SALICYLIC ACID AND ITS ROLE IN DEFENCE AGAINST *PLASMODIOPHORA BRASSICAE*

By

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List of abbreviations

°C	Degrees
µl	Microlitre
µm	Micrometer
µM	Micromolar
Cm	Centremeter
Col-0	Columbia
cpr1	Constitutive pathogenesis related
dH20	Deionised water
dnd1	Defence, no death
ECD	European Clubroot Differential
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
G	Grams
H_2O	Water
HCl	Hydrochloric acid
IR	Induced resistance
*Jar*1	Jasmonate insensitive
K_2HPO_4	Dipotassium hydrogen orthophosphonate
KAc	Potassium acetate
KH_2PO_4	Potassium dihydrogen orthophosphonate
L	Litre
Ler-0	Landsberg erecta
LSD	Least significant difference

M	Molar
mL	Millilitre
Min	Minutes
Mm	Millimetre
Ng	Nanogram
nM	Nanomolar
MS	Murashige and Skoog
npr1	Non-expresser of pathogenesis related
PC2	Physical containment level 2
PCR	Polymerase chain reaction
PDF	Plant defensin
PR	Pathogenesis related
RP-HPLC	Reverse-Phase-High Performance Liquid Chromatography
RT-qPCR	Real-Time-quantitative PCR
S	Second
SA	Salicylic acid
SAR	Systemic acquired resistance
SPAD	Soil plant analysis development
SPSS	Statistical Product and Service Solutions
Tul-0	Turk Lake
v/v	Volume/volume

Publications relating to this thesis

Journal publications:

The following publication is a combination of work from **chapters 3** and **4**:

David Lovelock, Caroline Donald, Xavier Conlan and David Cahill. Salicylic acid suppression of clubroot in broccoli (*Brassica oleracea* var. *italica*) caused by the obligate biotroph *Plasmodiophora brassicae*. Australasian Plant Pathology (DOI) 10.1007/s13313-012-0167-x.

Conference presentations:

David Lovelock, Arati Agarwal, Caroline Donald, Ian Porter, Rob Faggian and David Cahill (2009). Assessment of the role of Salicylic acid in *Brassica oleracea* (Broccoli). 17th Biennial Australasian Plant Pathology Society Conference, Newcastle, Australia.

David Lovelock, Arati Agarwal, Caroline Donald, Ian Porter and David Cahill (2010). Priming for SAR in Brassicaceae species against *Plasmodiophora brassicae*. 6th Australasian Soilborne Diseases Symposium, Sunshine Coast, Australia.

David Lovelock, Caroline Donald, Xavier Conlan and David Cahill (2011). Priming for resistance in the Brassicaceae family against *Plasmodiophora brassicae*. 18th Biennial Australasian Plant Pathology Society Conference, Darwin, Australia.

David Lovelock, Caroline Donald, Xavier Conlan and David Cahill (2012). Priming for resistance in the Brassicaceae family against *Plasmodiophora brassicae*. 2012 American Phytopathological Society, Annual Meeting, Rhode Island, USA.

Summary

The obligate biotroph Plasmodiophora brassicae is a devastating root pathogen responsible for millions of dollars of loss' in revenue around the world through the destruction of brassica crops. Within Australia, *P. brassicae* is prevalent in almost every state and is of particular importance as a number of economically important crops are susceptible to the pathogen. Current control methods do not adequately prevent infection of *P. brassicae* on susceptible hosts, nor is there a clear understanding of the role the phytohormone salicylic acid (SA) plays in a defence response.

As mentioned, not only has SA not been actively studied as a means of control, the mechanism by which SA may provide resistance against P. brassicae has not been widely studied. The interaction between *Arabidopsis thaliana* pathway mutants and *P. brassicae* has highlighted key aspects of the SA pathway which may be critical for a resistance response to occur. SA over-expressors *cpr1* and *dnd1* both show reduced galling from *P. brassicae* compared to the SA deficient *NahG* plants and wild-type Col-0. SA is known to induce *PR-1* when elevated concentrations are present and this is observed in the over-expresser lines of *A. thaliana* and in wild-types treated with SA at 0.5 and 1 mM.

