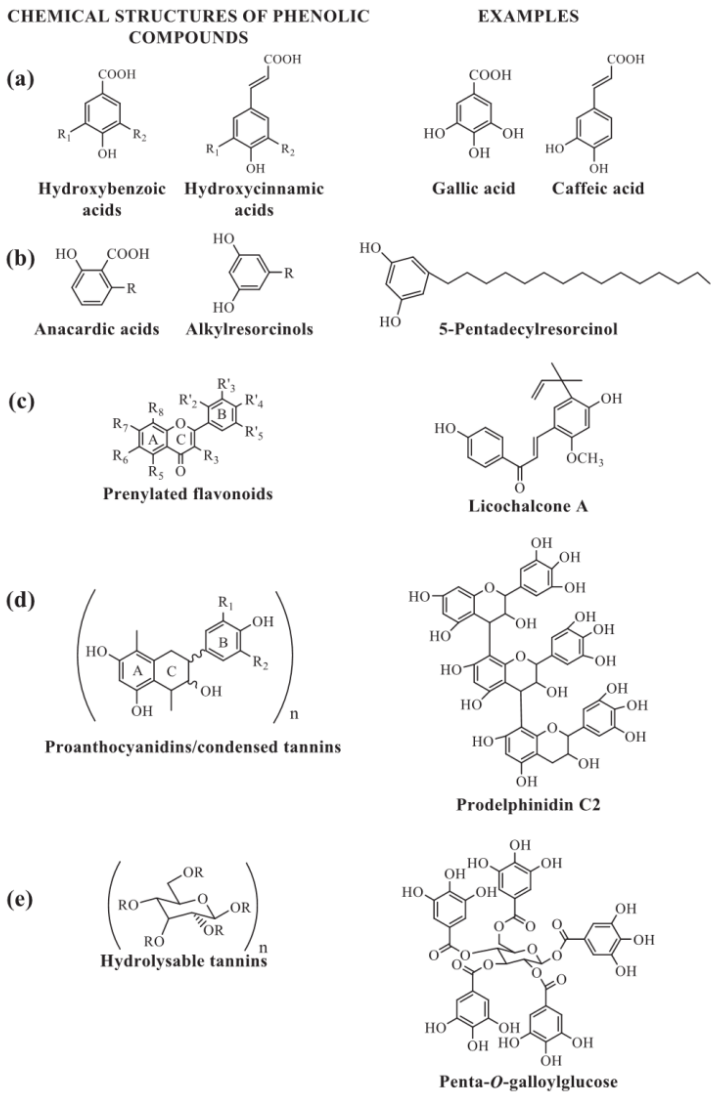


Figure 13 The above figure shows five commonly found phenolic compounds and examples of each (Sanchez Maldonado, Schieber et al. 2015).

Pine trees have being shown to be high in flavonoids and tannins and for this reason are commonly used in traditional and even modern medicines(Sokół-Łętowska, Oszmiański et al. 2007).

The aim of this thesis is to investigate if using natural the plant hormones methyl jasmonate and salicylic acid can induce a greater defence response within the kauri plant. Furthermore to see if this response, if present, is possibly capable of inhibiting the spread of phytophthora in Kauri. The hypothesis to be tested is that phenolic compound production will increase within the plant following hormone treatment and this in turn will have a greater effect on inhibiting the growth of phytophthora.

Chapter 2

Materials and Methods

2.1 Leaf treatment

Juvenile Kauri plants were purchased from Oratia Native Plant Nursery in Oratia West Auckland. The jasmonic acid solution used to treat the plants was made up by dissolving 23mg of methyl jasmonate in 200µl of 99% ethanol and 50µg of tween20. The mixture was then diluted with 25mls of deionised H_2O to give a final concentration of 4.1mM. Another stronger concentration was made up using 115mg of methyl jasmonate in 200µl of 99% ethanol and 50µg of tween20 also diluted with 25mls of deionised water to give a final concentration of 20.5mM. Solutions for salicylic acid were made using 15.1mg and 75.5mg respectively both dissolved in 200µl of 99% ethanol and 50µg of tween20 and diluted with 25mls of deionised water to give final concentrations of 4.4 mM and 21.9 mM.

Five Kauri plants for each treatment were covered in their entirety by the dissolved solutions using a pressurised atomiser device to ensure an even spread. Two control Kauri plants for each chemical were treated with a solution made up purely of 200µl of 99% ethanol, 50µg of tween20 and 25mls of deionised water. The trees were all kept in the same conditions and all constantly had water added to a container that the plants were resting in.

Leaves were collected from the plants before treatment and at 3 days, 6 days and 9 days after treatment. After each collection the leaves were bagged individually and placed into a -20^{o}C freezer. The leaves were kept frozen until all samples up to day 9 were collected and processed within the week of final collection.

<u>Sample preparation for assays</u>

50µg of plant leaf tissue was finely sliced using a sterile scalpel and transferred into a semi-micro glass test tube. 1mL of 80% acetone solution was then added to the tube. The sample was homogenised using a ULTRA-TURRAX T25 homogeniser with a 8mm attachment until an even blend was achieved.

Figure 14 The above image shows the ULTRA TURRAX T25 with a 8mm 8G dispensing unit attached used to homogenise the leaf samples.

Once homogenised the sample was transferred into a Eppendorf microcentrifuge tube. The remaining residue was rinsed from the tube using a further 400µl of 80% acetone solution. Using a vortex-genie with a multi head attachment the samples were then vigorously mixed for 15 minutes to extract the soluble phenolics.

After being vortexed the Eppendorf tubes were placed into the HERMLE Z 216 MK centrifuge and spun at 10,000 R.P.M. for 4 minutes. The supernatant was then pipetted off into a 5ml volumetric flask. A further 1ml of 80% acetone was placed into the eppendorf tube with the remaining pellet and was vortexed once again for 15 minutes. Once vortexed the sample was again centrifuged for 4 minutes at 10,000 R.P.M. and the supernatant pipetted off into the same flask. This process was repeated once more. The 5ml flask containing the leaf extract was then made up to the mark with 80% acetone solution. This solution is now considered the leaf soluble phenolic extract. The pellet is kept and dried at room temperature for insoluble phenolics and lignin assays.

Figure 15 The image above shows the HERMLE Z 216 MK centrifuge used during the phenolic assays.

2.2 Phenolic assays

2.2.1 Soluble phenolics assay

Gallic acid was used as a standard in this experiment in regards to phenolics. The gallic acid standard concentrations consisted of 0mg/L, 100mg/L, 200mg/L, 300mg/L, 400mg/L, 500mg/L, 600mg/L, 700mg/L, 800mg/L, 900mg/L and 1000mg/L. The range of standards was created rather wide as the expected results from the leaf extract was uncertain. 100µL aliquots of the blank solution (deionised H_2O), the gallic acid standards and the extraction sample solutions were pipetted into 10ml volumetric flasks. To each of these 10ml flasks 6ml of deionised H_2O was added followed by 500µL of Folin-Ciocalteu reagent. After 1 minute 1.5ml of 20% Na_2CO_3 was added to each flask. Each flask was then made up to the mark with deionised H_2O. The flasks were then left in a dark cupboard for 1hour before measuring the absorbance at 760nm using a Pharmacia Biotech Ultraspec 2000 Spectrophotometer.

Figure 16 The above image shows the Pharmacia Biotech Ultrospec 2000 Spectrophotometer used to measure absorbance's during the phenolic assays.

2.2.2 Insoluble phenolics assay

For this assay the pellet remaining from the soluble phenolics assay was dried at room temperature and used for the assay of insoluble phenolics (and also lignin). 1ml of 2N NaOH was added to the dry deposit left in the eppendorf tube to release phenolics esterified to the cell wall. The lid of the tube was loosened and the tube was then placed into a 70^{o}C water bath for 1hr. Due to the pressure build up within the tube it was important to loosen the lid, without loosening of the lid the sample can be compromised as the lid can explode open causing loss of sample inside. After the water bath the sample is left to cool and is centrifuged for 3 minutes at 10,000 R.P.M. The supernatant was then pipetted into a 10ml volumetric flask. The pellet was kept and dried at room temperature for the lignin assay.

To the flask 6ml of deionised H_2O was slowly added, after which 500µl of Folin-Ciocalteu reagent was added. After 1 minute has passed 1.5ml of 20% Na_2CO_3 was added to the flask. This solution was then left to stand for 1 hour in a dark cupboard after which the absorbance was read at 760nm using a quartz cuvette in a Pharmacia Biotech Ultraspec 2000 Spectrophotometer. The standards used for this assay were the same that were used in the soluble phenolics assay as the procedure was carried out in parallel.

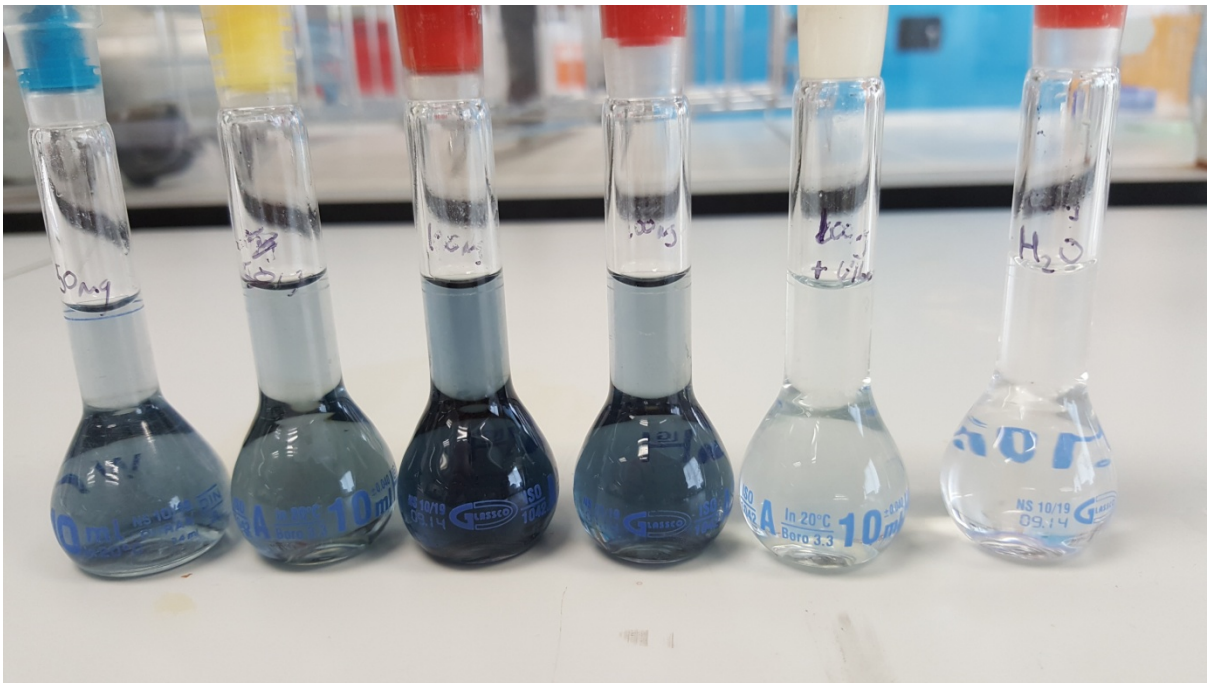


Figure 17 The above image is an example of the colour developed in the presence of phenolics during the soluble and insoluble phenolics assays.

2.2.3 Tannin assay

100µl of the original Kauri leaf extract was pipetted into an Eppendorf tube to which 900µl of tannin reagent was added. This reagent consisted of 50mg of $NH_4Fe(SO_4)_2.12H_2O$ in 90mL of butanol and 10mL of 12N HCl (Ojeda, Andary et al. 2002).

The eppendorf tubes were then placed into a 100^{o}C water bath and left for 20mins. After cooling to room temperature the tannin solution was pipetted into a 5ml volumetric flask and made up to the mark with butanol. This solution was then placed into a quartz cuvette and the absorbance was measured at 550nm with a Pharmacia Biotech Ultraspec 2000 Spectrophotometer.

Figure 18 The above image is an example of the colour developed in the presence of tannins during the tannin assay.

2.2.4 Lignin assay

The lignin assay was carried out on the residual material left after the soluble phenolics, insoluble phenolics and tannin extractions had been done. 5mg of the residual material was added to a 10ml glass sealable test tube. Inside a safety cabinet, 1mL of 25% acetyl bromide, made to concentration with diluted acetic acid, was then added and the test tube was sealed. The tube was then placed into a 70^{o}C water bath for 30mins. Once removed from the water bath the tubes were placed back into the safety cabinet and left to cool until at room temperature. During the cooling process 5ml of concentrated acetic acid was added to each tube. After the addition of acetic acid the tubes were each vortexed for 1 minute. 300µL of the digested lignin solution was added to a quartz cuvette. To this cuvette 400µL of 1.5 M NaOH was added followed by 300µL of 0.5 M hydroxylamine hydrochloride. The quartz cuvette was then mixed and diluted with 1.5mL of concentrated acetic acid. The absorbance of this solution was read with Pharmacia Biotech Ultraspec 2000 Spectrophotometer at a wavelength of 280nm (Chang, Chandra et al. 2008). The method works as shown below, where acetyl bromide (**4.10**) reacts with lignin (**4.11**) to form a lignin derivative (**4.12**) which is soluble under acidic conditions and absorbs at 280 nm due to the benzene rings.

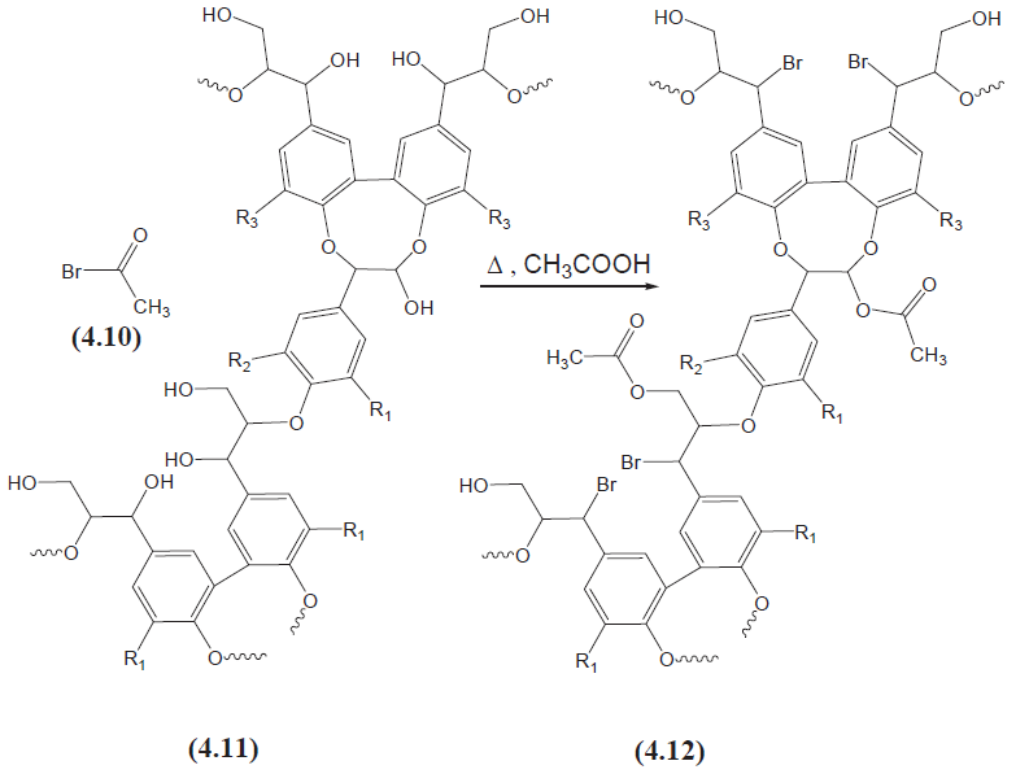


Figure 19 The image above shows the chemical reaction taking place during the lignin assay.

2.3 Culture experiments

2.3.1 Detached leaf assay

For this assay a branch was removed from a juvenile Kauri tree and placed, cut side down, into a solution of either deionised H_2O (control) or a treatment solution of 1% jasmonic acid or salicylic acid. The branch was left in the solution for 7 days in semi-controlled conditions to induce phenolic production. The leaves were then carefully removed from the branch and placed in a petri dish containing a paper towel moistened with 7ml of deionised H_2O . A 20 gauge needle was used to create a wound in the leaf near the petiole. A plate, inoculated in a lawn plate fashion with *P. cinnamomi* 7 days earlier, had a plug removed with a 20 gauge needle. The plug was then placed on the wound created on the leaf. The leaves were then left in the petri dish for 7 days in semi-controlled conditions. Three days into the experiment the paper towels looks relatively dry so a further 5mls of deionised H_2O was added to the edge of the paper towel of each sample. This was repeated again at day 5 on all samples.

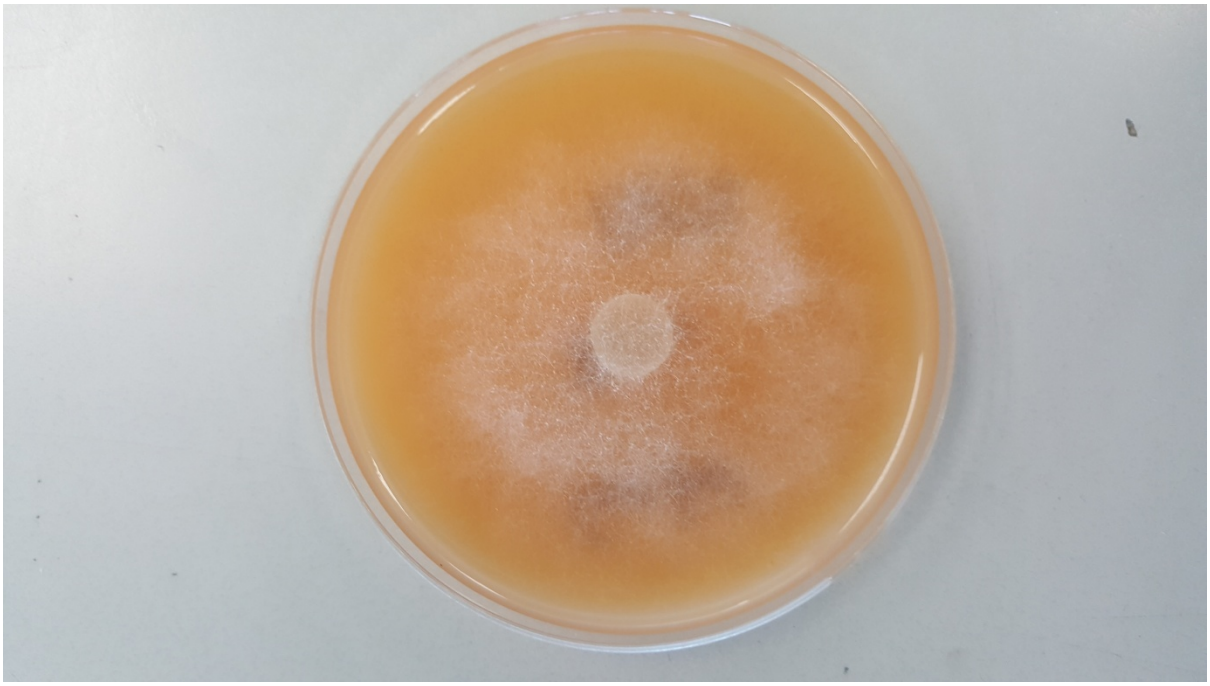


Figure 20 The above image shows the growth of *Phytophthora cinnamomi* coming out from the plug inoculum in the middle of the plate.

2.3.2 *Phytophthora* Culture experiments

The *Phytophthora cinnamomi* species used in the following experiments was obtained from Landcare Research (Isolate H401) and had previously been shown to be pathogenic on kauri (Horner & Hough, 2014). The *Phytophthora cinnamomi* was sub cultured every 5 days onto V8 agar to ensure a viable culture was maintained. The plates used to sub the culture were kept for a further 10 days so two viable cultures were available. These plates were kept in an incubator at 23^{o}C.

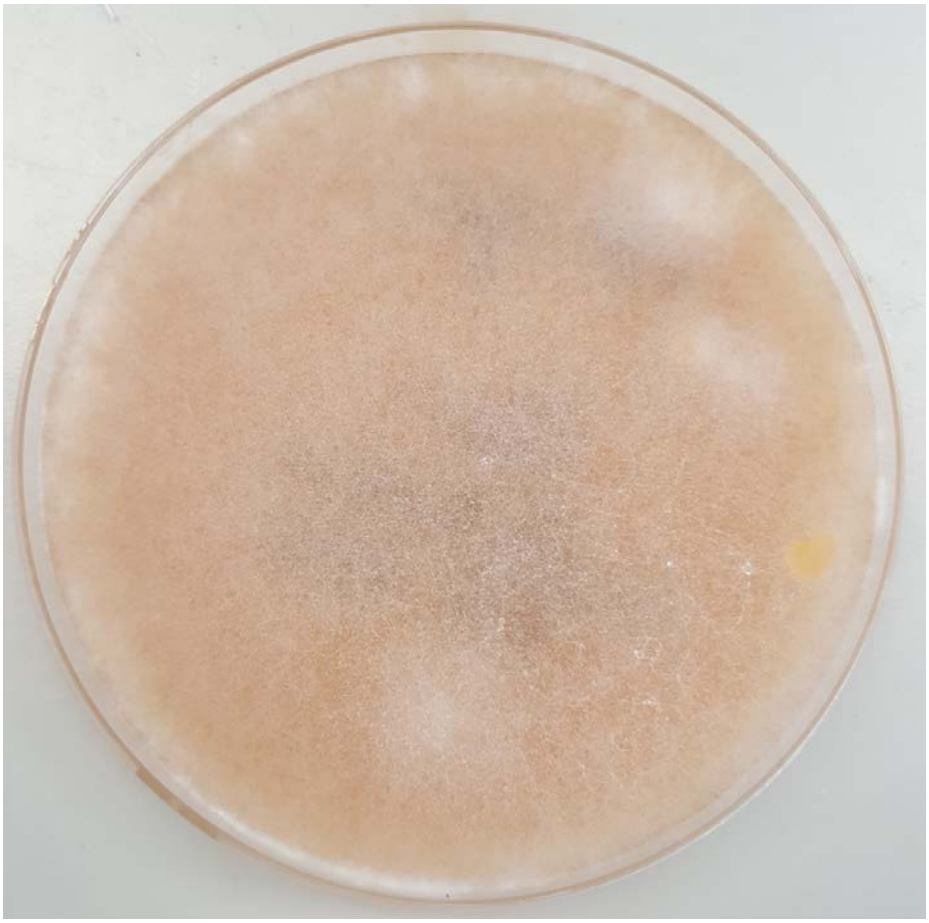


Figure 21 The above image shows *Phytophthora cinnamomi* growing on a V8 agar plate.

V8 agar

All culture experiments were carried out using V8 agar plates. These plates were made by first diluting 200ml of V8 juice with 800ml of deionised H_2O in a 2L Schott bottle. A magnetic stirring bar was added with the addition of 2g $CaCO_3$. The bottle was placed onto a magnetic stirrer and was mixed thoroughly until the $CaCO_3$ was fully dissolved and mixed evenly. 7.5g of BD agar technical was added to the solution and the mixture was further stirred. After 1 minute of further stirring the bottle was autoclaved at 15psi for 30 minutes. After autoclaving the solution was placed onto a heated stirring pad and mixed at a temperature of 60^{o}C, to avoiding premature setting, until plates were ready to pour.