Although SA is a key hormone in plant defence, concentrated applications of it can be detrimental to plant health. *Brassica oleracea* plants root-drenched with SA above 5 mM show phytotoxic responses through chlorosis of the leaves and reduction in plant biomass. Below concentrations of 5 mM there are limited if any developmental effects on *B. oleracea* and there is an activation of defence

responses through the up-regulation of *PR-1*. SA is now known to be systemically transported through plants as applications of SA to the roots are found to be concentrated in the shoots through high performance liquid chromatography analysis. The application of SA to *B. oleracea* successfully initiated a defence response in *B. oleracea* and has been shown in this research to reduce the root galling associated with *P. brassicae* in the glasshouse and field.

In conclusion, these results, the first in *B. oleracea*, indicate the importance that SA plays not only as a hormone in plant-pathogen defence, but as a possible application for *P. brassicae* control. Further research into the role SA plays in the defence against *P. brassicae* is needed to accurately identify target genes in a resistance response.

Chapter 1: Literature Review

1.1 General introduction

All plant species face an array of biotic and abiotic stresses on a daily basis and must respond with physiological (Jeun et al., 2000) and chemical changes in order to deal with these stimuli. Biotic stresses include a number of soil-borne pathogens such as; *Phytophthora, Pythium* (Okubara & Paulitz, 2005) *Sclerotinia, Fusarium* and *Plasmodiophora*. Abiotic stresses include the constant exposure to a changing environment where pressures such as drought, flood, temperature and salt stress are ever present and they can influence an interaction with a biotic stress. In most circumstances a plant is capable of defence against infection caused by fungi, oomycetes and other soil-borne pathogens through the direct inability of the pathogen to penetrate the first line of defence, such as wax and cuticle which cover epidermal cells (Agrios, 2005).

If however, a pathogen is able to penetrate and infect, the plant may initiate a defence response. Plants may induce structural defences to prevent spread into healthy regions or initiate a local or systemic response; both responses often result in the up-regulation of defence proteins (Dempsey & Klessig, 2012). In cases where a pathogen is not recognised and neutralised, the plant will almost always succumb to the pathogen, this susceptibility is true for both foliar and soil-borne pathogens.

Soil-borne pathogens comprise some of the most economically devastating pathogens including; *Phytophthora cinnamomi* Rands, the cause of root rot in a

large number of the world's native plant species including more than 2500 in Australia alone (Hardham, 2005). *Gaeumannomyces graminis* var. *tritici*, cause of take-all disease in wheat, has been attributed to millions of dollars in loss of revenues worldwide (Khaosaad et al., 2007; Macdonald & Gutteridge, 2012).

Plasmodiophora brassicae Woronin, is soil-borne biotroph and the causal agent of clubroot in a number of Brassicaceae species including broccoli, cabbage and cauliflower, has been isolated from many European countries, Japan, America and throughout parts of Australia. In Australia in the late 90's, particularly many areas of Victoria, revenue lost due to *P. brassicae* was estimated at close to $A13M, a result of stunted crop growth (Faggian et al., 1999). Clubroot can be considered a 'cancer' of the roots, in which virtually all nutrients and minerals are unable to be transported from the roots to the stems, resulting in under-development or stunting of the crop (Alix et al., 2007; Siemens et al., 2009).

Host defence is crucial for plant survival when dealing with insects and pathogens, hormones and secondary metabolites form part of a number of key molecules which a plant can utilise to actively defend itself. One strategy to defend against some biotrophic pathogens includes the production of the secondary plant metabolites, glucosinolates. There are a vast range of groups of glucosinolates; however there is some debate as to whether they are utilised by the pathogen in the formation of galls or actively up-regulated by the plant to combat the pathogen (Ludwig-Muller et al., 1997; Ludwig-Muller, 2009). Cell proliferation and root galling occur by the indirect aid of a couple of key plants hormones, auxin and cytokinin (Ludwig-Muller, 2009). Once the pathogen has

successfully penetrated the root hair, an increase in auxin concentration is thought to aid in cell proliferation. Secondary plasmodia differentiate to produce spores which fill and enlarge root cells and account for the large galls on the roots of highly susceptible plants (Ingram & Tommerup, 1972).

Controlling *P. brassicae* with chemicals has been utilised over the years, however no chemical exists that completely eradicates the organism. There are currently no chemicals in use that provide sustained protection against *P. brassicae*. There is however a select few Brassica crops that have been bred for resistance against the pathogen, including oilseed brassicas, *B. napus* (Donald & Porter, 2009). Two chemicals currently in use which have direct fungicidal activity against *P. brassicae* are fluazinam and calcium cyanamide (Donald *et al*., 2001; Donald *et al*., 2004).

Manipulation of soil pH has been widely seen as a strategy which can effectively control the infection rate of *P. brassicae*. As the pathogen infects favourably in a pH range of 5-6, liming soil increases the pH to above 7 and limits the pathogen's ability to infect (Narisawa *et al*., 2005). Decreasing the pH to below 5 does appear to reduce the occurrence of infection; however there is a suggestion that lowering it too much can affect plants ability to uptake important nutrients and minerals (Weaver & Hamill, 1985).

Successful control of a pathogen relies on the plants ability to first recognise and then respond to it. Systemic acquired resistance (SAR) is an important defence response against invading pathogens as it can quickly control the spread of the

pathogen before any lasting damage is done. It is believed that salicylic acid (SA) is one of the first signal molecules to be induced during an SAR response. For this research SA is believed to provide induced resistance (IR) at a local level.

1.2 Taxonomy of Plasmodiophorids

The Plasmodiophoridae family contains 10 genera, these are; *Ligniera*, *Membranosorus*, *Octomyxa*, *Plasmodiophora*, *Polymyxa*, *Sorodiscus*, *Sorosphaera*, *Spongospora*, *Tetramyxa* and *Woronina*. Plasmodiophorida comprises a range of pathogens including; *Plasmodiophora brassicae*, *Spongospora subterranean* f. sp. *nasturii*, *S. subterranean* f. sp. *subterranea*, *Polymyxa graminis*, *P. betae* and *Sorosphaera veronicae* (Cavalier-Smith & Chao, 2003; Hoppenrath & Leander, 2006; Goecke *et al*., 2012). All 10 genera are able to infect plants, some are economically important such as: *S. subterranean* f. Sp. *subterranea*, the causative agent of powdery scab of potato (Merz, 2008) and *Plasmodiophora brassicae*, the cause of clubroot on many Brassicaceae species.

1.2.1 *Plasmodiophora brassicae*

Classification of *P. brassicae* has always been a challenge for researchers as analysis of the pathogen's life-cycle has created difficulties in determining its exact place. *Plasmodiophora brassicae* has been likened to slime moulds and fungi due to the characteristics its shares with these, including; cruciform nuclear division, multinucleate plasmodia and long lived spores (Cavalier-Smith, 1993). Therefore in the past *P. brassicae* was grouped in the Kingdom Protozoa.

Plasmodiophora brassicae is currently classified under the Order 'Plasmodiophorida', Class: 'Phytomyxea', Kingdom: 'Rhizaria'. However, up until the early 1990's, before Cavalier-Smith (1993) classed *P. brassicae* as a Phytomyxea, it had been classified as a fungus and protist (Castlebury & Domier, 1998). A recent study from Goecke *et al*., (2012) has confirmed the classification of *P. brassicae* under Phytomyxea (Figure 1. 1).