Phenolic compounds

Five standard phenolic compounds were chosen to test for inhibition of growth of the *P. cinnamomi* along with phenolic extracts from the treated Kauri. The five compounds were, phloroglucinol, cinnamic acid, *p* - hydroxybenzoic acid, gallic acid and hydroquinone. A solution of each of these phenolics were made at a concentration of 0.1M. These were prepared as follows:

phloroglucinol - 0.162g was dissolved in 10ml of deionised H_2O

cinnamic acid - 0.148g was dissolved in 10ml of deionised H_2O

p - hydroxybenzoic acid - 0.138g was dissolved in 10ml of deionised H_2O

gallic acid - 0.170g was dissolved in 10ml of deionised H_2O

hydroquinone - 0.110g was dissolved in 10ml of deionised H_2O

V8 culture plates

Sterile petri dishes were labelled prior to the addition of any chemicals or agar. 200µL of the 0.1M phenolic compounds were each added to their own independent petri dish. Quickly after which 20ml of molten agar was poured in the petri dishes and mixed via a circular motion to give a final concentration of 1mM. This was repeated in triplicate to obtain concurrent results.

Along with the phenolic compounds the extraction solutions from the control plants and the plants treated with jasmonic acid were also tested for *Phytophthora* growth inhibition. The initial total phenolic compound concentration in the extracts was determined using the Folin-Ciocalteu assay and a standard calibration curve using gallic acid for the standards. The concentrations of both the control leaf extract and treated leaf extract were adjusted to 0.1M gallic acid equivalents (GAE). 200µL of these 0.1M GAE plant extracts were aliquoted to individual petri dishes and 20ml of molten agar was added and the dishes mixed. In addition to this 200µL of raw (unadjusted) extract from control leaves and leaves treated with both concentrations of jasmonic acid were added to individual petri dishes and 20ml of molten agar was added and dishes mixed.

Each plate, once set, was inoculated with a 6mm agar plug taken from a V8 agar plate previously inoculated with *Phytophthora cinnamomi* five days earlier. This plug was created by using a sterile 6mm cork borer. All plugs were taken from the same culture plate to ensure an even distribution of growth was present. The plates were incubated at 23^{o}C in an incubator. At 5 days the growth diameter was measured. This was done by measuring the diameter of the circular growth out from the plug.

Chapter 3

Results

3.1 Phenolics

3.1.1 Methyl jasmonate

Each of the treated Kauri plants acted as its own control with Day 0 (untreated) as the reference point. The treatments were replicated 5 times (5 plants) and the mean responses are shown in the figures in this section. (The responses of each individual plant can be found in the Appendix).

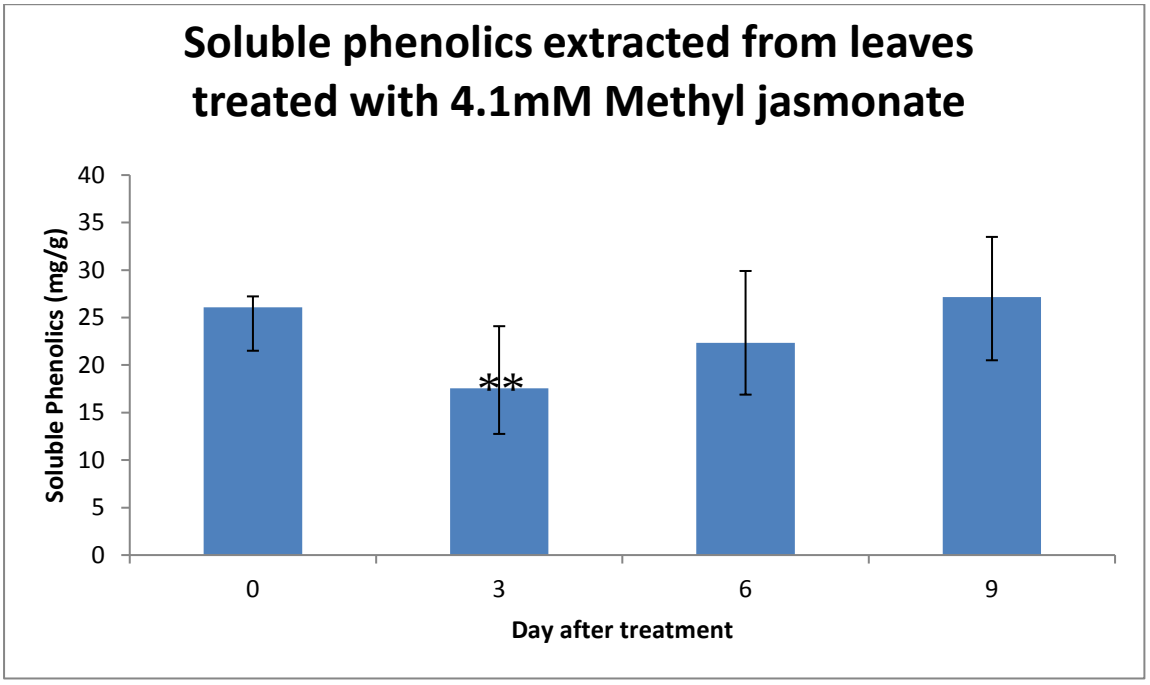


Figure 22 Chart showing amount of soluble phenolics present in Kauri leaves after treatment with 4.1 mM of Jasmonic acid, over a period of 9 days. n=5, ** $p < 0.01$ (significantly different compared to Day 0).

The data shows that there is a significant decrease in soluble phenolics between day 0 and day 3 with a lower concentration treatment of jasmonic acid. Although the soluble phenolics begin to increase again after day 3, day 6 and day 9 do not show any significant difference from day 0. A significant difference is seen however between day 3 and day 9 indicating that there is an increase occurring between this time period.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.004	0.273	0.713
Day 3	0.004		0.285	0.018
Day 6	0.273	0.285		0.276
Day 9	0.713	0.018	0.276	

Table 2 shows the p-values between days for soluble phenolics content for leaves treated with 4.1mM jasmonic acid.

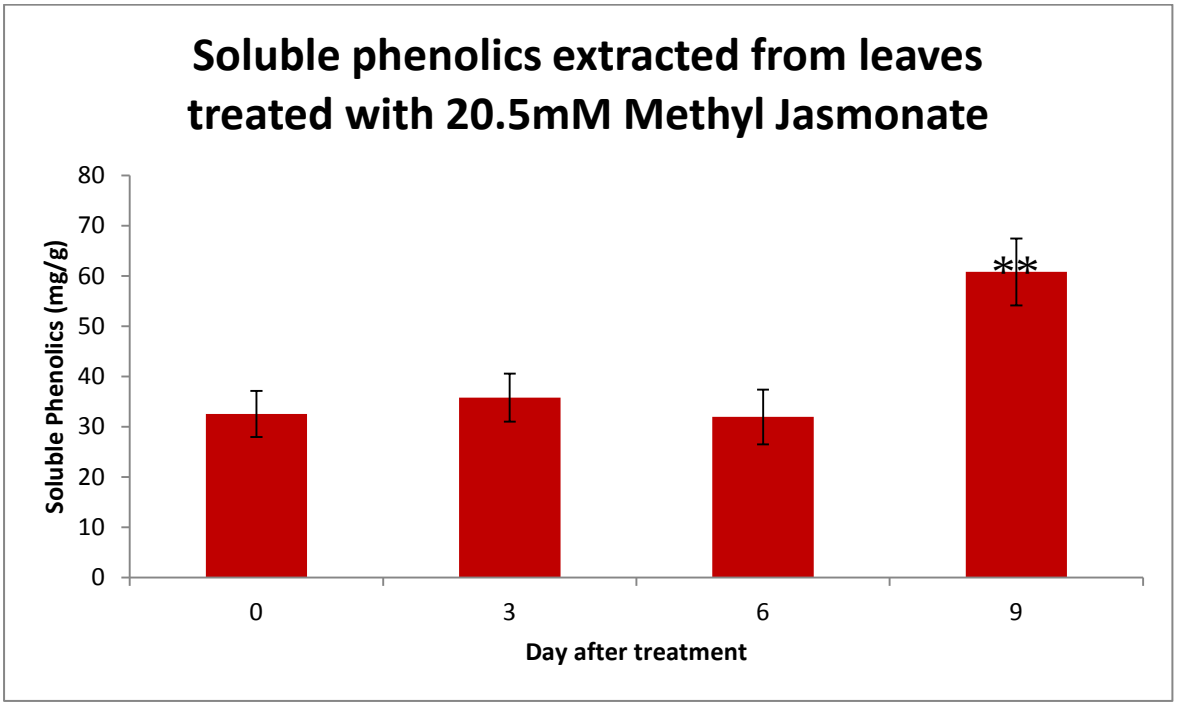


Figure 23 Chart showing amount of soluble phenolics present in Kauri leaves after treatment with 20.5 mM of Jasmonic acid, over a period of 9 days. n=5, ** $p < 0.01$ (significantly different compared to Day 0).

Kauri leaves treated with a high concentration of jasmonic acid showed a significant increase in soluble phenolic compounds between day 0 and day 9. No other significant difference can be seen between day 0 and either day 3 or 6. There is a significant increase in soluble phenolics between both day 3 and 6 and day 9.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.271	0.859	< 0.0001
Day 3	0.271		0.237	< 0.0001
Day 6	0.859	0.237		< 0.0001
Day 9	< 0.0001	< 0.0001	< 0.0001	

Table 3 shows the p-values between days for soluble phenolics content for leaves treated with 21.9mM jasmonic acid.

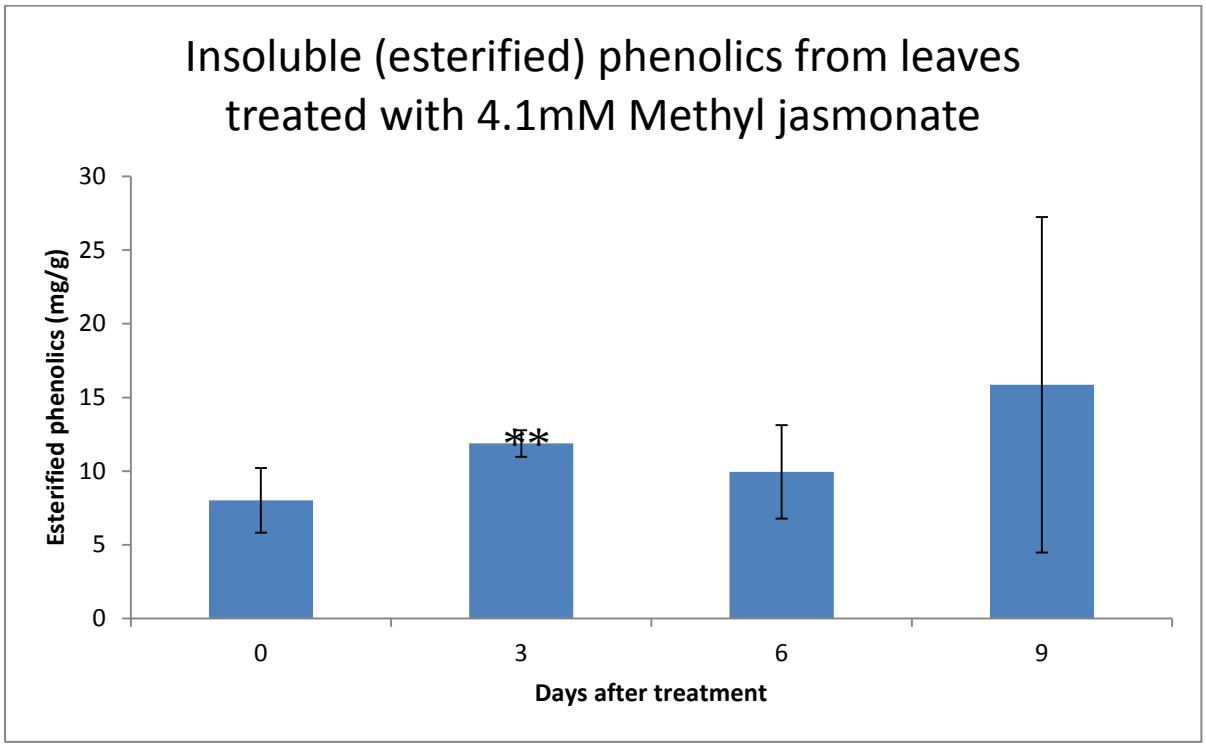


Figure 24 Chart showing amount of esterified phenolics present in Kauri leaves after treatment with 4.1mM of Jasmonic acid, over a period of 9 days. n=5, ** p< 0.01 (significantly different compared to Day 0).

When treated with a low concentration of jasmonic acid it appears that there are no significant changes occurring between day 0 and day 9. The chart indicates visually that there is an increase between day 0 and day 9 however statistically this has not occurred. A possible reason for no significance being found is due to the large range in values in day 9 as indicated by the error bar at day 9. A significant difference however was found between day 0 and day 3. This data counter acts the findings from soluble phenolics treated with the same concentration in which day 0 was significantly greater that day 3 (Fig 22). One explanation is that the soluble phenolics are being converted into esterified (insoluble) phenolics due to the hormone treatment (i.e. the cell wall is being strengthened).

	Day 0	Day 3	Day 6	Day 9
Day 0		0.000	0.264	0.130
Day 3	0.000		0.190	0.436
Day 6	0.264	0.190		0.263
Day 9	0.130	0.436	0.263	

Table 4 shows the p-values between days for esterified phenolics content for leaves treated with 4.1mM jasmonic acid.

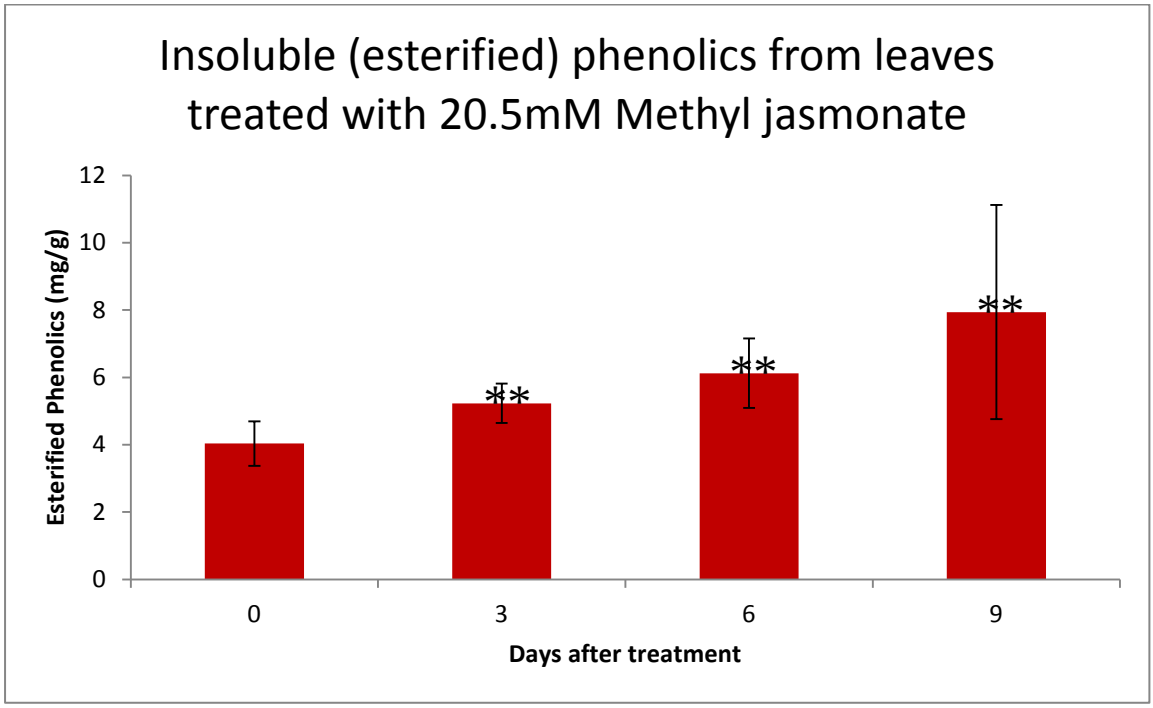


Figure 25 Chart showing amount of esterified phenolics present in Kauri leaves after treatment with 20.5mM of Jasmonic acid, over a period of 9 days. n=5, ** p< 0.01 (significantly different compared to Day 0).

Esterified phenolics in leaves treated with 20.5mM of jasmonic acid showed a greater increase in content. A statistically significant increase can be seen between day 0 and all other days. This increase appears to continue between each day that data was recorded however it was not statistically significant.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.003	0.000	0.007
Day 3	0.003		0.090	0.061
Day 6	0.000	0.090		0.225
Day 9	0.007	0.061	0.225	

Table 5 shows the p-values between days for esterified phenolics content for leaves treated with 20.5 mM jasmonic acid.

"Total Phenolics" are the sum of "Soluble Phenolics" and "Esterified Phenolics".

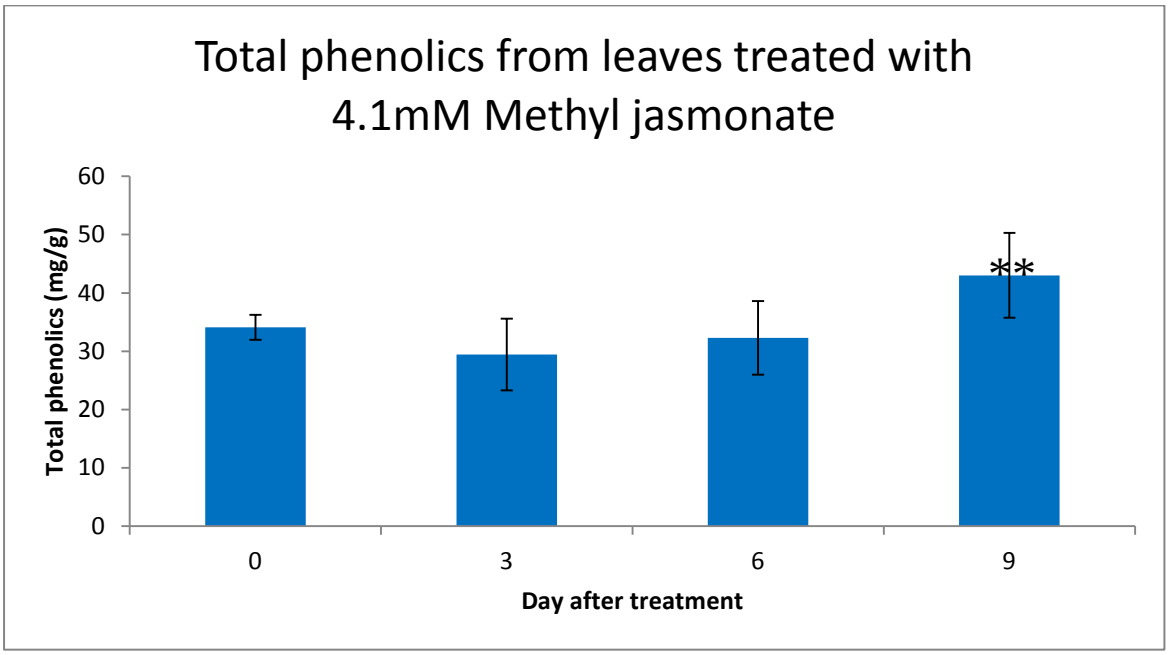


Figure 26 Chart showing amount of total phenolics present in Kauri leaves after treatment with 4.38mM of Jasmonic acid, over a period of 9 days. n=5 ,** p< 0.01 (significantly different compared to Day 0).

When the total phenolics are calculated a significant increase is seen between day 0 and day 9. A significant increase is also seen between day 3 and day 9 and well as day 6 and day 9.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.107	0.539	0.009
Day 3	0.107		0.467	0.001
Day 6	0.539	0.467		0.013
Day 9	0.009	0.001	0.013	

Table 6 shows the p-values between days for the total phenolic content shows the p-values between days for the total phenolic content for leaves treated with 4.1mM jasmonic acid.

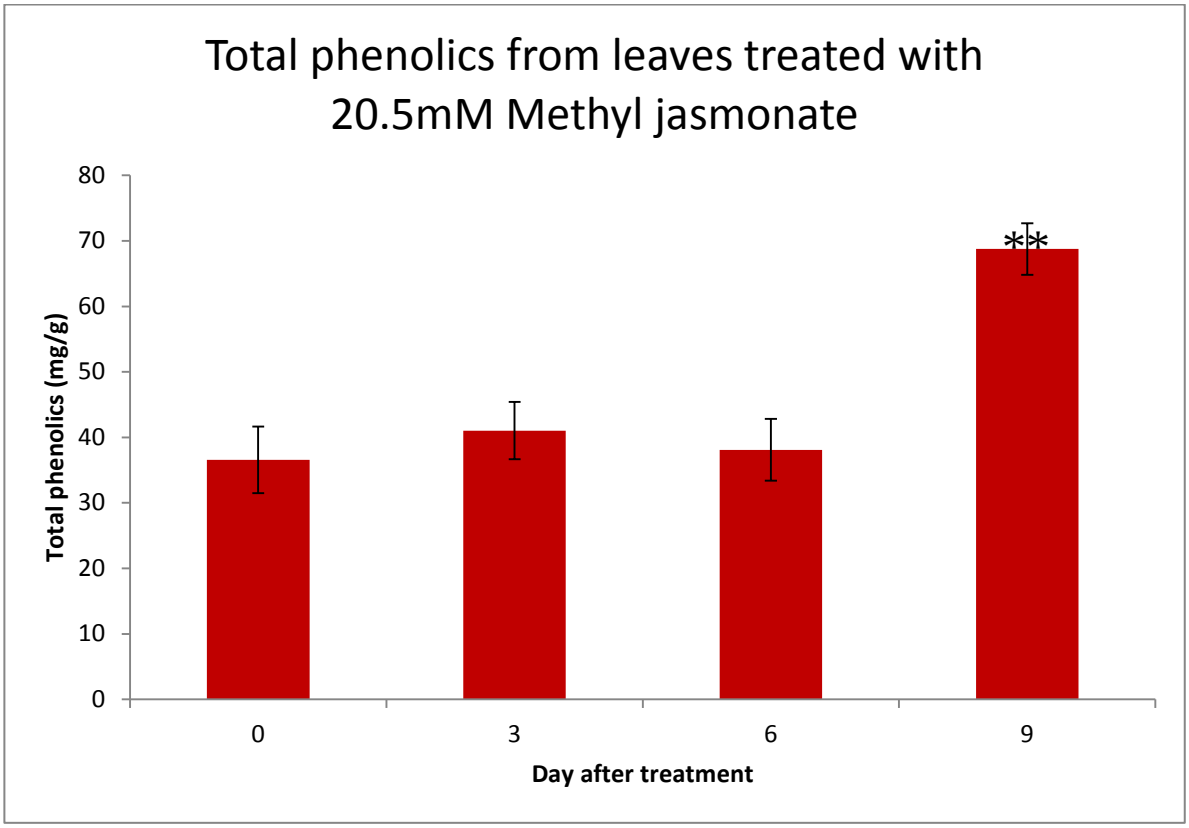


Figure 27 Chart showing amount of total phenolics present in Kauri leaves after treatment with 21.9mM of Jasmonic acid, over a period of 9 days. n=5, ** p< 0.01 (significantly different compared to Day 0).