The exact origin of the soil-borne pathogen *Plasmodiophora brassicae* is not known, what is known however, is that Michael Stephanovitch Woronin first isolated it in the mid-to-late 1800's in Russia. The pathogen was therefore named *Plasmodiophora brassicae* Woron. It became quite clear that the pathogen was not only a problem in Russia but many parts of Europe and even as far away as the United States (Woronin, 1878). *Plasmodiophora brassicae* is now worldwide endemic with reports in Europe, Africa, North and South America and many Asian and Oceanic nations, including Australia (Kuginuki et al., 1999; Donald et al., 2002; Tewari et al., 2005). Unfortunately the last worldwide survey addressing the loss in crop numbers was undertaken over 30 years ago by Crête (1981). It had been estimated through this survey that the crop losses due to *P. brassicae* equated to between 10-15% worldwide (Dixon, 2009b). It is difficult to know if current losses in crops worldwide would be similar to those of 30 years ago, as although there have been some preventative measures put in place, such as through the use of fungicides and liming, there has most likely been an increase in the production of Brassica crops due to a rising population.

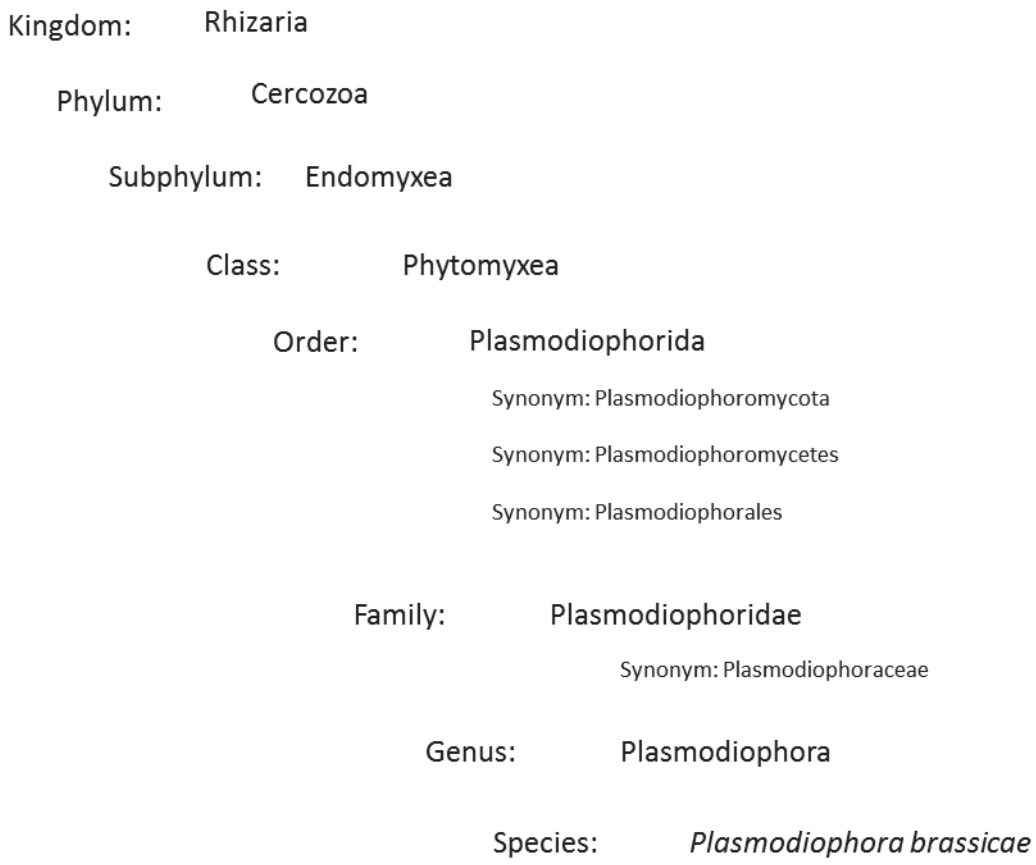


Figure 1. 1: Taxonomy of *Plasmodiophora brassicae*

Taxonomy indicates that *P. brassicae* belongs to Kingdom Rhizaria and although its life cycle closely resembles that of a number of water moulds such as *Phytophthora*, it is not considered an oomycete and therefore not classified as a protist (Hoppenrath & Leander, 2006; Goecke *et al*., 2012). The pathogens characteristic cruciform nuclear division ensures its classification.