The total phenolics data from leaves treated with 21.9mM show a definite difference between day 0 and day 9. Not only is this difference seen visually but it can be seen statistically as well. A significant difference is also seen between day 3 and day 9 as well as day 6 and day 9.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.136	0.622	< 0.0001
Day 3	0.136		0.307	< 0.0001
Day 6	0.622	0.307		< 0.0001
Day 9	< 0.0001	< 0.0001	< 0.0001	

Table 7 shows the p-values between days for the total phenolic content for leaves treated with 20.5 mM jasmonic acid.

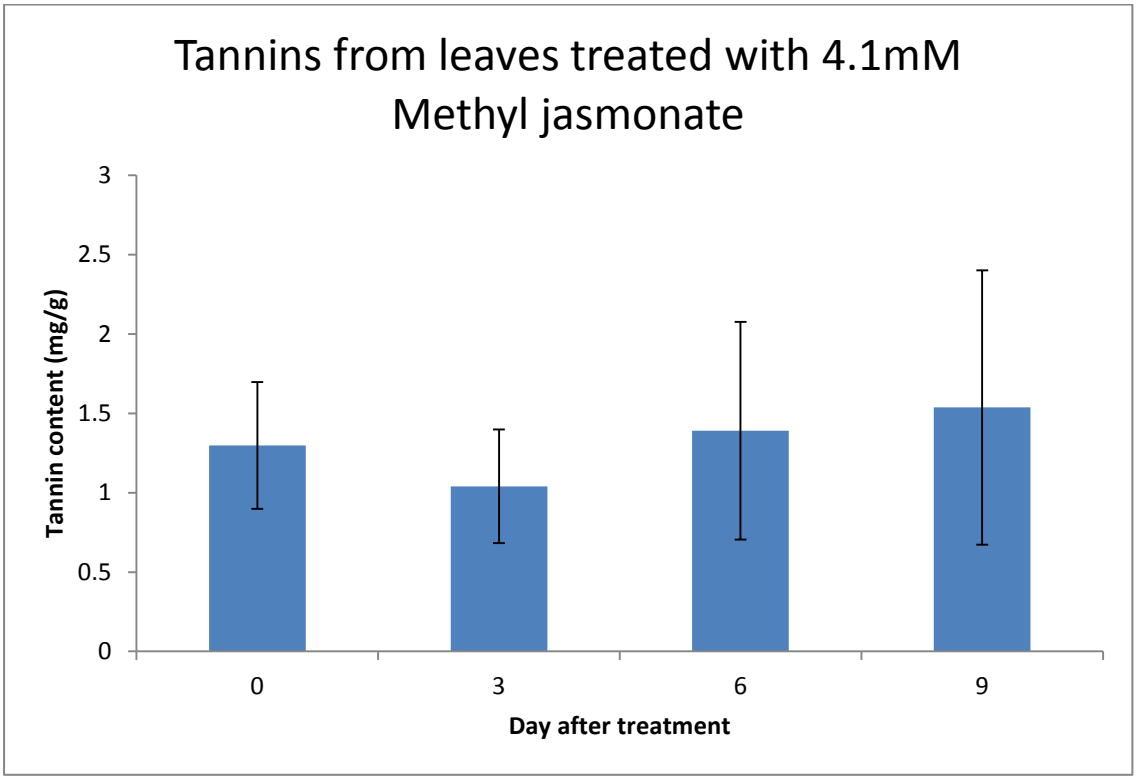


Figure 28 Chart showing amount of tannins present in Kauri leaves after treatment with 4.1mM of Jasmonic acid, over a period of 9 days. n=5

Tannin content does not significantly change between any of the days. However the chart appears to show a similar visual trend to that of the soluble phenolics chart (Fig 22) where Kauri was treated with the same concentration of jasmonic acid. This could indicate that treatment with jasmonic acid increases tannin content proportionally with other soluble phenolics found in Kauri leaves but not enough to reach statistical significance.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.284	0.795	0.574
Day 3	0.284		0.313	0.236
Day 6	0.795	0.313		0.766
Day 9	0.574	0.236	0.766	

Table 8 shows the p-values between days for the tannin content for leaves treated with 4.1mM jasmonic acid.

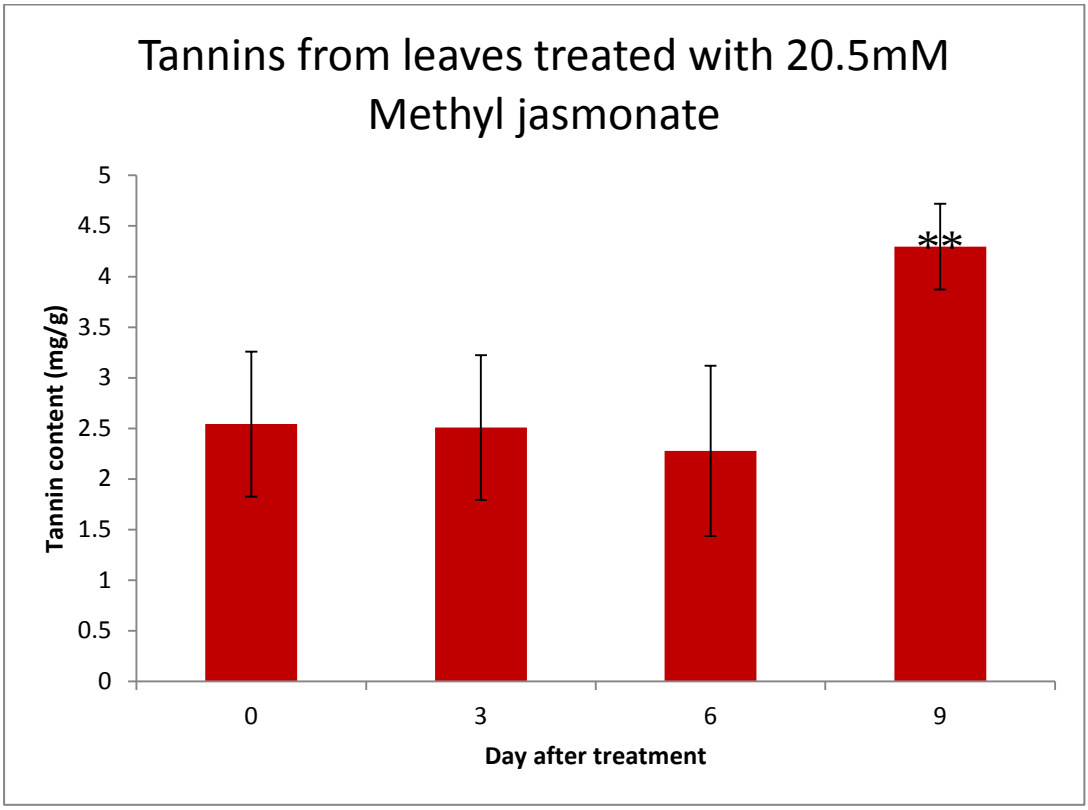


Figure 29 Chart showing amount of tannins present in Kauri leaves after treatment with 20.5mM of Jasmonic acid, over a period of 9 days. n=5, ** p< 0.01 (significantly different compared to Day 0).

Leaves treated with 20.5 mM jasmonic acid solution showed a significant increase in tannins between day 0 and day 9. As with the lower concentration treatment, the same trend can be seen between the soluble phenolics and the tannin content. In this case the trend being that day 9 contains a significantly greater content than any other day recorded.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.939	0.593	<0.0001
Day 3	0.939		0.642	<0.0001
Day 6	0.593	0.642		<0.0001
Day 9	<0.0001	<0.0001	<0.0001	

Table 9 shows the p-values between days for the tannin content for leaves treated with 21.9mM jasmonic acid.

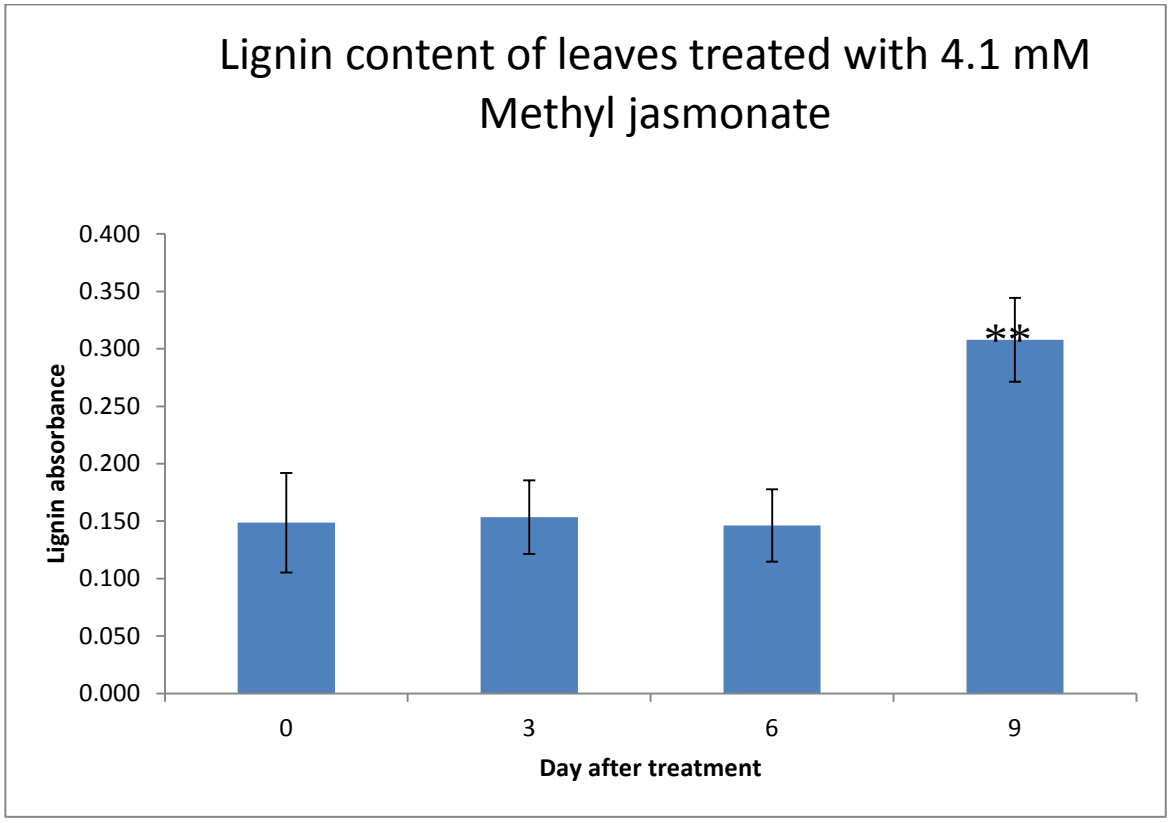


Figure 30 Chart showing lignin content (absorbance units) present in Kauri leaves after treatment with 4.38mM of Jasmonic acid, over a period of 9 days. n=5, ** p< 0.01 (significantly different compared to Day 0).

Treatment with a low concentration of Jasmonic acid appears to have quite a large impact on the lignin concentration in Kauri leaves. Although it may have taken 9 days to notice a large increase, statistically the change is significant. No change was noticed in the lignin content between day 0, 3 and 6.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.842	0.920	< 0.0001
Day 3	0.842		0.720	< 0.0001
Day 6	0.920	0.720		< 0.0001
Day 9	< 0.0001	< 0.0001	< 0.0001	

Table 10 shows the p-values between days for the lignin content (absorbance units) for leaves treated with 4.1mM jasmonic acid.

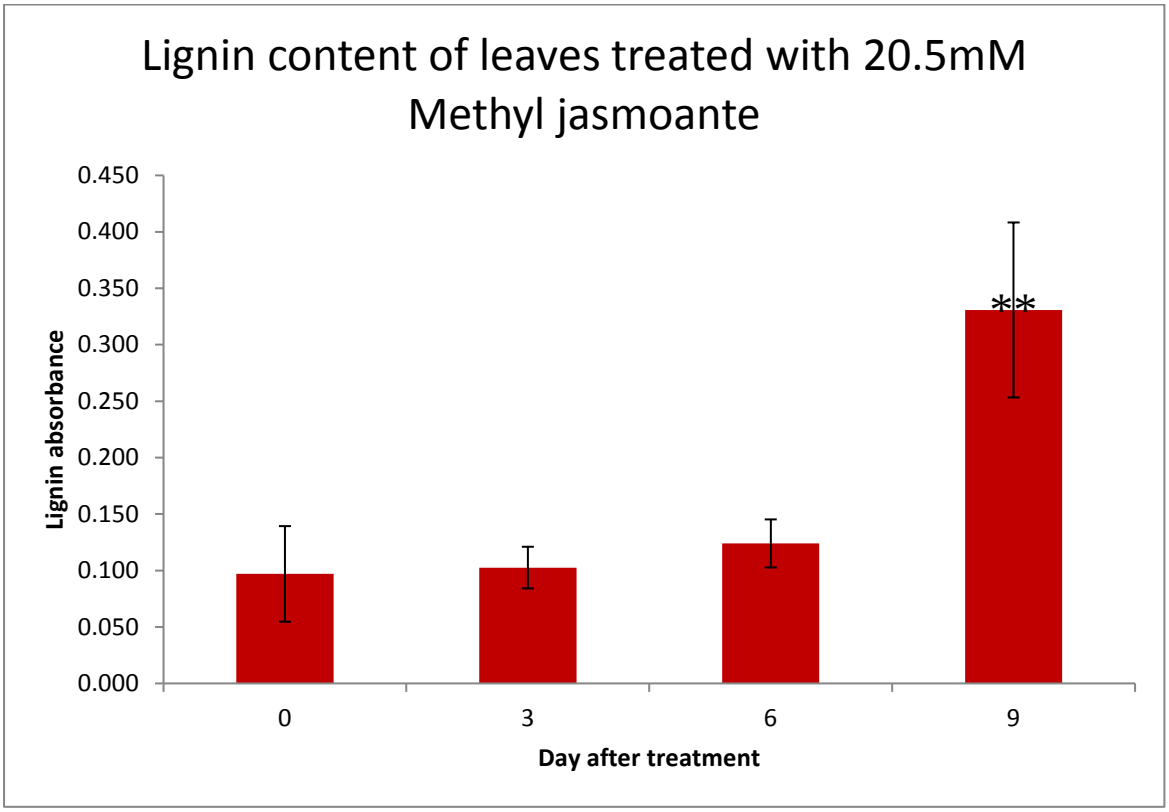


Figure 31 . Chart showing lignin absorbance present in Kauri leaves after treatment with 20.5mM of Jasmonic acid, over a period of 9 days. n=5, ** p< 0.01 (significantly different compared to Day 0).

It appears that the lignin content is not dependent on the concentration of jasmonic acid that the leaf is treated with. Even with a higher concentration of jasmonic acid it appears that the increase still reaches a peak around 9 days. Also, as with the lower concentration, day 0,3 and 6 appear to have no significant difference in the absorbance.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.786	0.203	< 0.0001
Day 3	0.786		0.089	< 0.0001
Day 6	0.203	0.089		< 0.0001
Day 9	< 0.0001	< 0.0001	< 0.0001	

Table 11 shows the p-values between days for the lignin content (absorbance units) for leaves treated with 20.5mM jasmonic acid.

3.1.2 Salicylic acid

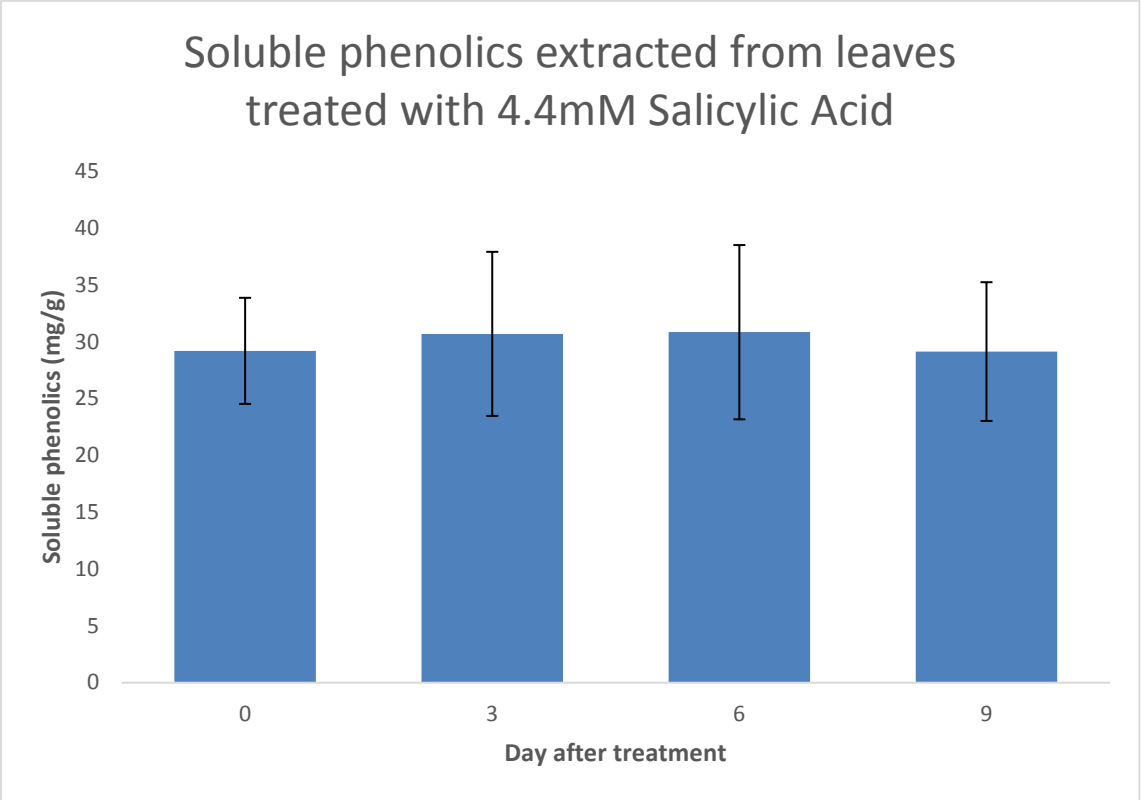


Figure 32 Chart showing amount of soluble phenolics present in Kauri leaves after treatment with 4.38mM of salicylic acid, over a period of 9 days.

The chart appears to show no significant pattern with no significant difference shown between any of the days.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.697	0.682	0.985
Day 3	0.697		0.975	0.712
Day 6	0.682	0.975		0.697
Day 9	0.985	0.712	0.697	

Table 12 shows the p-values between days for the soluble phenolics for leaves treated with 4.4mM salicylic acid.

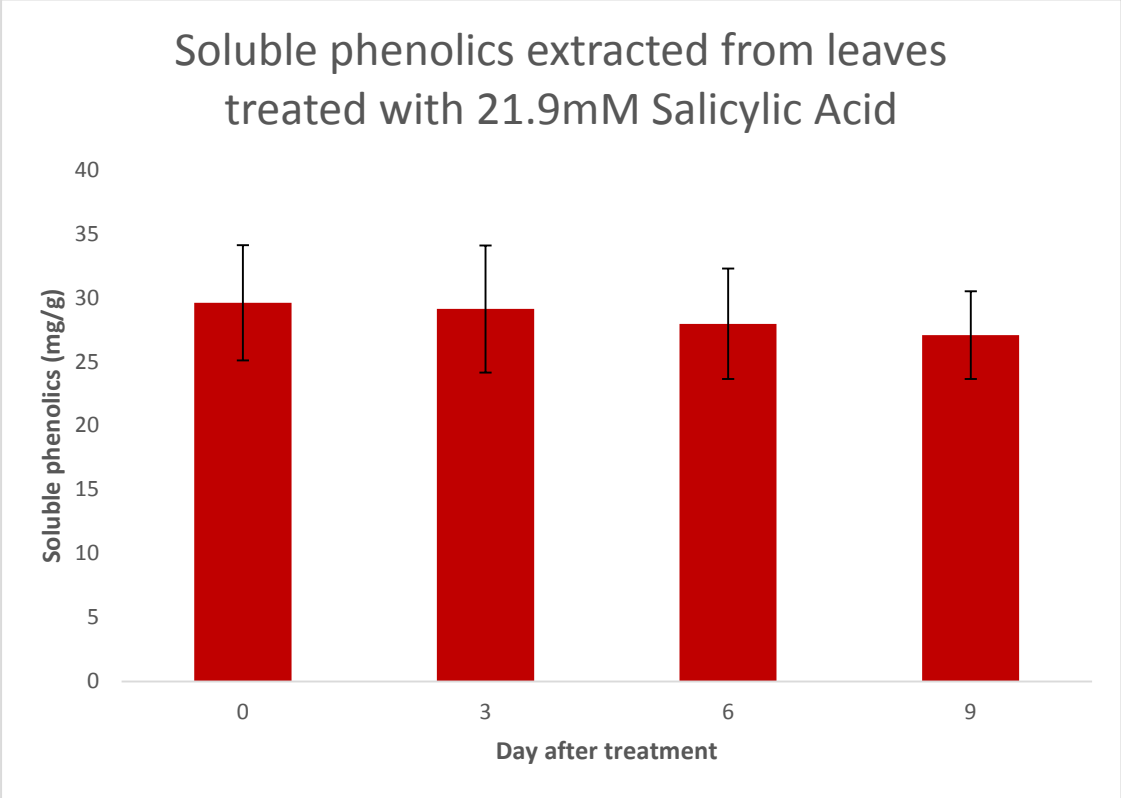


Figure 33 Chart showing amount of soluble phenolics present in Kauri leaves after treatment with 21.9mM of salicylic acid, over a period of 9 days.

Treatment with a higher concentration of salicylic acid appears to have caused a slight visual decrease in the soluble phenolics content up to day 9 however this is not significant statistically. There is no significant difference between any of the days.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.870	0.555	0.319
Day 3	0.870		0.694	0.451
Day 6	0.555	0.694		0.721
Day 9	0.319	0.451	0.721	

Table 13 shows the p-values between days for the soluble phenolics for leaves treated with 21.9mM salicylic acid.