Plasmodiophora brassicae is known to infect many economically important Brassicaceae crops throughout Australia and the world, including *Brassica oleracea*, *Brassica rapa*, *Brassica napus* and *Raphanus sativus* (Cao et al., 2008; Osaki et al., 2008). Importantly *P. brassica* infects a very well-known Brassicaceae plant *Arabidopsis thaliana*, with much work being undertaken using *A. thaliana* (Siemens et al., 2002).

1.2.2 European Clubroot Differential

Use of the European clubroot differential (ECD) host plants to classify *P. brassicae* began in the mid-late 1970's. It has now been used to identify at least 128 different pathotypes of the pathogen (Buczacki *et al*., 1975; Kuginuki *et al,* 1999) (Table 1. 1). The ECD is now recognized as the standard rating system for *P. brassicae* isolates around the world. The rating system was designed around 15 host *Brassicae* species, 5 each from *B. rapa*, *B. napas* and *B. oleracea*, with a score or binary value assigned to each of the 5 lines. The order of the host plants does not change as the binary value is fixed to each of the plants, the values appear as follows; 2^0, 2^1, 2^2, 2^3, 2^4. These scores then equate to denary values of; 1, 2, 4, 8 and 16. *P. brassicae* collection isolates are screened against all 15 lines and values are totaled for any of the lines that are susceptible. This is important information as each code is unique, as only one combination of numbers can give rise to a particular ECD code. Within Australia one of the most common isolates found has the ECD code 16/2/31 and is known to infect a large population of crops in the *Brassica oleracea* species (Donald *et al,* 2006).

Table 1. 1 : European clubroot differential (ECD) codes of 3 common *P. brassicae* isolates found in Australia and their respective resistant and susceptible hosts as defined by their ability to infect 5 varieties of *B. rapa*, *B. napus* and *B. oleracea*. Binary and Denary values give rise to the ECD codes which identifies an isolates pathogenicity. Table modified from Donald *et al*., (2006).

	Brassica rapa					*Brassica napus*					*Brassica oleracea*					
Original Host	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	ECD code
Chinese cabbage	?	?	R	R	**S**	**S**	**S**	**S**	**S**	**S**	**S**	?	**S**	**S**	R	16/31/13
Cabbage	R	R	?	R	**S**	**S**	**S**	R	R	?	**S**	**S**	**S**	**S**	?	16/3/15
Broccoli	R	R	R	R	**S**	R	**S**	R	R	R	**S**	**S**	**S**	**S**	**S**	16/2/31
Binary Value	2^0	2^1	2^2	2^3	2^4	2^0	2^1	2^2	2^3	2^4	2^0	2^1	2^2	2^3	2^4	
Denary Value	1	2	4	8	16	1	2	4	8	16	1	2	4	8	16	

'S' indicates susceptible interaction, 'R' indicates a resistance interaction, '?' indicates an interaction which is best described as tolerance.

1.2.3 *P. brassicae* in Australia

Plasmodiophora brassicae was first recorded in Australia in 1891 and is believed to have been transported to Australia from infected nursery stocks from Europe (Donald *et al*., 2006). *Plasmodiophora brassicae* has now been isolated in most states of Australia with Victoria and New South Wales recording a large variation in pathotypes. A few isolates have been recorded in more than 2 states, suggesting a possible spread through either infected feed stock or nursery plants. Of the most damaging isolates, ECD 16/2/31 infects a number of crop species in the Brassicaceae family including *B. oleracea*, *B. rapa* (Figure 1. 2) and *B. napus* and has been isolated in Victoria, New South Wales, Queensland and Western Australia.

1.2.4 Life cycle of *P. brassicae*

The life cycle of *P. brassicae* is still open to conjecture, however it is widely accepted that it occurs in two stages, the first occurring in the soil and root hair cells, whilst the second stage occurs in the cortical cells of the root cortex (Kageyama & Asano, 2009) (Figure 1. 3). Resting spores of *P. brassicae* will only germinate in the soil if the appropriate stimuli are present; these include appropriate plant exudates from a species, such as *B. rapa* and *B. oleracea* and sufficient moisture in the soil. With appropriate stimuli the resting spore germinates to release a primary zoospore and is able to move freely by the use of two flagella through the water (Ingram & Tommerup, 1972; Dixon, 2009a).

Figure 1. 2: Effect of *P. brassicae* in the field.

Chinese cabbage (*B. rapa*) crops in Victoria affected by *P. brassicae* showing characteristic wilting due to the pathogen (inside red section) while unaffected crops remain healthy. Picture obtained from Caroline Donald.

Upon reaching a susceptible host, the zoospore encysts, thereby losing both its flagella and developing a secondary membrane. A germ tube-like structure develops from the encysted spore and penetrates into the root hair, upon which the parasite/amoeba is injected into the root hair (Ingram & Tommerup, 1972; Ludwig-Muller & Schuller, 2008).

Nuclear division of the amoeba occurs, which results in the formation of primary multinucleate plasmodium, this multinucleate plasmodium then cleaves to form zoosporangia. The formation of zoosporangia is regarded as the completion of the first stage of the infection cycle (Ingram & Tommerup, 1972; Ludwig-Muller & Schuller, 2008; Kageyama & Asano, 2009).

It is believed the zoosporangia release the majority of their zoospores into the soil these spores then penetrate the cortical tissue and the the second stage of infection begins in the form of binucleate secondary plasmodium. Through a series of mitotic divisions the secondary plasmodium transform into multinucleate secondary plasmodia, prolific cell division accompanies this stage of infection. In what can be regarded as the final stage of infection, the sporulating plasmodium is cleaved and form resting spores which fill the majority of the root galls (Ingram & Tommerup, 1972; Ludwig-Muller & Schuller, 2008).

Figure 1. 3: Life cycle of *P. brassicae*.

(**A**) Resting spores germinate into bi-flagellate primary zoospores and (**B**) upon finding a susceptible host encyst and penetrate root hairs.

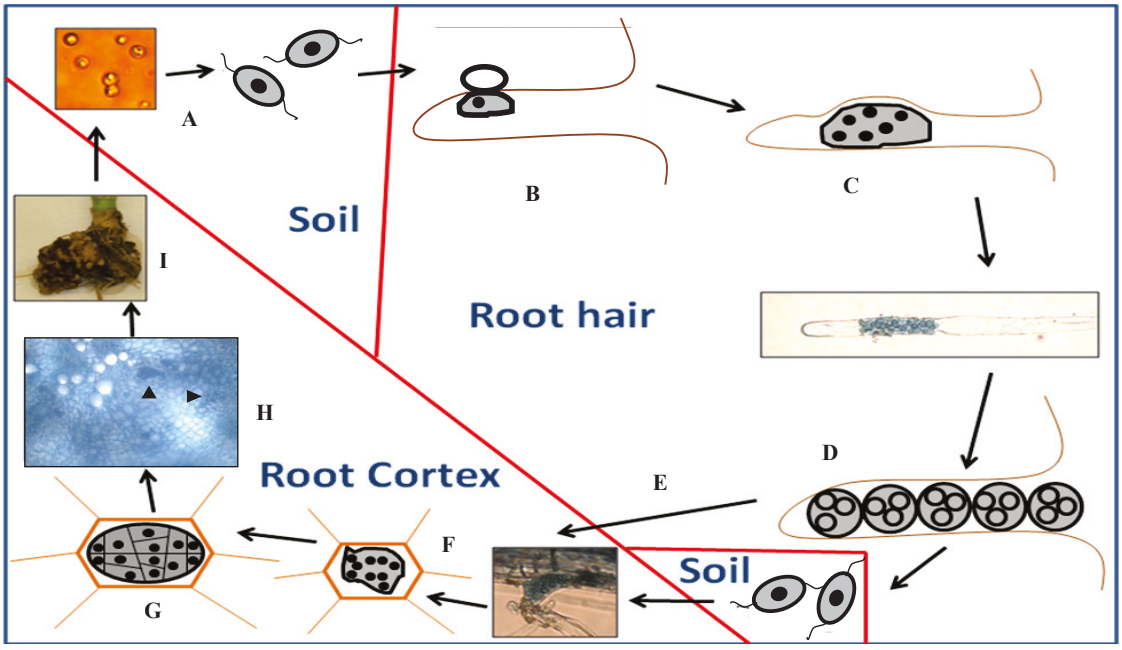
(**C**) Uninucleate primary plasmodium develops within the root hairs and (**D**) multinucleate zoosporangia develop via nuclear division.