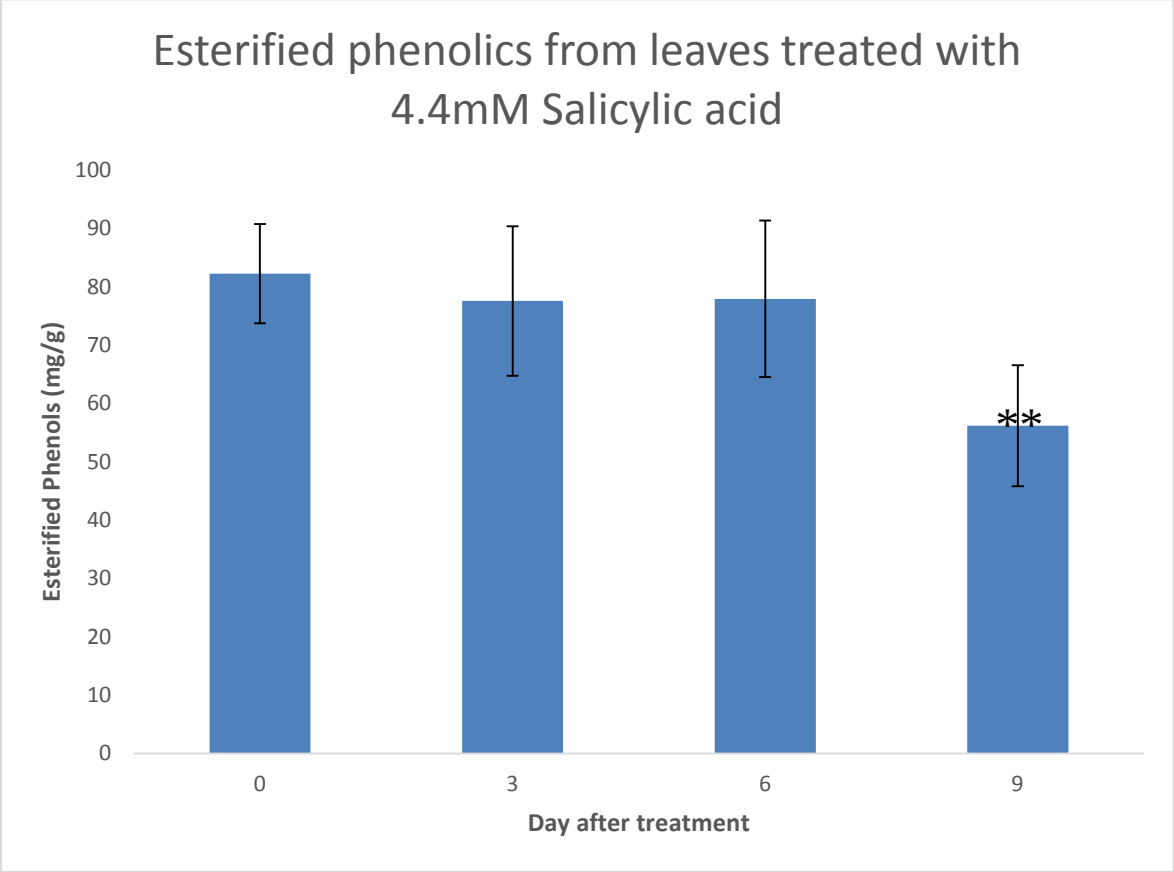


Figure 34 . Chart showing amount of esterified phenolics present in Kauri leaves after treatment with 4.38mM of salicylic acid, over a period of 9 days. n=5, ** p< 0.01 (significantly different compared to Day 0).

The esterified phenolics appear to show a slight decrease up until day 6, after which point a significant decrease occurs when compared with day 0 in that day 9 shows to be significantly lower than all other days. Although visually a decrease seems to appear in the first 6 days, it is not significant.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.497	0.543	< 0.0001
Day 3	0.497		0.966	0.004
Day 6	0.543	0.966		0.004
Day 9	< 0.0001	0.004	0.004	

Table 14 shows the p-values between days for the esterified phenolics for leaves treated with 4.4 mM salicylic acid.

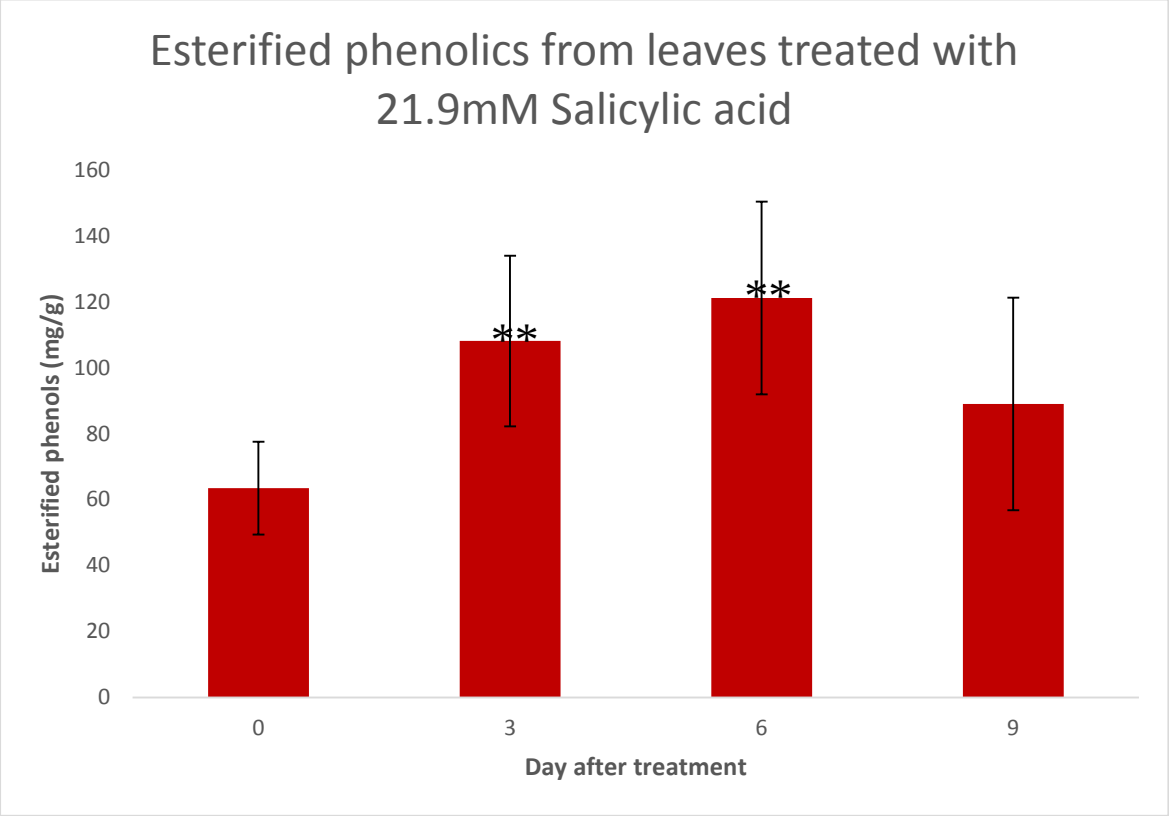


Figure 35 Chart showing amount of esterified phenolics present in Kauri leaves after treatment with 21.9mM of salicylic acid, over a period of 9 days. n=5, ** p< 0.01 (significantly different compared to Day 0).

When the Kauri leaves were treated with a higher concentration of salicylic acid there is a significant increase occurring. The content of esterified phenolics increase between day 0 and 3, and day 0 and 6. After day 6 a decrease in esterified phenolics occurs.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.001	< 0.0001	0.104
Day 3	0.001		0.455	0.302
Day 6	< 0.0001	0.455		0.099
Day 9	0.104	0.302	0.099	

Table 15 shows the p-values between days for the esterified phenolics for leaves treated with 21.9mM salicylic acid.

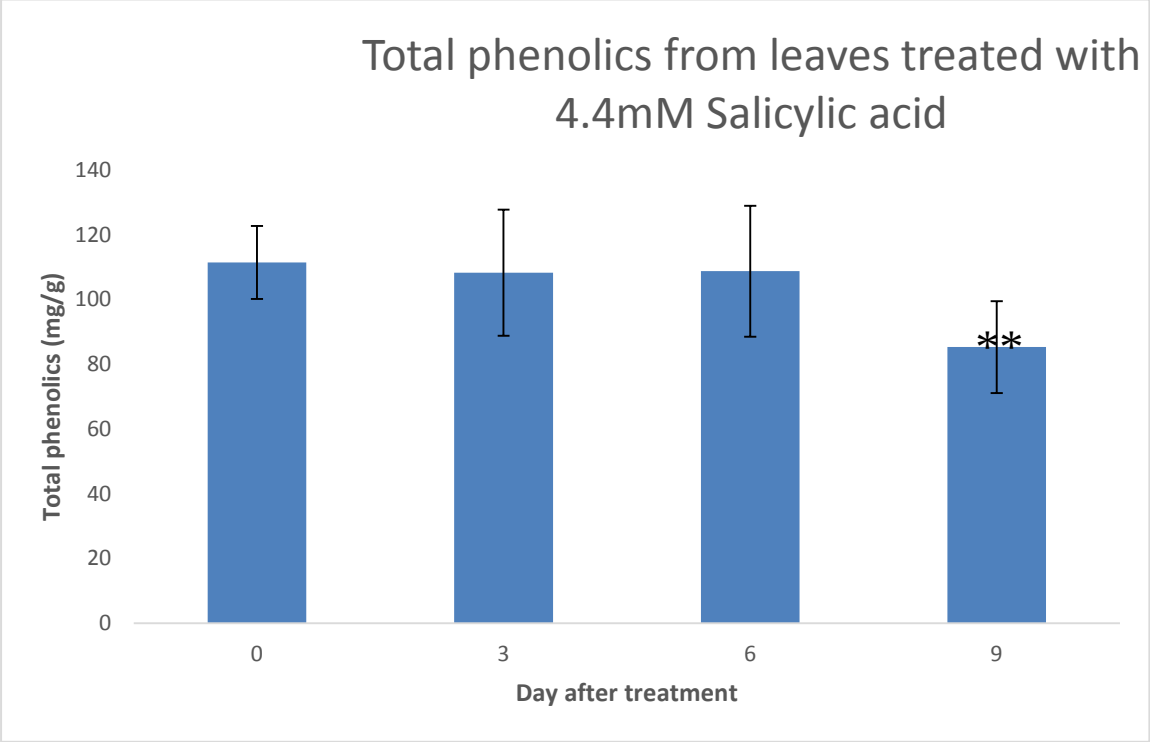


Figure 36 Chart showing amount of total phenolics present in Kauri leaves after treatment with 4.38mM of salicylic acid, over a period of 9 days. n=5, ** p< 0.01 (significantly different compared to Day 0).

With all the phenolics combined the trend shows a decrease occurring between day 0 and day 9. Between the first 6 days no significant change occurs.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.752	0.796	0.001
Day 3	0.752		0.968	0.033
Day 6	0.796	0.968		0.034
Day 9	0.001	0.033	0.034	

Table 16 shows the p-values between days for the total phenolics for leaves treated with 4.4mM salicylic acid.

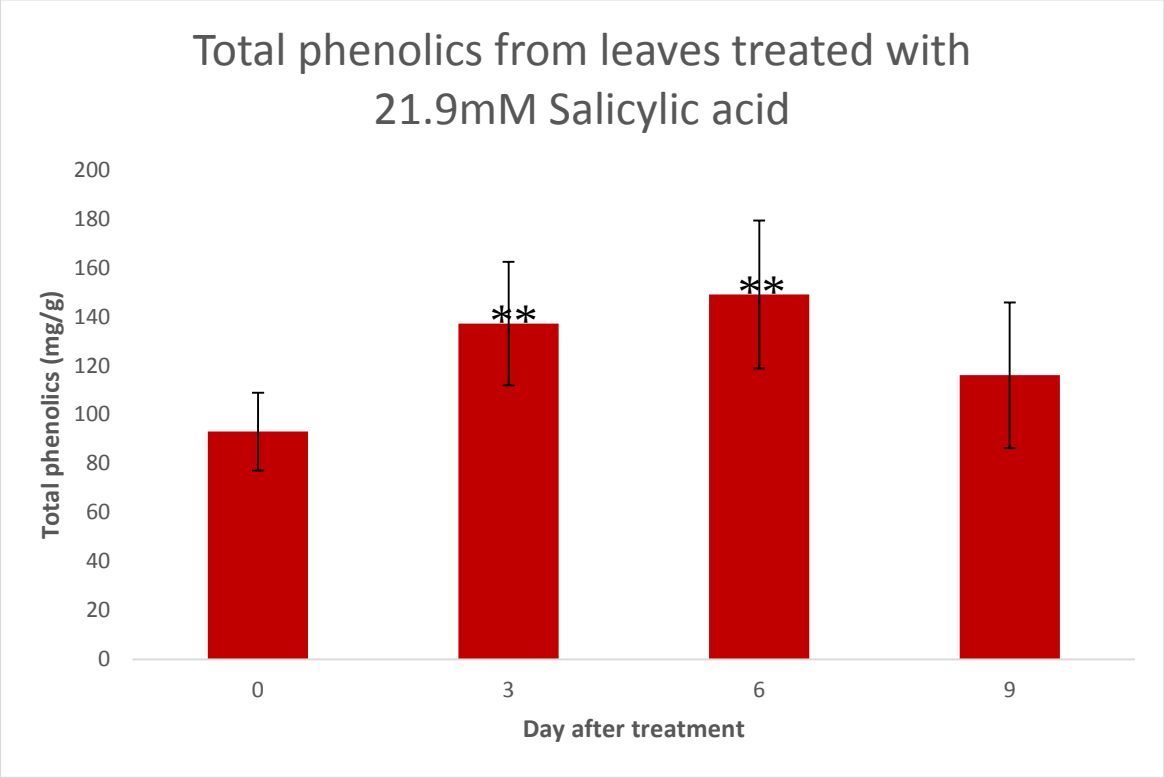


Figure 37 Chart showing amount of total phenolics present in Kauri leaves after treatment with 21.9mM of salicylic acid, over a period of 9 days. n=5, ** p< 0.01 (significantly different compared to Day 0).

The combined (total) phenolics from leaves treated with a higher hormone concentration show an increase in total phenolic concentration until a visual decrease is seen on the graph. The increase seen between day 0 and day 3 and 6 is significant.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.001	0.000	0.128
Day 3	0.001		0.499	0.226
Day 6	0.000	0.499		0.082
Day 9	0.128	0.226	0.082	

Table 17 shows the p-values between days for the total phenolics for leaves treated with 21.9mM salicylic acid.

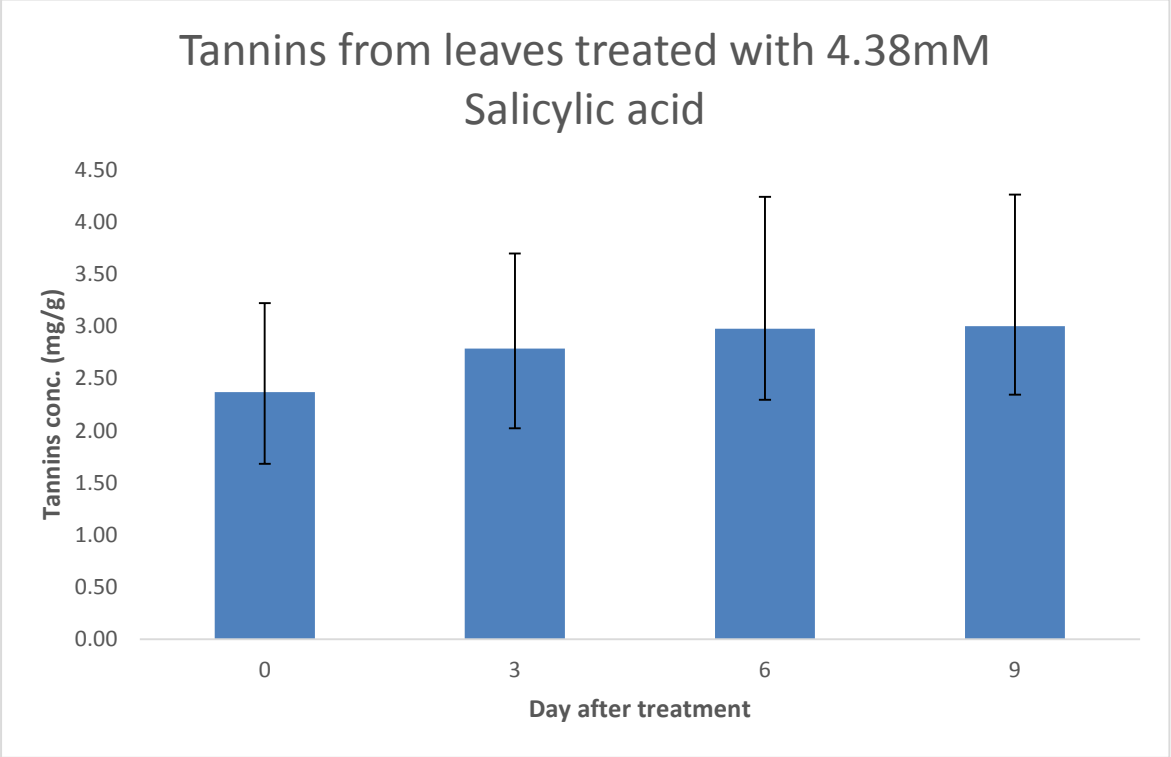


Figure 38 Chart showing amount of tannin present in Kauri leaves after treatment with 4.38mM of salicylic acid, over a period of 9 days. n=5

Over all the days the tannin showed no significant changes however visually there appears to be a continual increase between day 0 and day 9.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.456	0.375	0.354
Day 3	0.456		0.786	0.758
Day 6	0.375	0.786		0.975
Day 9	0.354	0.758	0.975	

Table 18 shows the p-values between days for the tannin content for leaves treated with 4.4 mM salicylic acid.

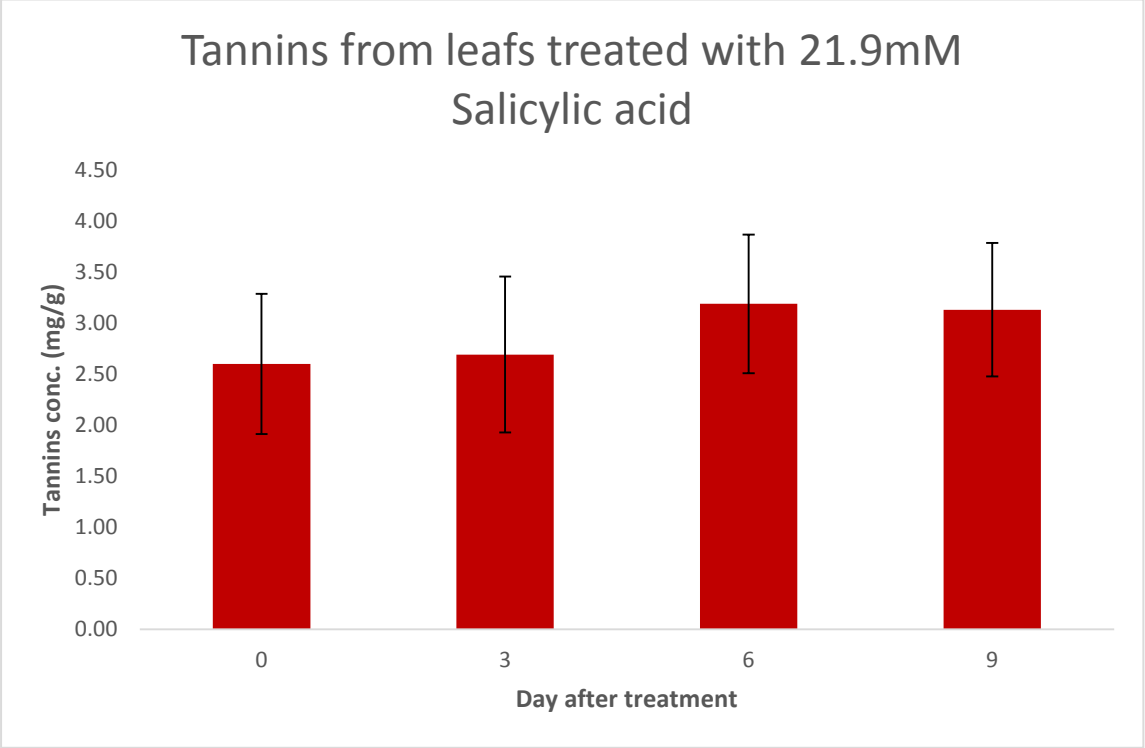


Figure 39 Chart showing amount of tannin present in Kauri leaves after treatment with 21.9mM of salicylic acid, over a period of 9 days. n=5

As with the tannin content from Kauri leaves treated with a low concentration of salicylic acid, no significant differences can be seen. Although the chart does appear to show an increase between day 3 and day 6 and 9. This pattern appears to be the same with the lower concentration treated leaves, that is to visually show an increase after day 3.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.841	0.173	0.211
Day 3	0.841		0.278	0.329
Day 6	0.173	0.278		0.892
Day 9	0.211	0.329	0.892	

Table 19 shows the p-values between days for the tannin content for leaves treated with 21.9mM salicylic acid.

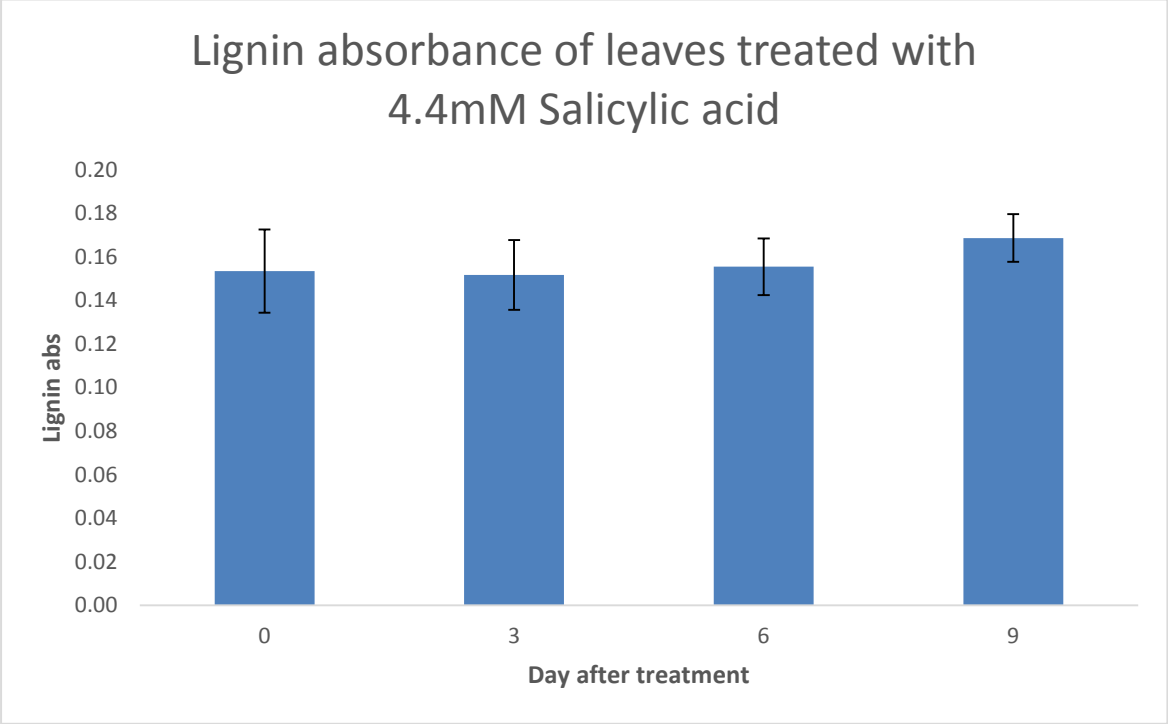


Figure 40 Chart showing lignin content (absorbance units) present in Kauri leaves after treatment with 4.4 mM of salicylic acid, over a period of 9 days. n=5

The lignin content did not significantly increase after treatment with 4.4 mM salicylic acid when compared to Day 0 (untreated). However the content at Day 9 almost achieved significance when compared to Day 3 (p = 0.05).

	Day 0	Day 3	Day 6	Day 9
Day 0		0.872	0.846	0.122
Day 3	0.872		0.680	0.050
Day 6	0.846	0.680		0.082
Day 9	0.122	0.050	0.082	

Table 20 shows the p-values between days for the lignin absorbance for leaves treated with 4.4 mM salicylic acid.