(**E**) Secondary zoosporangia are released into the soil and (**F**) infect the cortical cells of the host plant where mitotic divison occurs giving rise to multinucleate secondary plasmodium.

(**G**) Sporulating plasmodia then form in the root cortex (**H**) giving rise to resting spores

(**I**) which increase galling in the root system and are eventually released back into the soil.

Modified from Ingram & Tommerup (1972), Ludwig-Müller & Schuller (2008) and Kageyama & Asano (2009).


A
B
C
D
E
F
G
H
I
Soil
Root hair
Root Cortex
Soil

1.2.5 Characteristics of infection

Michael Woronin first described the characteristics of *P. brassicae* infection as, "The disease of cabbage… is generally portrayed by characteristic swellings of various shapes and sizes on the roots of affected plants…" Root galls (Figure 1. 4) are still the major characteristic of *P. brassicae* infection, their formation is due to a number of contributing factors; the mass production of spores from within the root cortex and cell proliferation of the root that accompanies the increase of spore numbers (Ingram & Tommerup, 1972; Devos et al., 2006). There is very good evidence that the hormones indole-3-acetic acid (IAA), auxin and cytokinin may contribute to root gall formation (Ludwig-Muller & Schuller, 2008; Siemens et al., 2008; Siemens *et al*., 2006).

1.2.6 Root gall formation

Root galls are formed in the final stages of infection and are accompanied by cell proliferation within the root cortex, which may be caused by increases in intracellular levels of auxins and cytokinin (Siemens et al., 2008). This increase in cell numbers allows for the mass production of *P. brassicae* spores, a result of cleavage of secondary plasmodium (Ingram & Tommerup, 1972). The root gall restricts the uptake of vital nutrients and minerals as it interferes with the vascular tissue of the root cortex in particular the phloem and xylem (Figure 1. 5). Once the plant becomes starved of vital minerals and nutrients it begins to die as many of its molecular processes begin to fail. Eventually the root and shoot of the plant wilt and may die leaving the root gall in the soil to rot. Once the gall has become rotten, the spores are released into the soil where they seek out susceptible host plants when conditions are right.

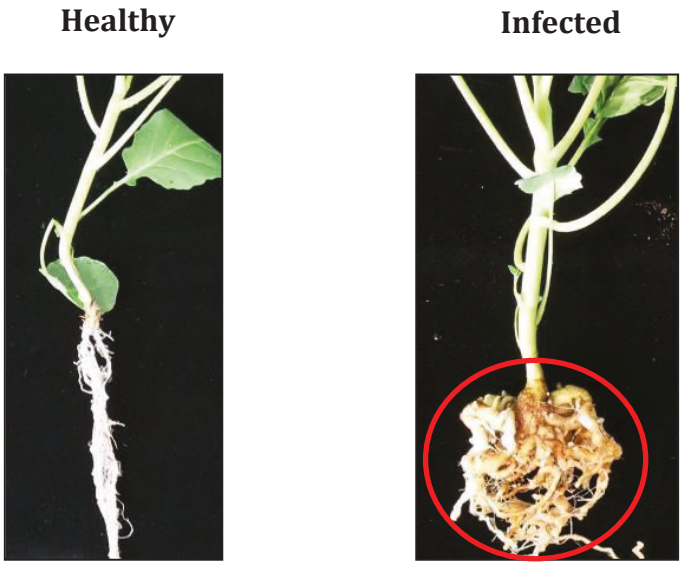


Figure 1. 4: Root galling on *B. oleracea* root.