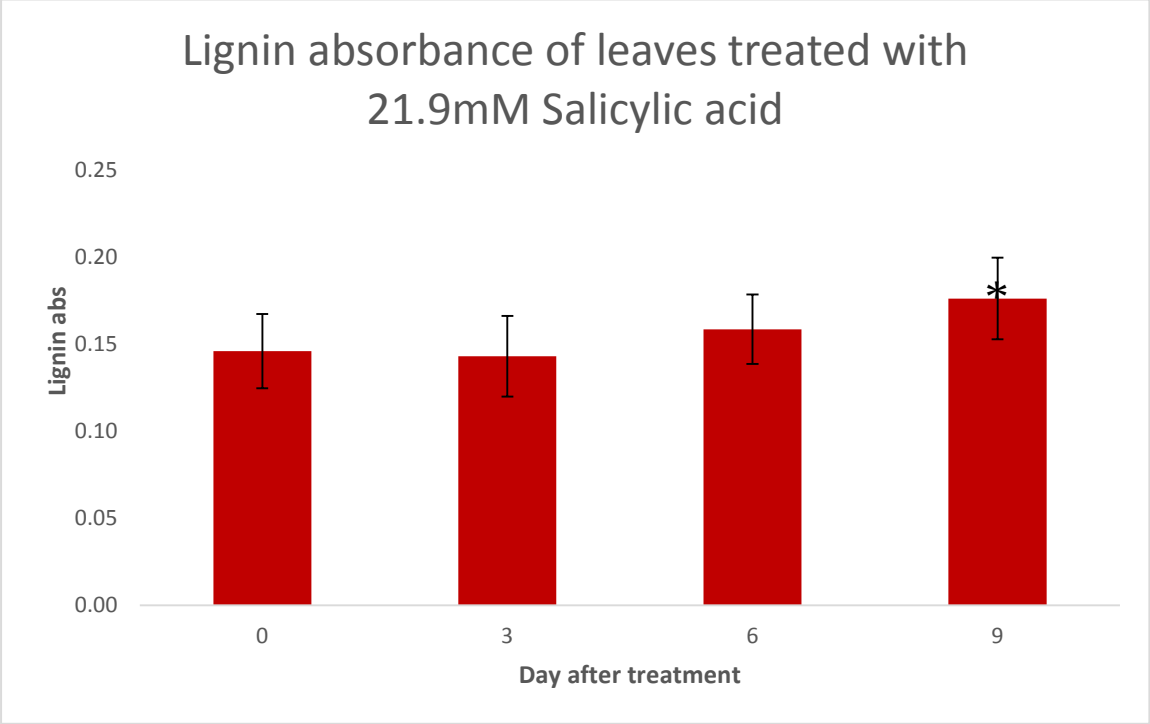


Figure 41 Chart showing lignin content (absorbance units) present in Kauri leaves after treatment with 21.9mM of salicylic acid, over a period of 9 days. n=5, * $p < 0.05$ (significantly different compared to Day 0).

Kauri plants treated with a higher concentration of salicylic acid showed a significant increase in lignin absorbance between day 0 and day 9. There was also a significant increase in absorbance between day 3 and day 9. There were no other significant changes between the other days.

	Day 0	Day 3	Day 6	Day 9
Day 0		0.831	0.334	0.033
Day 3	0.831		0.253	0.024
Day 6	0.334	0.253		0.200
Day 9	0.033	0.024	0.200	

Table 21 shows the p-values between days for the lignin absorbance for leaves treated with 21.9mM salicylic acid.

3.2 Culture Results

3.2.1 Detached Leaf Assays

Figure 42 The above images show control Kauri leaves before (left) and 7 days after infection with *Phytophthora cinnamomi (*right). The indicated zones (red circle) on the right show where the *P. cinnamomi* was inoculated and it appears plant tissue death has occurred. The leaf on top in the left photo has changed colour during the treatment shown by the red colour of the leaf on the top in the right picture.

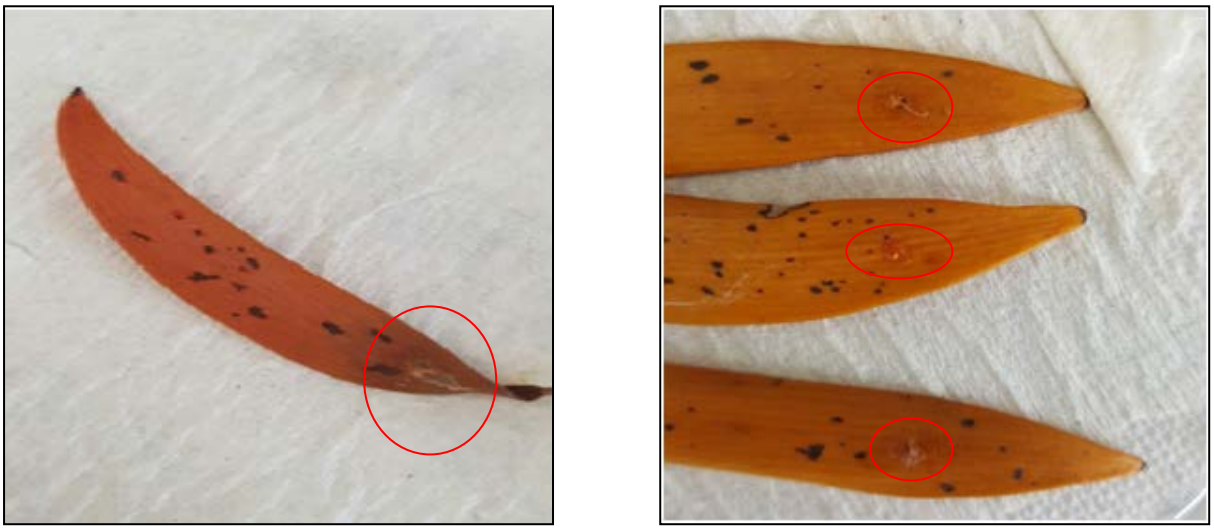


Figure 43 The above images show a red coloured control Kauri leaf (the leaf was red at the start of treatment) 7 days after infection with *Phytophthora cinnamomi* (left), the indicated zone show what appear to be plant tissue death around a wound formed to infect leaf. Control leaves 7 days after treatment with a plug of un-inoculated V8 agar (right) show little death around wound sites as shown in the indicated zones.

Figure 44 The above images show Kauri leaves treated with jasmonic acid before infection (left) and 7 days after infection (right). The indicated zones show wounds created on the leaf indicating the infection sites.

Figure 45 The above images show Kauri leaves treated with salicylic acid before infection(left), the indicated zone shows the wound that has been infected with *P. cinnamomi* and 7 days after infection (right). It appears that after 7days little plant death has occurred with no obvious colour changes around the infection site.

Treatment	Infection Zone 7 days after inoculation (mm)
Control leaf 1	2
Control leaf 2	2
Control leaf 3	1
Jasmonic acid leaf 1	1
Jasmonic acid leaf 2	1
Jasmonic acid leaf 3	1
Salicylic acid leaf 1	1
Salicylic acid leaf 2	1
Salicylic acid leaf 3	1

Table 22 shows the diameter of the infection zone around the inoculation site 7 days after the inoculation.

Looking at the control leaves it appears that when Kauri leaves are infected with *P. cinnamomi* it causes a slight region of decolourisation around the infection site. The regions of decolourisation are relatively small and therefore getting accurate measurements was difficult. Although there was not a great difference between the treated leaves and control leaves there none the less appears to be a more severe response in the untreated control leaf than those leaves treated with hormones.

Another factor that has to be taken into account is the original colour of the Kauri leaf or the reddening that occurred during the 7 days of leaf incubation. With the leaves that were red in colour, the measuring of the zone became more difficult. This was due to the decolouration zone having a very similar colour to that of the natural leaf. Due to the majority of leaves present, at the time this experiment took place, it was impossible to obtain a branch that contained enough green leaves to carry out all the assays. It is for this reason that the controls were carried out on both green leaves and red leaves so that a contrast could be obtained to more easily compare results.

In comparison, it appears the zone around the infection site appears to be more clearly in a circular fashion in the green leaf. With the red control leaf the infection zone appears to be more spread out around the end of the leaf and a uniform zone is not quite as obvious when first viewed.

Even with the small zones present, the zone does appear to be more damaging on untreated control leaves. The leaves that were treated with jasmonic acid and salicylic acid both

appeared to show less damage or death around the infection zone. The zone itself appears to be made up of the initial wound made during the infection process.

One possible reason for the small zones could be due to the fact that *P. cinnamomi* does not commonly spread or infect plant leaves. Its most common form mode of infection begins at the root of the plant and causes death and infection via internal means. The increased content of phenolics and lignin in the treated leaves most likely inhibited the growth of *P. cinnamomi*, however, further experiments such as the results that follow, are required to give a more definitive answer.

3.2.2 Culture plate assays

	Growth		
Phenolic compound	Day 5 (mm)	Day 7 (mm)	Literature value day 7 (mm)
Hydroquinone	0	10	57.6
Cinnamic Acid	18	54	0
p-hydrobenzoic acid	55	78	57.6
Gallic Acid	60	85	65.2
Phloroglucinol	71	85	100.6
Acetone	85	85	
Control (V8)	85	85	
Control leaf 1mmol	65	85	
Treated leaf 1mmol	48	85	

Table 23 shows the diameters of the P. cinnamomi culture 5 and 7 days after inoculation onto agar.

Category	Standardized difference	Pr > Diff	Significant
Control (V8) vs Hydroquinone	52.721	0.000	Yes
Control (V8) vs Cinnamic Acid	41.557	0.000	Yes
Control (V8) vs Treated leaf 1mmol	22.949	0.000	Yes
Control (V8) vs p-hydrobenzoic acid	18.483	0.000	Yes
Control (V8) vs Gallic Acid	15.630	0.000	Yes
Control (V8) vs Control leaf 1mmol	12.426	0.000	Yes
Control (V8) vs Phloroglucinol	8.684	0.000	Yes
Control (V8) vs Acetone	0.000	1.000	No

Table 24 shows the comparison in growth at 5 days after inoculation between the control plates and the culture plates with phenolic compounds and leaf extracts incorporated.

Category	Standardized difference	Pr > Diff	Significant
Control (V8) vs Treated leaf 1mmol	0.000	1.000	No
Control (V8) vs Phloroglucinol	0.000	1.000	No
Control (V8) vs Gallic Acid	0.000	1.000	No
Control (V8) vs Control leaf 1mmol	0.000	1.000	No
Control (V8) vs Hydroquinone	178.701	0.000	Yes
Control (V8) vs Cinnamic Acid	73.784	0.000	Yes
Control (V8) vs p-hydrobenzoic acid	16.361	0.000	Yes
Control (V8) vs Acetone	0.000	1.000	No

Table 25 shows the comparison in growth at 7 days after inoculation between the control plates and the culture plates with phenolic compounds and leaf extracts incorporated.

Contrast	Standardized difference	Pr > Diff	Significant
Control leaf 1mmol vs Treated leaf 1mmol	70.386	< 0.0001	Yes

Table 26 shows the comparison in growth at day 5 after inoculation between plates treated with phenolics extracted from control leaf and a treated leaf, both at a 1mmol concentration.

Control leaf and treated leaf have had the concentration of the phenolic compounds adjusted using acetone to ensure a constant concentration of 1mmol for comparison reasons. This had been done so that a difference may be seen due to the individual compound concentration within the leaves rather than the total concentration of phenolics. Each treatment has been done in triplicate and the average is shown above.

The culture results indicate that hydroquinone and cinnamic acid have the greatest inhibition on the growth of *Phytophthora cinnamomi* . It appears that acetone, which was used in the extraction of the phenolics, had no effect on the growth and with no additional chemicals the growth filled the plate.

The extracted solution from the untreated control leaf appears to have inhibited the growth of Phytophthora and the leaf treated with jasmonic acid shows a greater effect. After seven days most phenolics appear to have little effect on the growth with the exception of gallic acid, cinnamic acid and hydroquinone.

The greater effect on inhibition of growth shown by the treated leaf could indicate that within the leaf a greater concentration of more potent phenolics are present. Having the same concentration of total phenolics between the control leaf and the treated leaf shows that it is unlikely that the total volume of phenolics present is the reason for inhibition.

Literature results are similar to the results obtained. Results from previous research has shown cinnamic acid to be very effective in inhibiting the growth of *P. cinnamomi*. The research also showed Phloroglucinol to have little effect on inhibition(Cahill and McComb 1992).

Compared with the known phenolic compounds, the phenolics extracted from the kauri leaves showed average inhibition at the same concentration. As expected the higher concentration of extracts had a greater effect.

	Growth
Agar treatment	Day 5 (mm)
Treated leaf 4.1mM Methyl Jasmonate	61
Treated leaf 20.5mM Methyl Jasmonate	42
Control leaf	70
Acetone	84

Table 27 shows the diameters of the *P. cinnamomi* culture 5 days after inoculation onto agar.

Contrast	Standardized difference	Pr > Diff	Significant
Control vs Treated leaf 20.5mM	54.147	< 0.0001	Yes
Control vs Treated leaf 4.1mM	17.278	< 0.0001	Yes
Control vs Acetone	0.836	0.836	No
Acetone vs Treated leaf 20.5mM	53.310	< 0.0001	Yes
Acetone vs Treated leaf 4.1mM	16.530	< 0.0001	Yes
Treated leaf 4.1mM vs Treated leaf 20.5mM	31.152	< 0.0001	Yes

Table 28 shows the statistical comparisons between the leaves treated with high and low concentration jasmonic acid solutions compared with each other and a control leaf extraction.

V8 agar plates where poured containing 20ul of the crude phenolic extract from leaves treated with 4.1mM and 20.5mM of jasmonic acid. A leaf without treatment was used as a control and a plate containing acetone was also used.

The results indicate that the extract from the leaf treated with the highest concentration of jasmonic acid had the greatest inhibition of the P. cinnamomi. Although it was not as effective at inhibiting the growth, the extract from the plant treated with 4.1mM jasmonic acid also showed some impact.

Chapter 4

4.1 Discussion

The data can be broken down into two fundamental parts. Firstly the results from the leaf phenolic assays and secondly the *Phytophthora cinnamomi* cultures. Starting at the beginning, the data shows what was expected and stated in the hypothesis. For phenolic compounds an increase in soluble phenolics was shown in plants treated with 20.5mM jasmonic acid. Plants treated with a lower concentration (4.1mM) appeared to show little increase in the amount of soluble phenolics. In terms of esterified phenolics, the amount present within the leaves tends to show a similar pattern to soluble phenolics when treated with a high concentration of jasmonic acid. From day 0 onwards a significant increase can be seen at day 3,6 and 9. A lower concentration of jasmonic acid showed a significant increase at day 3 however this increase soon ceased in comparison to day0. Day 9 has such a large range that it was not statistically possible to show a significant difference. A few plants (as can be seen in the appendix) showed an increase at day 9 however this was not seen consistently throughout the plants. The lack of esterified phenolics in other samples could be due to loss during processing. The presence of esterified phenolics in some samples and not others could also be due to soluble phenolics failing to be fully extracted during the initial processing steps.

Total phenolic concentration was done to try and eliminate any errors that may have occurred during the extraction process. By this I mean any soluble phenolics that were not extracted during initial processing would show up in the esterified phenolic assay. By calculating the total phenolics the relevance of esterified or soluble types can be ignored. In a sense a clearer picture was generated with the total phenolics results. A much more consistent trend appears to be seen with plants treated with 4.1mM jasmonic acid. An increase can be seen at day 9, plants treated with 20.5M jasmonic acid show the same image that soluble phenolics produced.

Tannin content was shown to be fairly consistent within leaves treated with a lower concentration of jasmonic acid. With no significant differences being found between any of the days. With rather low values a large range was also found which could indicate that no consistent reactions were taking place. A higher concentration treatment produced a statistically higher tannin content at day 9. This mirrors the results shown in total phenolics which coherently makes sense as tannins would be included in this assay.

An increase in lignin content was observed in both hormone treatments. The figure below, taken from (Wout Boerjan 2003), outlines Lignin Biosynthesis in plant cells starting from the amino acid Phenylalanine in the top left of the figure. Lignin biosynthesis proceeds through a variety of soluble phenolic compounds (e.g. coumaric, caffeic, ferulic and sinapic acids). Two key enzymes which control the overall process are phenylalanine ammonia lyase (PAL, top left in figure) which controls the overall synthesis of the soluble phenolics and Peroxidase (bottom of figure) which initiates lignin polymerisation.

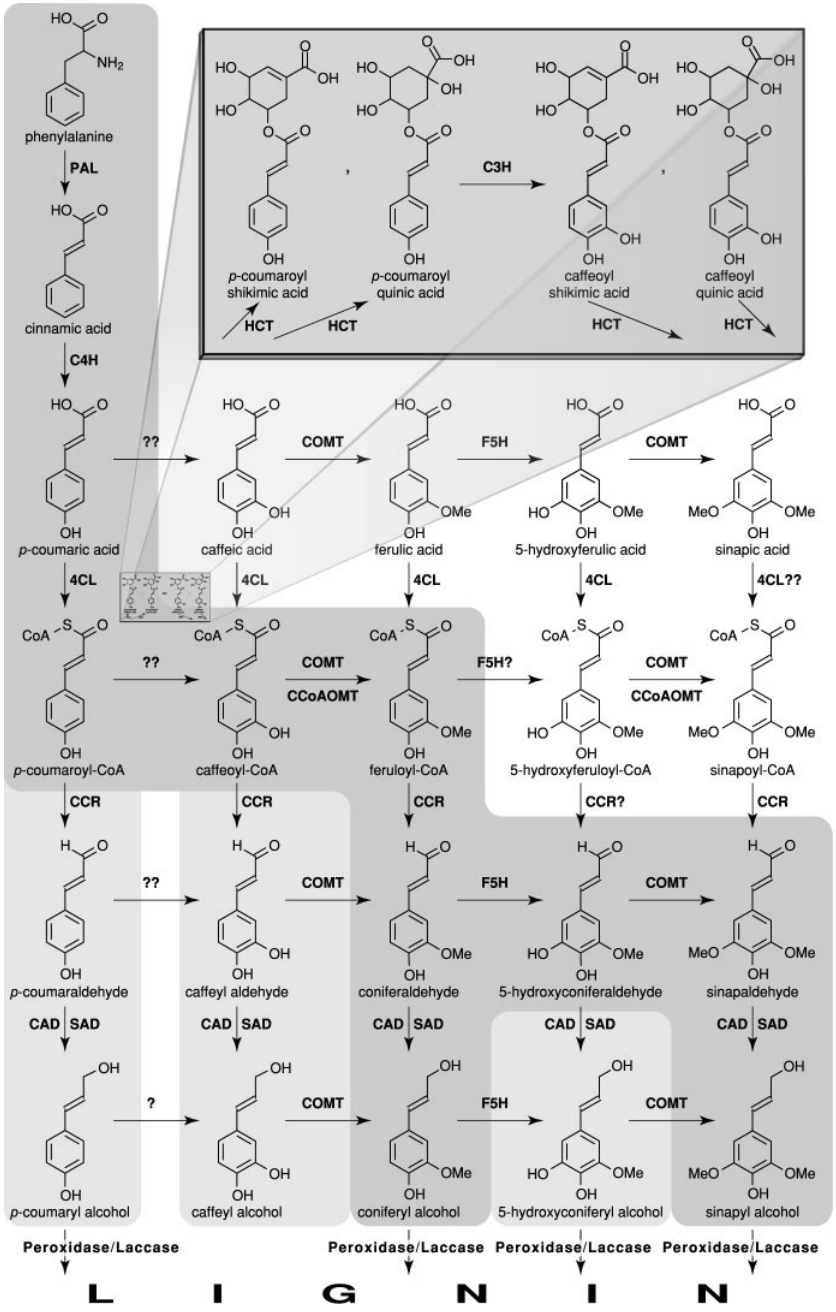


Figure 46 The above image shows the chemical pathway in which lignin is produced (Wout Boerjan 2003).

One possible explanation for the results observed in the current project is that the initial decrease in soluble phenolic content in the kauri leaf cells (Day 0 vs Day 3) occurs as the hormone treatments activate lignin production and the soluble phenolics present are converted to lignin (and also esterified to the plant cell wall). This change is consistent with the activation of peroxidase in Kauri observed by Joseph (Joseph 2014). The later increase in the soluble phenolics that was seen may occur as PAL is also activated by the hormone, the delay being caused by the fact that de novo synthesis of the phenolics is occurring by the shikimate pathway that leads to phenylalanine. Higher hormone concentrations may lead to faster overall activation of this process. Thus the overall effect of the hormone treatment is to strengthen the plant cell wall by attachment of phenolics to the kauri cell wall via esterification and the creation of lignin. This may help to protect against micro-organisms such as Phytophthora.

Salicylic acid treatments of the Kauri plants lead to some interesting results. In terms of soluble phenolics it appears that there is no change in the content caused by the addition of salicylic acid in low concentration. With the addition of higher concentrations of salicylic acid it appears that at day 9 the concentration of soluble phenolics decreases when compared with the original content of the leaf at day 0.

In terms of esterified phenolics there appears to be a remarkable decrease in both the plants treated with a lower concentration of salicylic acid and the control plants between day 0 and day 9. As this is occurring also within the external control plants it could indicate an environmental change has occurred causing the dramatic drop in esterified phenolics. Another possible reason is due to the harvesting of the leaves for testing, this may have triggered a defence or repair response that caused a decrease in the esterified phenolics although this did not occur in the plants treated with jasmonic acid including the controls. The plants treated with a higher concentration of salicylic acid showed an increase in esterified phenolics in day 3 and 6 however at day 9 the concentration decreases and shows no significant difference from day 0. The response that is occurring in the external control plants and plants treated with a lower concentration of salicylic acid appears to be overcome by a treatment of higher concentration salicylic acid. The results shown mimic those shown in an experiment conducted in soy bean plants which show, when infected with *P. megasperma,* an increase in cell wall bound phenolics (esterified phenolics) (Graham and Terrence 1991). The treatment with higher concentrations of hormones has appeared to trigger this defence response within the Kauri leaves.

Previous research has shown that salicylic acid can impair a plants resistance to wounding. The impairment is in the form of suppressing the defence mechanism that jasmonic acid has on a plant. Although this experiment was undertaken on a different species of plant the principle may be the same. By increasing the amount of salicylic acid present, the defensive mechanism of the plants are possibly compromised and in this case the production of phenolic compounds could be negatively affected(Yang, Tang et al. 2011) .

Overall it appears that Kauri plants treated with higher concentrations of salicylic acid do have an increased total phenolic compound content, although this increase was witnessed at different days on different plants.

The tannin content within the Kauri leaves was not significantly changed with a higher or lower concentration treatment of salicylic acid. These results would indicate that salicylic acid does not have a major role in the production of tannins within the plant or perhaps that the mechanism involved with tannin production is not altered via leaf treatments.

Infecting leaves with *P. cinnamomi* offered results that, while not on their own, indicated hormone treatment can slow down the spread of this pathogen. Control leaves indicated that cell death does appear to occur around the inoculation site where *P. cinnamomi* was introduced. This cell death was more obvious in the green leaves however a area of death could also be seen on the red leaves as well.

The infected leaves taken from trees that were grown in solutions containing the plant hormones appeared to show little death if any around the infection site. Although this experiment was more visual than statistical, the results did tend to show that the hormone treatments impacted the spread of *P. cinnamomi* within the leaves.

The plate culture results showed a clear difference in growth inhibition between the control plates and the plates treated with phenolic compounds and extracts, which was proven to be statistically significant with Tukeys test. When compared with literature values a similar pattern emerged with the exception of one phenolic compound. At day 5 cinnamic acid, *p*-hydrobenzoic acid, gallic acid and phloroglucinol provided a similar trend in terms of growth inhibition. Hydroquinone showed unusual results in the sense it had the greatest effect on growth inhibition(Cahill and McComb 1992). It is possible that the strains could be different with the strain used in this research having less resistance to the effect of hydroquinone. Another possibility is that the hydroquinone chemical used for this research was not pure with a possible chemical contaminant present in the stock bottle.

Culture results for V8 agar with leaf extract incorporated harboured results indicating some sort of increased inhibition occurring when compared with control plates and with each other. A statically significant difference was found between the control plates and extract plates, this shows that the plant extract has had an inhibitory effect on the *Phytophthora cinnamomi* culture. A statistically significant difference was also seen between the growth diameter of control leaf extract and treated leaf extract when used at the same concentration. This could indicate that the composition of the extract obtained from the treated leaf differs from that of the control leaf. As both the control leaf extract and the treated leaf extract solutions were adjusted with acetone to have the same concentration it can be concluded that increased phenolic concentration alone in the treated plants is not the only cause for inhibition.

When comparing the growth inhibition between plates containing extract from plants treated with a 4.1mM and 20.5mM concentration of jasmonic acid a significant difference was found. This compliments the results found by the phenolic assays. The phenolic assays showed an increase in the amount of total phenolics when treated with a higher concentration of jasmonic acid. In response to the higher phenolic concentration it appears the culture growth has been inhibited. Although it cannot be concluded from the gained data that it is definitely the concentration of total phenolics having an impact on the growth it can be concluded that this may be a possibility.

Looking at previous investigations carried out with extracts from conifers, it has been found that once extracted various naturally occurring chemicals do have an effect in hindering certain species of *Phytophthora.* Extracts taken from the heartwood of several conifers were found to have inhibition on *P. ramorum* growth at different severities depending on the tree the extracts were obtained from (Daniel K. Manter 2008). While other research showed that the extracts of pine and spruce trees can inhibit mycelial growth and reduce sporulation of *P. cactorum* (Minova, Sešķēna et al. 2015). This is promising for this research as it shows the viability of plant extracts in the inhibition of *Phytophthora.*

It has been shown previously that phenolic compounds tend to accumulate in higher concentration in trees with known resistance to *Phytophthora* species (Nagle, McPherson et al. 2011). This data compliments the findings from this research in the sense that the higher concentration of phenolics are shown to inhibit the growth of *P. cinnamomi.* It would tend to show that in naturally resistant trees, the ability to produce greater amounts of phenolic compounds has helped to protect them against *Phytophthora* infestation.

Comparing all the data obtained during this thesis with data previously collected from literature it appears that there is a strong relationship between phenolic compound content within a plant and the resistance that is shown towards *Phytophthora*. The high phenolic content in Kauri leaves, which was identified after hormonal treatment, was shown to inhibit the growth of *P. cinnamomi* during lab studies in controlled conditions. Previously increased phenolic content in plant leaves was shown to exist in plants with resistance to *P. infestans (Rubio-Covarrubias, Douches et al. 2006)*. Even more closely related was a study showing that plants with roots infected by *P. cinnamomi* contain a greater content of phenolic compounds. Once extracted from the roots it was found that a solution containing this increased phenolic content inhibited the germination of zoospores. In comparison, extract from roots that had not been infected showed lower phenolic content and did not inhibit the germination of zoo spores (Grant 1984). Both lots of data tend to indicate that perhaps increased content of phenolic compounds is the key to *Phytophthora* resistance.

In terms of the connections between hormone treatment of plants and *Phytophthora* infection, phenolic compound increases can also be made. An increase in lignin production was shown during the hormone treatment of Kauri trees during this thesis, previous research has shown plants infected with *P. cinnamomi* also show increase lignin concentration (Cahill, Bennett et al. 1993). Although this is admittedly a relatively weak link, it none the less shows that in both instances an increase can be seen within the lignin content, that combined with the knowledge that lignin plays an important factor in plant defence may show why treatment with plant hormones can increase plant resistance.

Reading through various papers and via this research it appears that, although it is possible to slow down and inhibit the growth and spread of *phytophthora*, there are few results to indicate that eradication is possible. Treatment with various chemicals, such as metalaxyl-M and phosphorus acid which are commonly used in nurseries, have shown to be very effective with controlling the spread of *Phytophthora.* These chemicals are still however only used to control the spread and not destroy the pathogen, for this reason other preventive factors ,such as good soil drainage and control of infected plants, are still required (Reglinski, Spiers et al. 2010). Experiments showed that a single application was sufficient in the prevention of the spread of *Phytophthora* and that subsequent treatments only offered slightly better results.

Although this treatment was useful in the prevention of *P.cactorum* it also showed the limitations of such chemical treatments in terms of application. When measurements of metalaxyl were carried out on the soil samples around the plants treated during seedling emergence, it was found

that the product was breaking down and after about 30 days half the product was inactive. Due to this breakdown, metalaxyl was seen to be ineffective against root rot. Treatment with metalaxyl six months after seedling emergence, one week after root pruning, however showed to be more effective in suppressing root rot.

Another aspect to look at is the effect of treatment on adult trees. Radiata pine has a fast growth rate when compared to Kauri, pine reaching maturity within 25-35 years compared to Kauri taking 50 years to just break a forest canopy but with the ability to live for over 1000 years. For this reason it is important to find a way to not only treat juvenile Kauri trees but also mature trees. It would be wise then for future research to also explore treatments of various age trees.

Other treatments trialed involved biological agents. *Trichoderma* spp. were also introduced into the soil with the knowledge that this beneficial fungi is effective in the control of some diseases. However, this treatment was also found to be ineffective in preventing *Phytophthora* outbreaks.

Treating pine seedlings with methyl jasmonate was also found to inhibit growth. When the pine seedling were treated with a doss of phosphorous acid however no growth inhibition was found. The stunted growth of the seedling with methyl jasmonate treatment could be due to most of the plants energy going into strengthening the cells and trying to prevent a large scale infection instead of producing chemicals to induce growth. As the protective measures provided by treatment with methyl jasmonate offer little resistance to *P. cactorum* this would seem more detrimental to the tree.

During this experiment several options were available in terms of what part of the plant should be used to measure the phenolic content. Previously phenolic compounds have extracted from root (Golmakani, Mohammadi et al. 2014), leaf and bark samples (Veggi, Prado et al. 2014) each sample type giving results in term of phenolic compounds. Due to time constraints measuring at each site was not possible. To use root samples would most likely mean to kill the tree each time a sample was needed or at best cause a greater amount of injury than other sites. Bark samples would also cause greater harm than leaves and may also have a greater effect on following day samples. For these reasons it was determined that leaf samples would offer the best results in terms of survival rate of the plants and still offer enough sample mass to test for phenolic compounds. Using leaves also offered the ability to carry out replicates for most experiments thus increasing the accuracy and reliability of the statistics.

One possibility for future experiments could be to treat the roots of the plants with either soil treatments or directly. As *Phytophthora* most commonly infects via the roots this could be a possible way to treat the plant more directly to the site of infection. Although the phenolic compounds extracted from the plants had no effect in destroying the pathogen it is possible the plant hormones could directly have an effect against *Phytophthora.* Possible culture

assays similar to the ones carried out on the phenolic compounds is another possible to see if anything effect can be had against the pathogen.

Kauri trees are detrimentally effected by *P. agathidicida* rather than *P. cinnamomi*. It would seem more prudent then to use *P. agathidicida* during these experiments. If *P. agathidicida* was going to be used physical containment would be required through the course of the work. As this research was more about measuring changes occurring within the plants during hormone treatments and not the direct effect of the hormones on the pathogen it was possible to substitute the pathogen with one that required less containment.

Using *P. cinnamomi* in place of *P. agathidicida* allowed for more freedom during the experiments. Any results obtained which involved *Phytophthora* would be seen as an indication rather than a certainty. Another advantage of using *P. cinnamomi* over *P. agathidicida* was that due to amount of research already carried out on *P. cinnamomi* it was possible to find some literature values and previous research with which to compare my own results. As *P. agathidicida* has only recently been identified it was difficult to find relevant data that would correlate with these experiments.

If inhibition had been seen during this experiment involving *P. cinnamomi* further experiments may have been more beneficial, however, it is doubtful that treatments that have not completely inhibited *P. cinnamomi* would effected *P. agathidicida.* This is due to fact that treatments most commonly used to prevent the spread of *P. cinnamomi* have not been effective against *P. agathidicida* however effective treatments are often effective throughout other species of *Phytophthora.*

One aspect not covered during this research was the broader effect of plant hormone treatment on Kauri. Although detached leaf assays were carried out it could be beneficial in future experiments to study the plants for longer periods of time to determine if the plant hormones are impacting on the virulence *Phytophthora* is having on the plant. It may possibly be that other defensive chemicals such as terpenes are being produced in greater amounts. This could provide beneficial protection throughout the root system of the plant.

4.2 Conclusion

Through this research it is apparent that Kauri trees do respond to treatment with plant hormones. Treatments with jasmonic acid and salicylic acid in concentration of 20.5mM both showed increases in lignin with phenolic compounds shown to increase in both treatments however reducing in salicylic acids treatments after the increase. Although the exact type of phenolic compounds was not investigated during this thesis it can be seen that jasmonic acid did have an effect on the tannin content however salicylic acid did not.

Lower treatment with the same hormones showed be to less effective on the phenolic content. It is possible that a greater concentration of hormone could in turn have a greater increase on phenolic content however doing so would most probably cause damage to the plant itself.

Culture results indicated that extracts from leaves treated with jasmonic acid did have an effect on inhibiting the growth of *P. cinnamomi.* These extracts however did not stop the growth of the culture, the most potent phenolic compound in this research, hydroquinone, also showed inhibition however this too also failed to completely stop the growth.

As previous experiments showed that some treatments had various effects depending on when they were introduced it may be that plant hormones show similar trends. The research carried out in this thesis was on juvenile plants, possibly if the same experiments were undertaken on trees of various age and growth stage, greater inhibition might be seen.

Future research could possibly be carried out on what effects plant hormones have directly on *Phytophthora.* Other possibilities for future study could include investigating the site at which the hormones are introduced. As infection from *Phytophthora* is most common via the roots perhaps treatment at this site might provide greater protection.

Although the results indicated that hormone treatment by itself cannot stop the spread of *P. cinnamomi* it could be possible that a combination of plant hormones with other chemicals, such as commonly used metalaxyl or phosphorous acid, may have a great impact on inhibiting the pathogen growth.

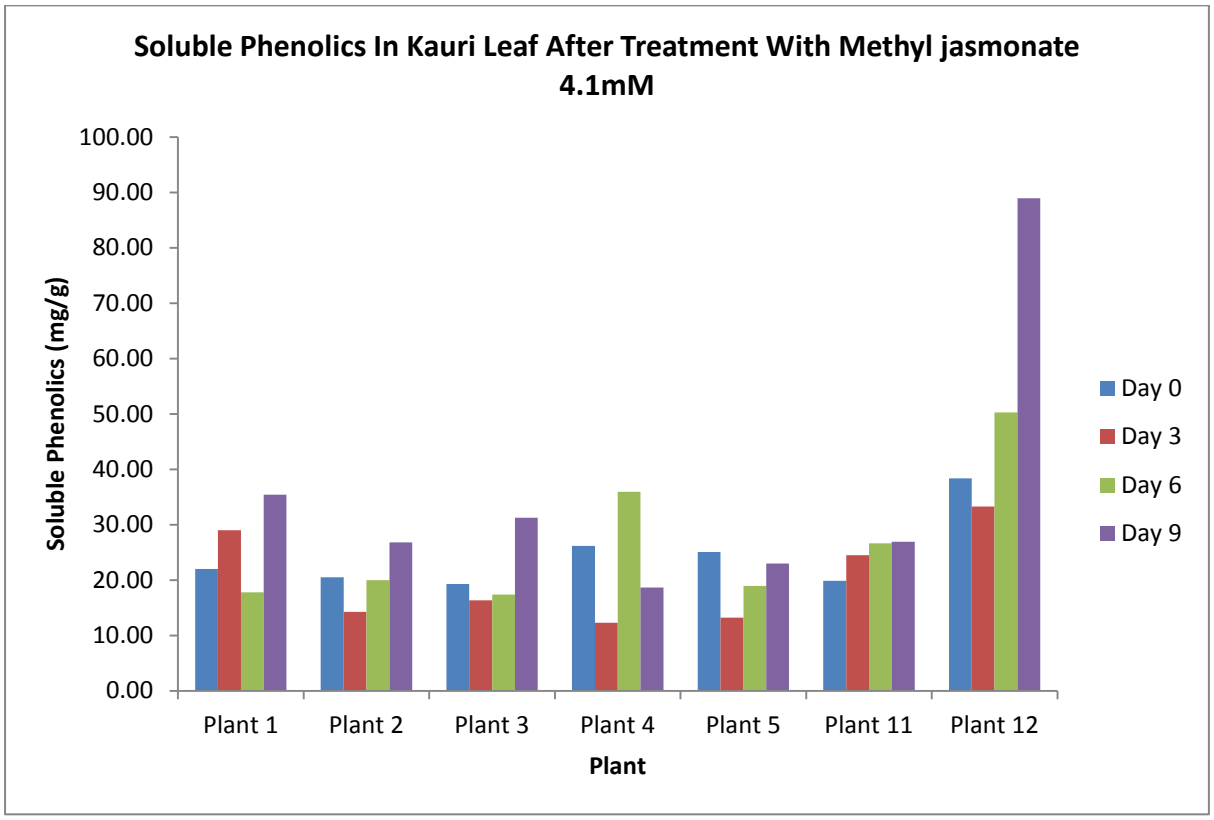
To conclude it appears that the treatment of Kauri plants with naturally occurring hormones would not be effective in *Phytophthora* eradication. This mirrors previous research in pine which has shown that foliar methyl jasmonate treatment is not effective in eradicating infection with *P.cactorum* (Reglinski, Spiers et al. 2009). With a single treatment of phosphorous acid having the desired effect, even follow up treatments, or combined

treatments are not necessary. Although some of the current chemicals used do tend to break down over time within the soil, if introduced to the plant at the right stage it provides the desired effect.

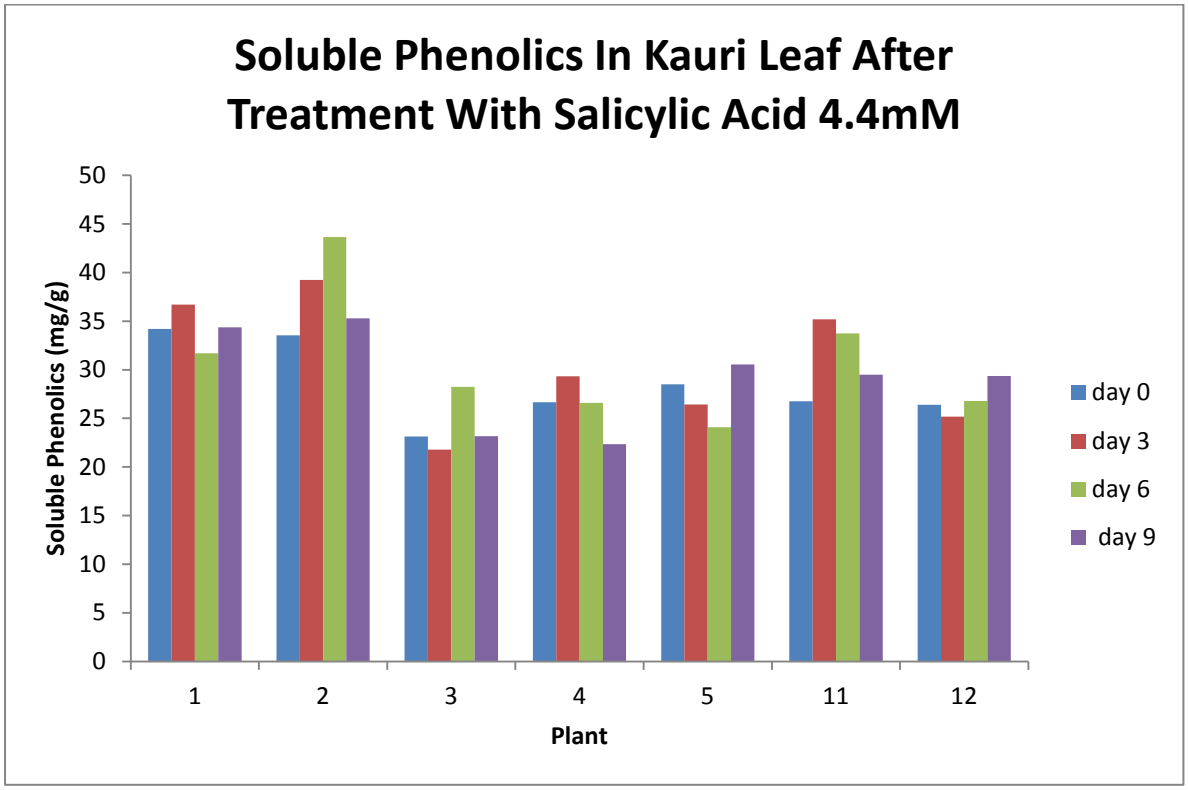
At this stage it would seem like the most effective means to prevent the spread of *P. agathidicida* lies with prevention rather than cure. That is to say stopping the spread through physical means such as trampers and movement of contaminated tissue from infected sites to sterile sites. As soil conditions also appear to have an effect in terms of water drainage it would indicate that where possible new growth should be encouraged in well drained areas. This does not help mature trees as it would be difficult and not viable to move entire root systems are also increase the chance of spreading the disease. It is however possible that the information obtained through this thesis could possibly be used as a foundation for further research, one that may combine hormone treatment with possible chemical or biological treatments to obtain a synergistic effect.

Appendix

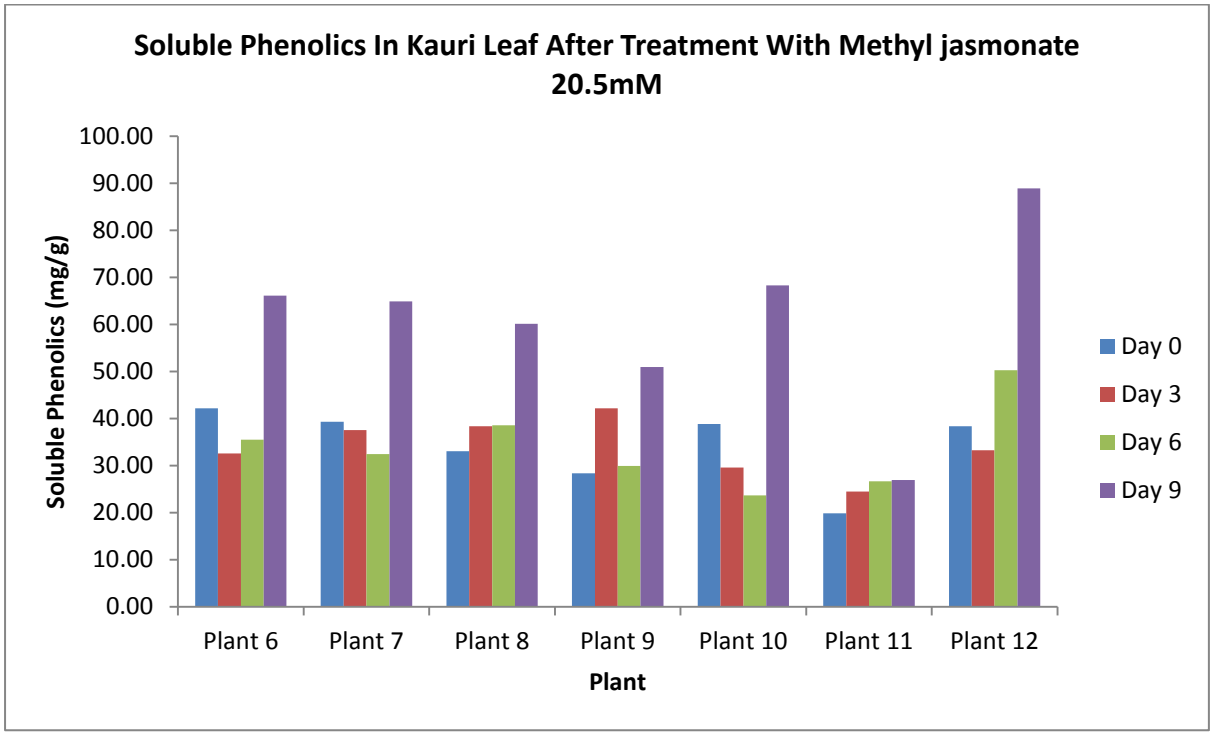
In the following figures plants 1 to 10 have been treated with hormone whereas plants 11 and 12 have been sham treated by spraying with the appropriate solvent and removing leaves.



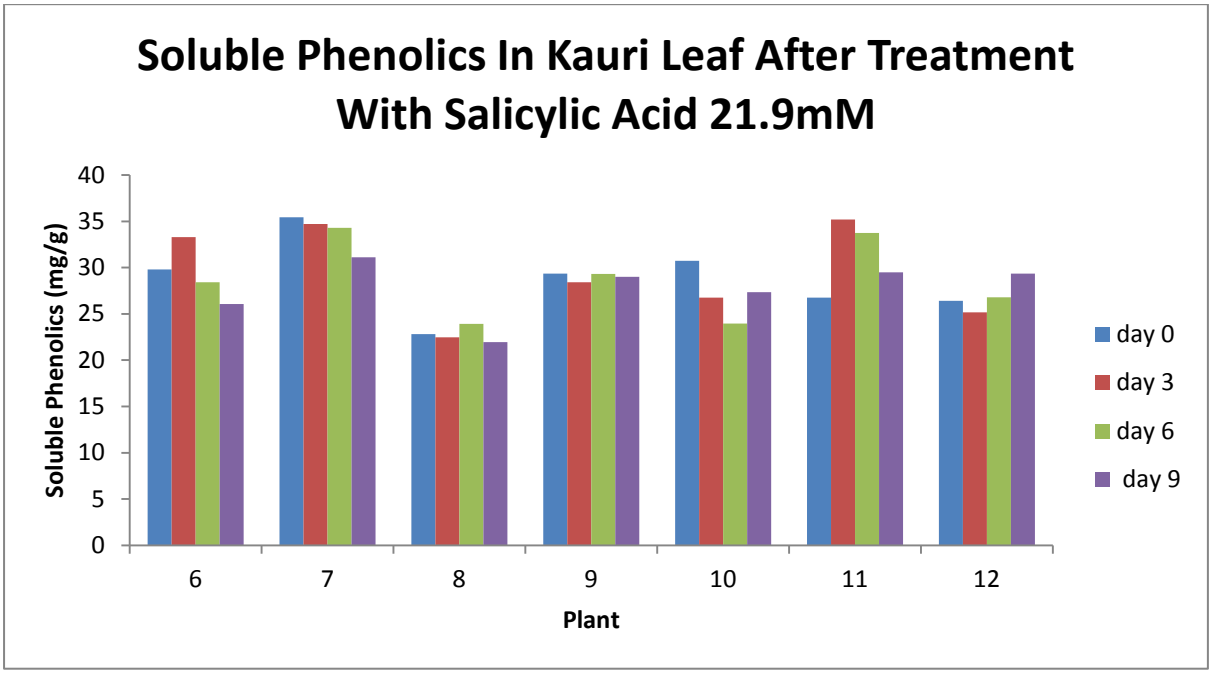
When treated with a lower concentration of Jasmonic acid there appears to be little change in the soluble phenolic concentration. Plant 1,2 and 3 appear to have slightly raised concentration on day 9 however the control plant 12 also shows a large increase.



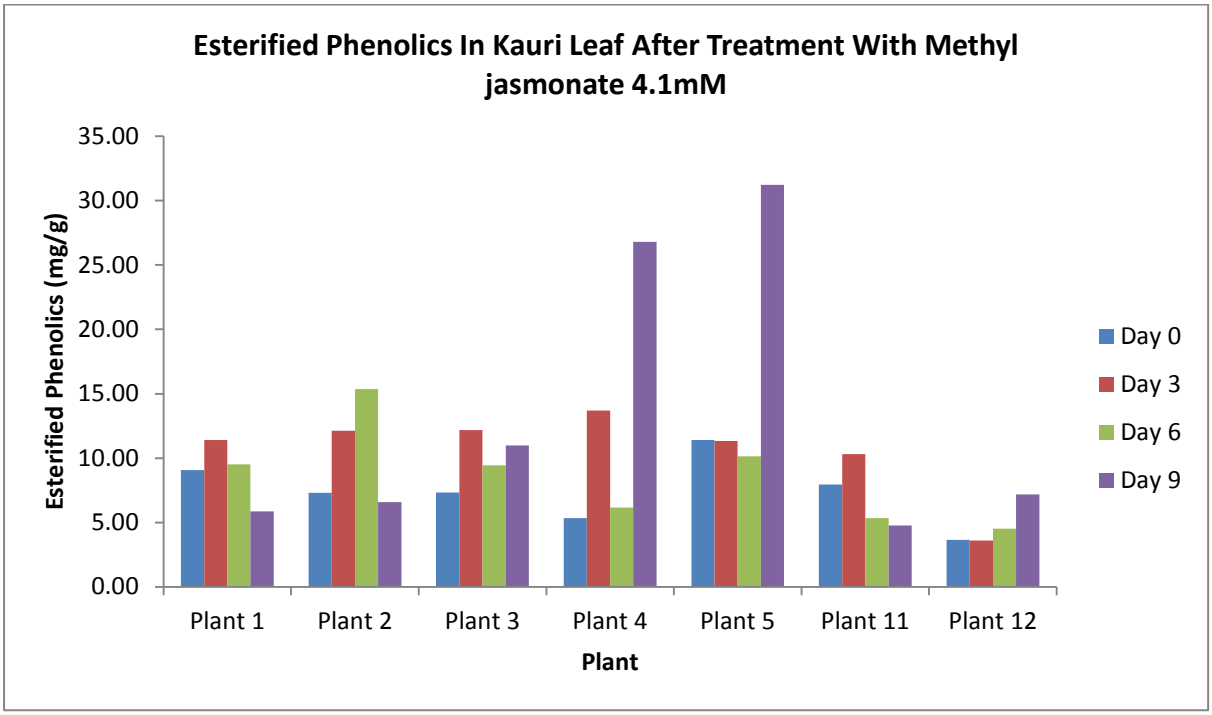
No pattern has emerged from the graph above. The only noticeable difference can be seen in plant 2 with a slight increase at day 3 to 6 and in control plant 11 which also shows an increase at day 3 with a sudden decrease at day 6.



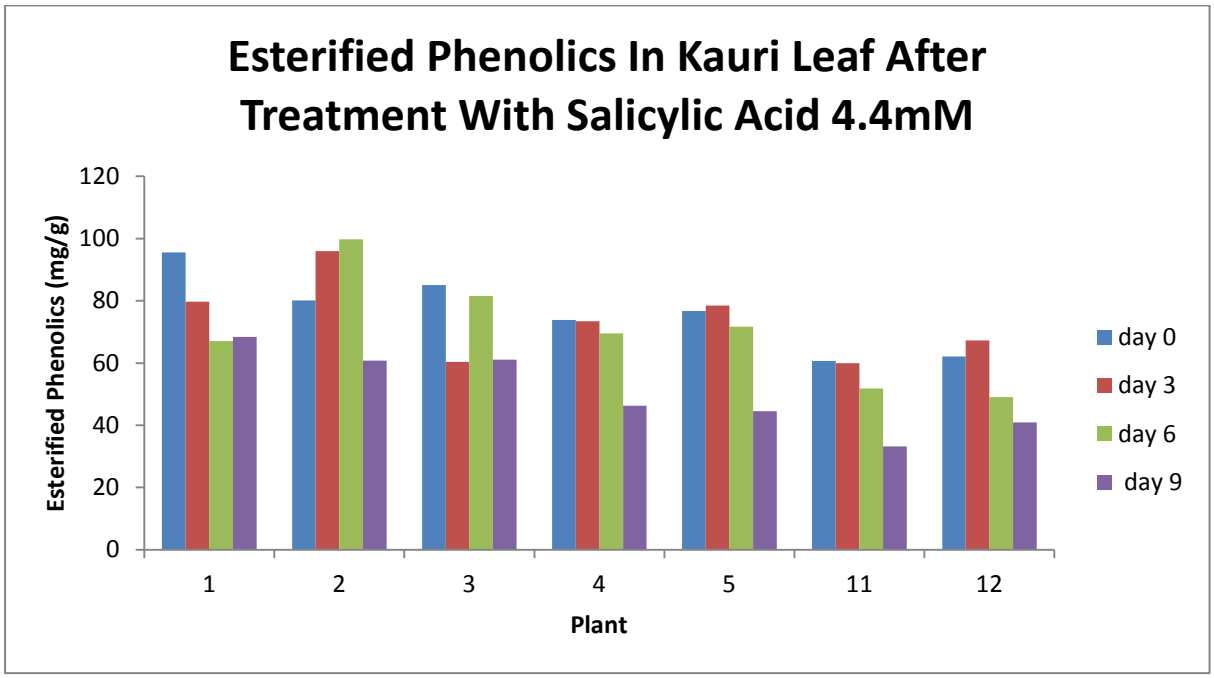
The data shows a increase in the concentration of Jasmonic acid when a concentration of $0.5molL^{-1}$ is used. Plant 6,7,8,9, and 10 all show increases when compared to day 1. Plant 9 also shows a slight increase at day 3. Control plants shows a large increase at day 9 however control plant 11 show minimal increase.



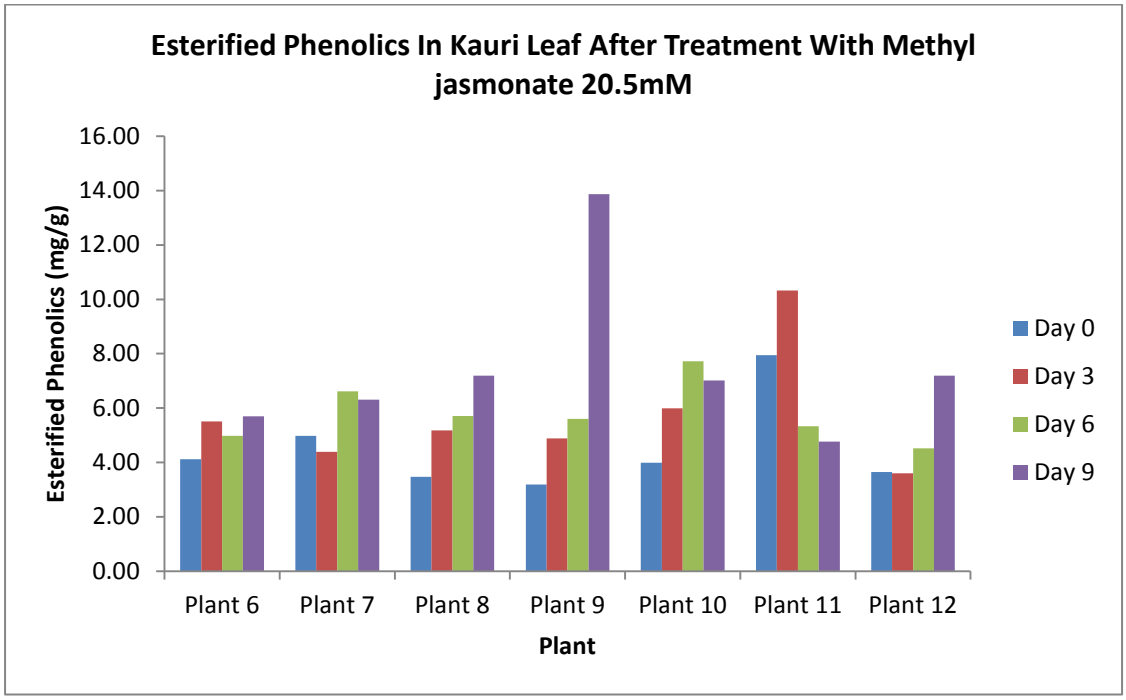
As with the leaves treated with a lower concentration of salicylic acid there appears to be no pattern. The amount of phenolics present appears to be relatively stable amongst all days for each treated plant.



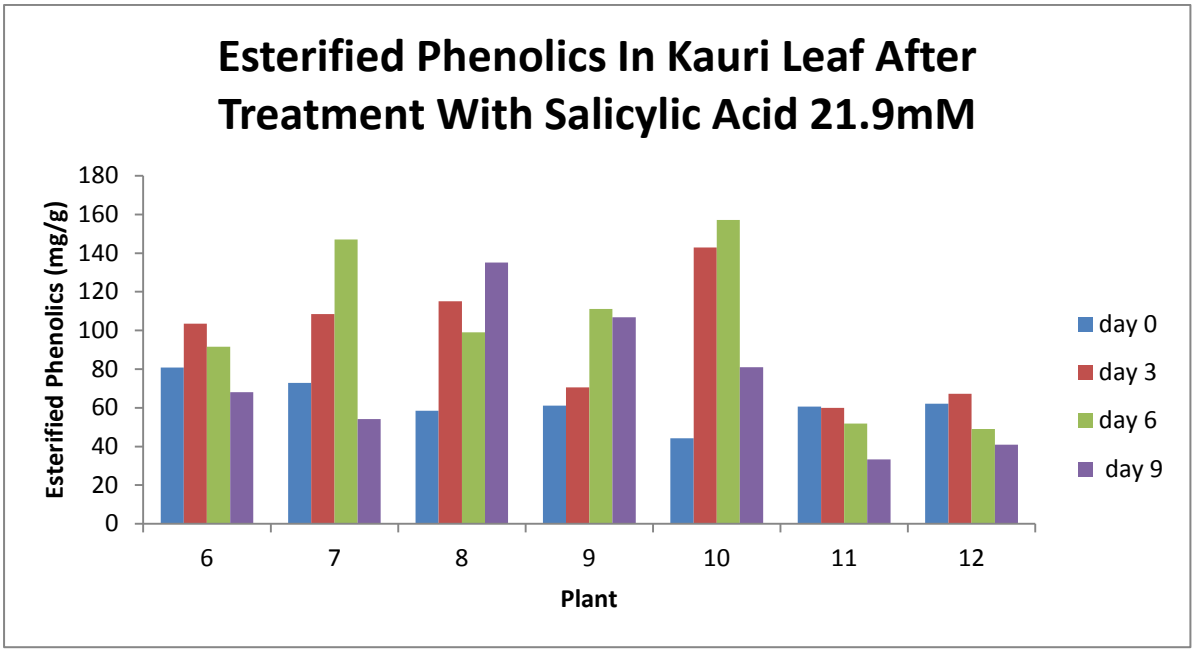
Esterified phenolics appear to increase after day 0 and in the case of plant 1 and 2 it appears to decrease at day 9. Plant 4 and 5 show a large increase at day 9 however there does not appear to be a consistent pattern amongst the results.



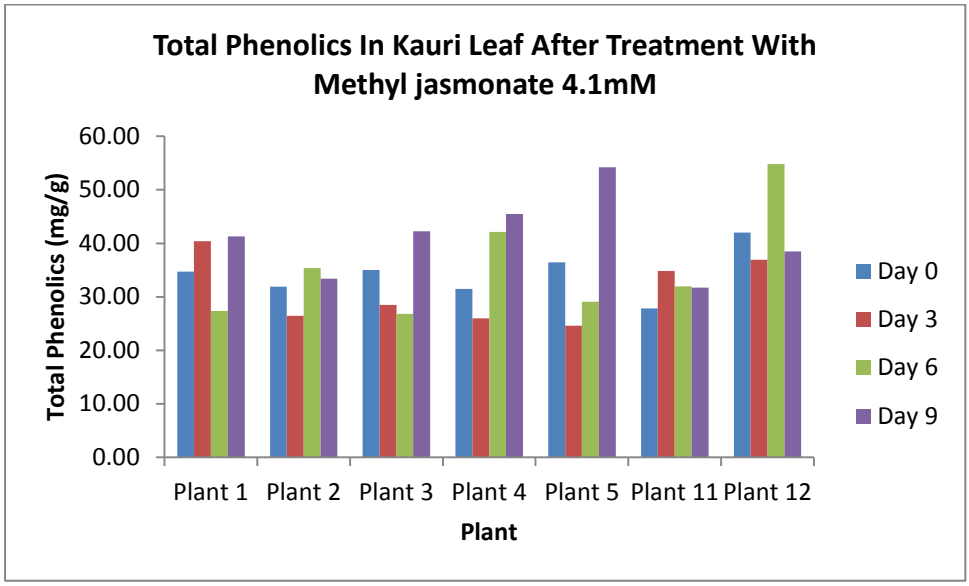
The data appears to show a decrease in the amount of esterified phenolics at day 9 with all the plants including the control plants. Plant 2 shows and increase at day 3 and day 6.



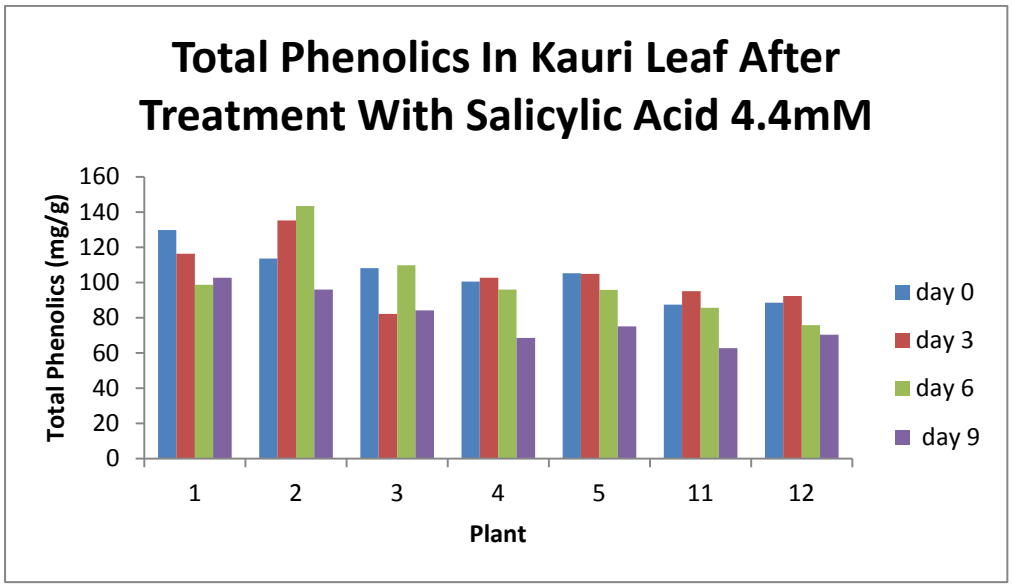
An increase is apparent in most plants after day 0 however the most significant changes appear to occur in plant 8,9 and 10. Control plant 11 shows a decrease in the concentration whilst control plant 12 shows a increase at day 9.



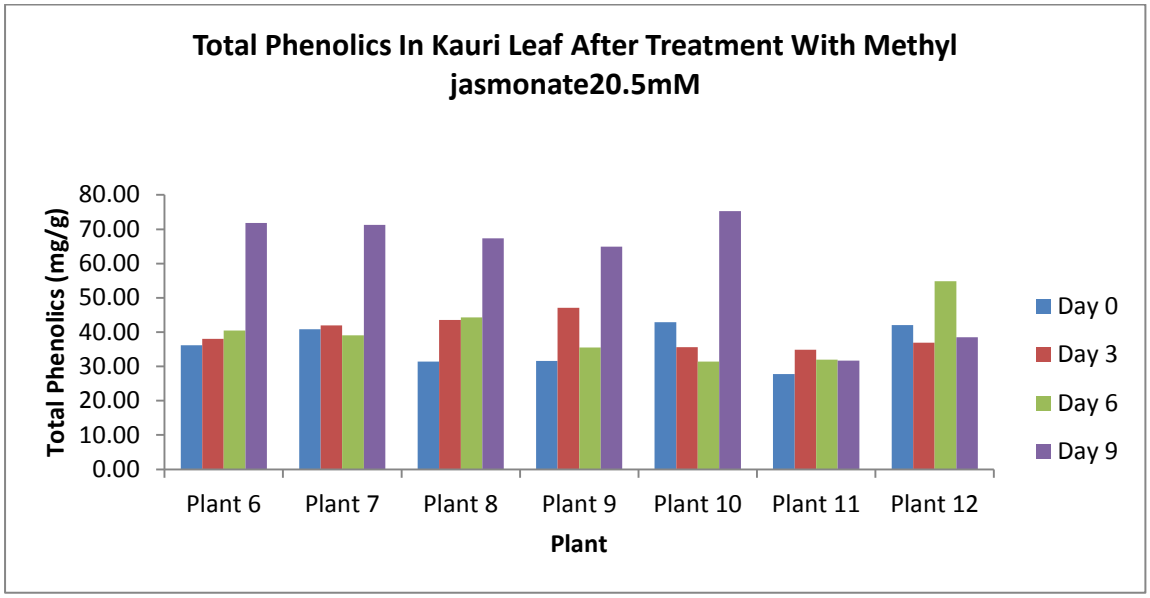
When treated with a higher concentration of Salicylic Acid it appears that an increase in esterified phenolics does occur. Plant 6,7,8,9 and 10 all show an increase at day 3 with that increase continuing in plant 7,8,9 and 10. Plants 6,7 and 10 however show a decrease at day 9.



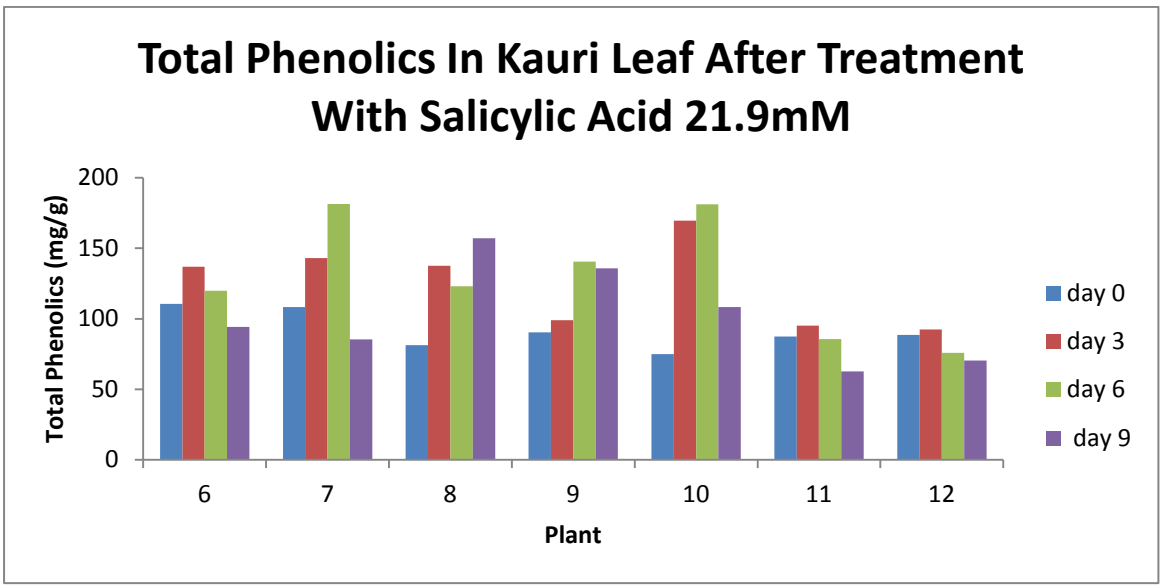
There does not appear to be a pattern of increase in the total phenolics when treated. A slight increase can be seen in all treated plants between day 0 and day 9 however it does not look significant at first glance with the exception of plant 4 and 5.



Within the total phenolic data for a lower concentration it appears the only plant to show and change is plant 1 with a decrease in phenolics and plant 2 with an increase in phenolics. Plants 3,4 and 5 and control plants 11 and 12 all show a decrease in total phenolics at day 9 when compared with day 0.

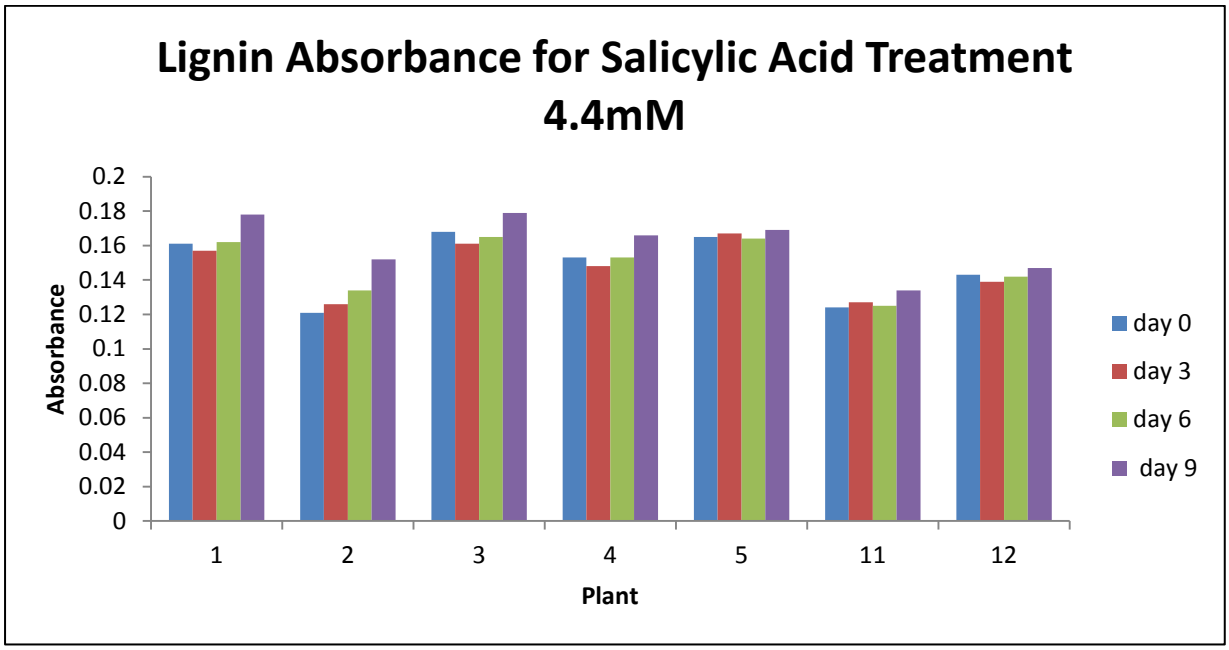


When looking at the total phenolics for the plants treated with a higher concentration of Jasmonic acid it seems that an increase can be seen in all treated plants between day 0 and day 9. The increase looks significant and cannot be seen in the either control plant 11 or 12.



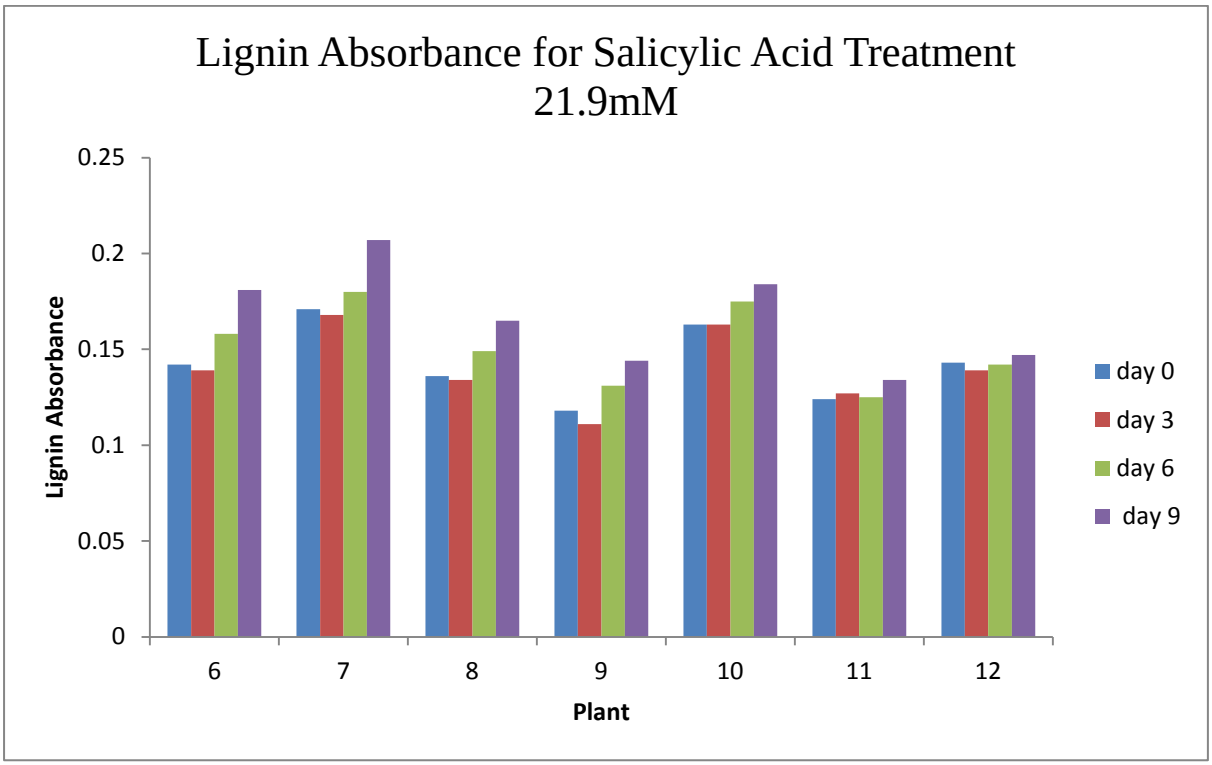
The data is indicative of a an increase in phenolic production with a higher concentration of salicylic acid. It appears there is an increase in all treated plants which appears to peak at day 3 or day 6 with the exception of plant 8. In the case of plants 6,7 and 10 there is a noticeable decrease at day 9, this can also be seen to a smaller effect in control plant 11.

Lignin



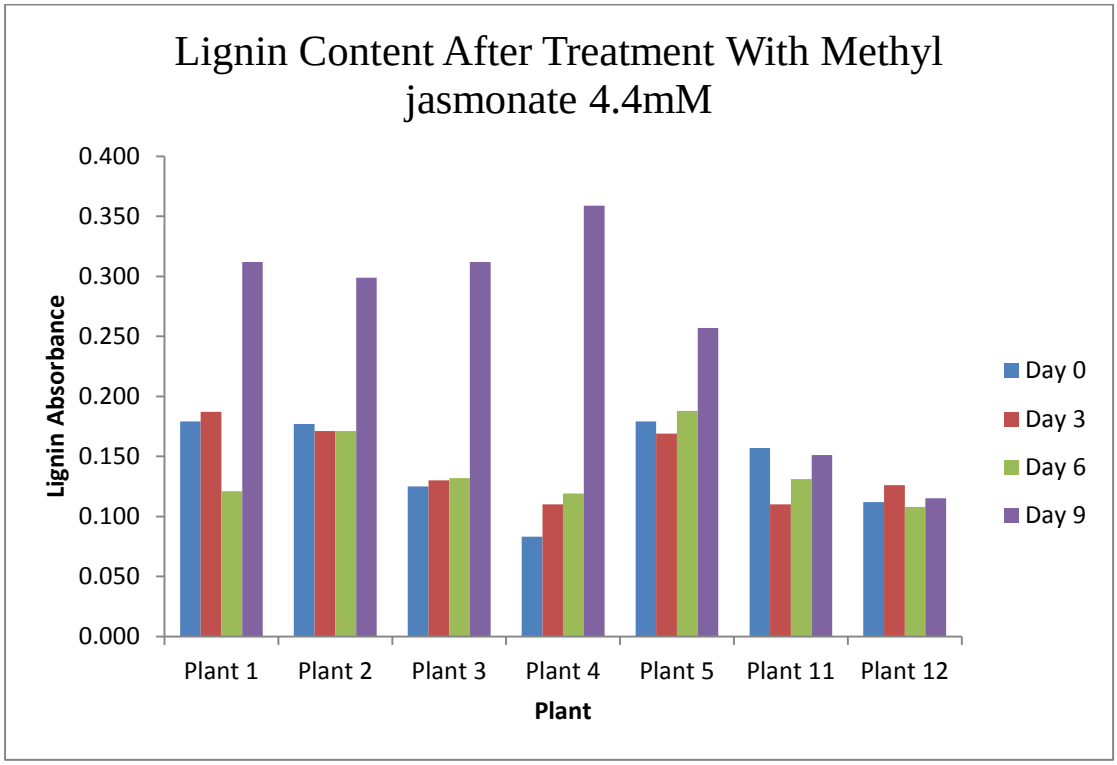
The above graph shows the absorbance of lignin in the Kauri leaves after treatment with a low concentration of salicylic acid

The lignin content appears to be the same for all plants, perhaps slightly increasing over the 9 days with the exception of plant 2 which appears to slightly higher increase. Both control plants indicate a similar trend with a very minimal increase between day 0 and day9.



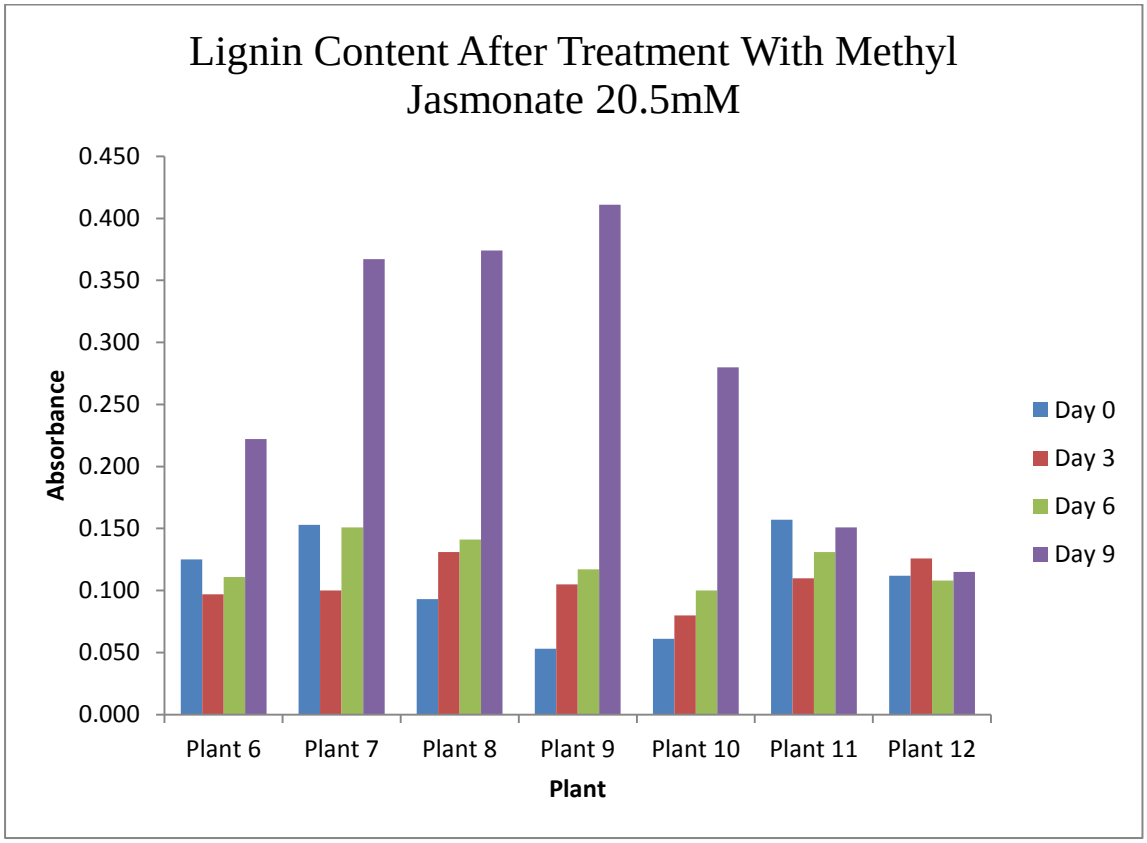
The above graph shows the absorbance of lignin in the Kauri leaves after treatment with a high concentration of salicylic acid

There appears to be a increase occurring over the 9 days and it appears to occur in all the treated plants. Although it looks like there is a slight increase in the control plants it seems to be very minimal.



The above graph shows the absorbance of lignin in the Kauri leaves after treatment with a low concentration of jasmonic acid.

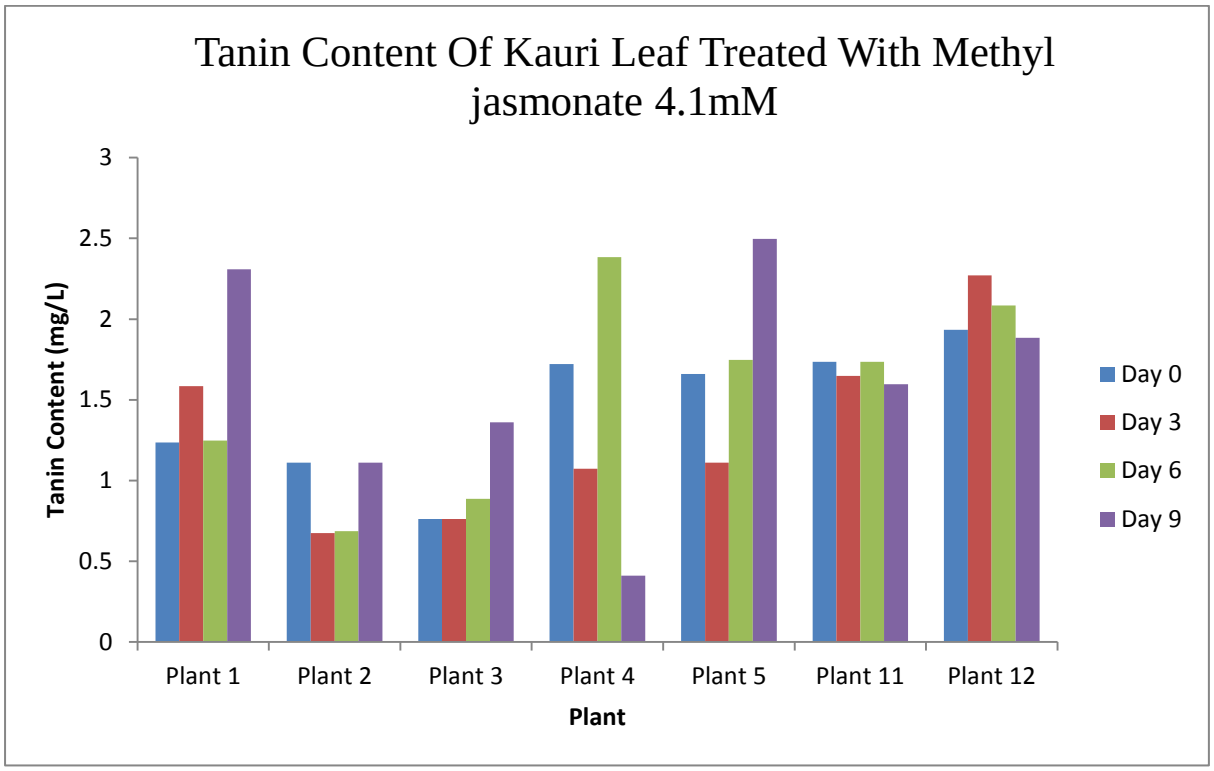
An obvious increase in the lignin content is apparent between the first 6 days and day 9. The concentration appears to be a lot greater in the treated plants when compared with the control plant. There appears to be little difference between the first 6 days in all plants with the exception of plant 1 with a slight decrease in day 6.



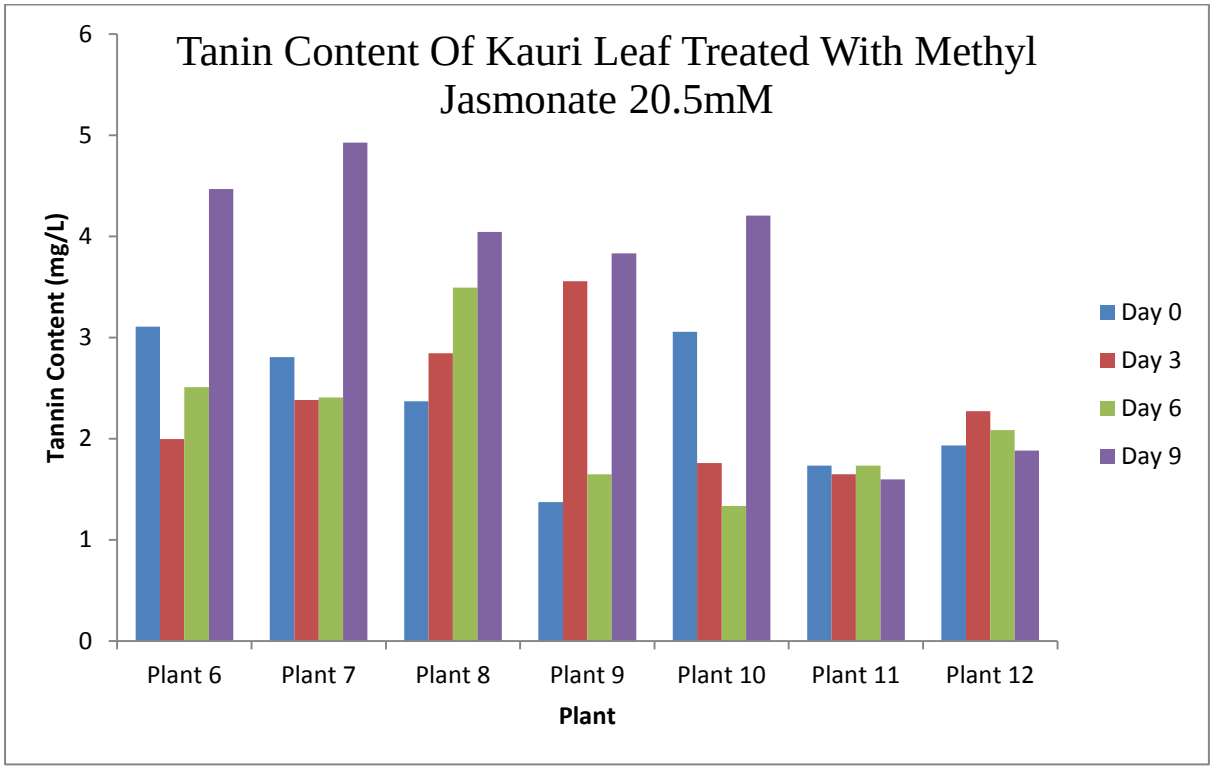
The above graph shows the absorbance of lignin in the Kauri leaves after treatment with a high concentration of jasmonic acid

With a higher concentration of jasmonic acid similar results were obtained in comparison to lignin content with a lower concentration of jasmonic acid. Day 9 indicates a much larger content of lignin throughout all the treated plants. The two control plants appear to be unchanged throughout the nine days.

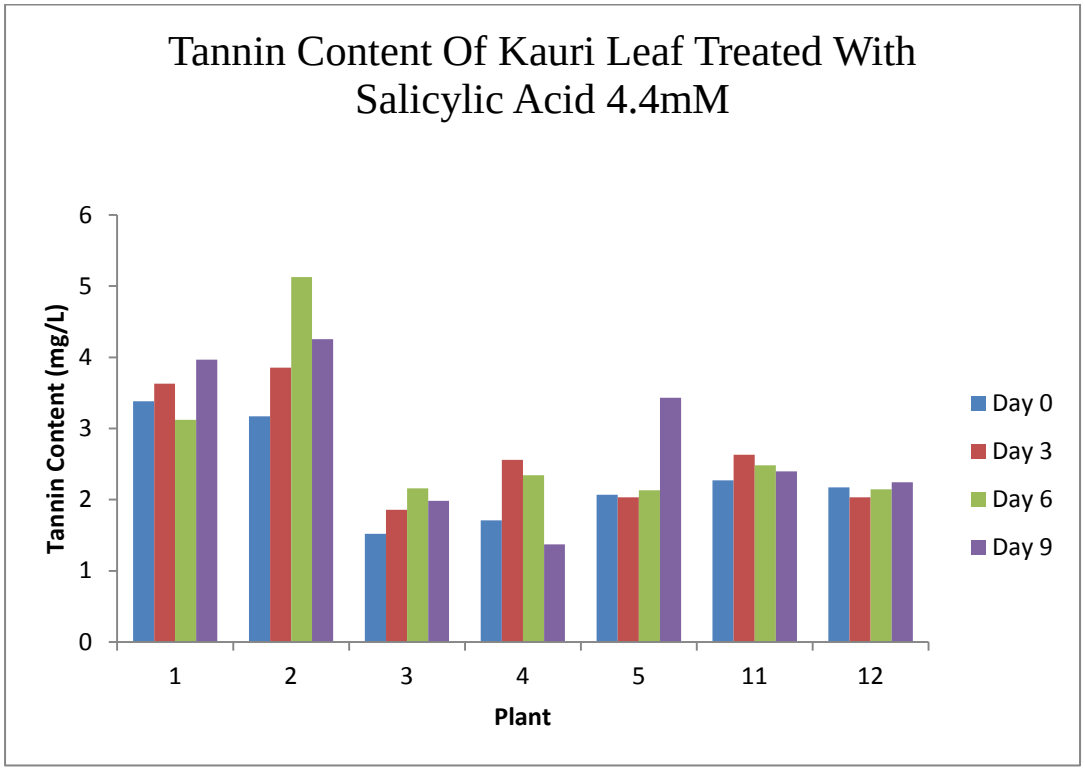
Tannins



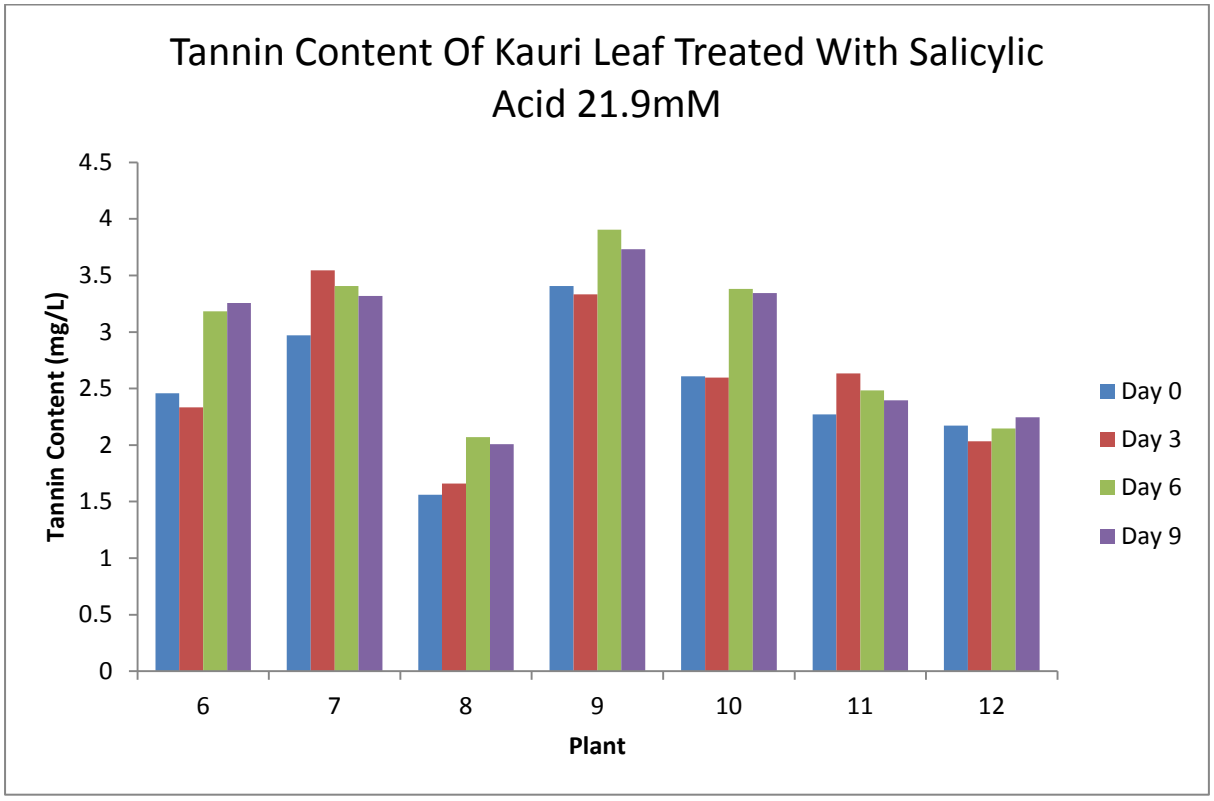
The tannin content increases at day 9 within plant 1 and plant 5 and slightly with plant 3. Plant 4 shows a decrease in tannin content at day 9 and an increase at day 6. Plant 2 appears to have a similar content between day 0 and day 9. The controls appear to be unremarkable over the 9 days.



The plants treated with a higher concentration of jasmonic acid appear to have a clear trend. The tannin content looks to increase greatly in all plants between day 0 and day 9. The tannin content looks to have doubled in plant 9 between day 0 and 9 however the content on day 3 is almost level with day 9. The control plants show little change over the 9 day time period.



A low concentration of salicylic acid has appeared to have little effect on the tannin content of the kauri trees. The data indicates only plant 5 had an increase in tannin content at day 9. Plant 2 had its greatest content at day 6 then decreased at day9. The control plants indicate no great change.



As with the plants treated with a lower concentration of salicylic acid, there appears to be no large increase in the tannin concentration. A slight increase can be seen in plant 6 at day 6 and also in plant 7 at day 3. Plant 8, 9 and 10 also seem to show an increase at day 6 which then decreases again at day 9. Control plants 11 and 12 remain relatively unchanged in the tannin concentration.

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