Characteristic root galling caused by *P. brassicae* on a 6 week old *B. oleracea* root (red circle) compared to a healthy, uninfected root.

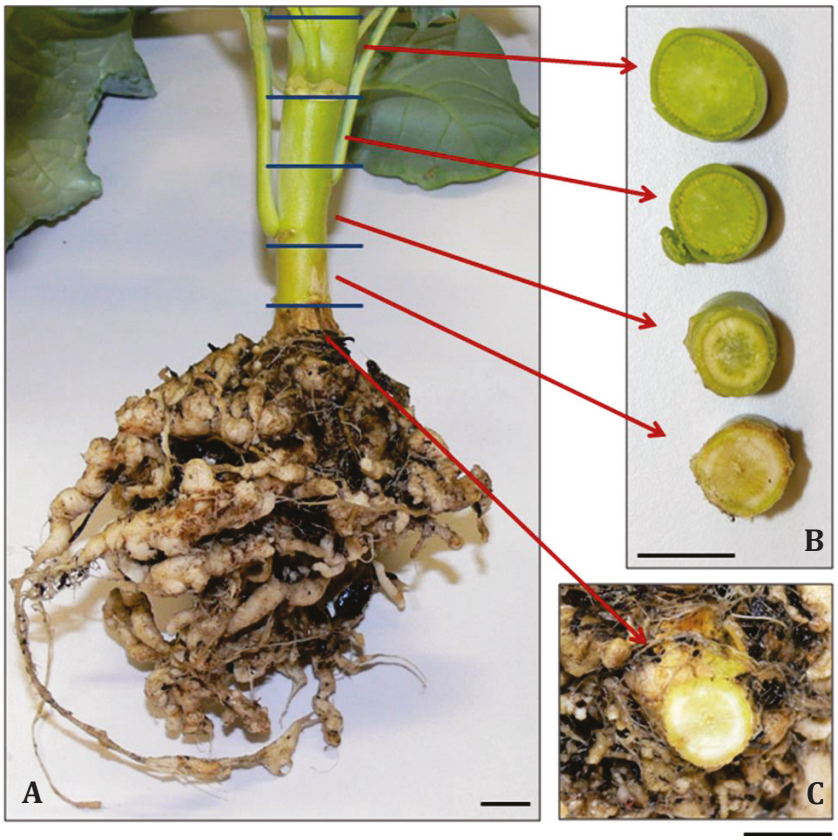


Figure 1. 5: *P. brassicae* infection of root and shoot

(**A**) Typical *P. brassicae* infection does not only affect the root system of *B. oleracea* with root galling of host plants, it causes a reduction in water and nutrient uptake to the shoots through disruption of the phloem and xylem as seen in the discolouration at the base of the root and lower part of the stem (**B** & **C**).

1.2.7 Means of controlling *P. brassicae*

Currently there are few ways to effectively combat *P. brassicae*; however a number of chemical fungicides have been developed in the hope of combating the pathogen. Fluazinam is a fungicide which targets the zoospores of *P. brassicae*, limiting their ability to infect. Fluazinam has been utilised in many crop species and has been assessed against a number of other soil-borne pathogens (Donald *et al*., 2001).

Calcium cyanamide has been used for a number of years with some success in the control of *P. brassicae*, like fluazinam it has direct fungicidal activity against the pathogen itself and also increases soil pH (Donald et al., 2004). For calcium cyanamide to be fully effective, the soil must be treated roughly 2 weeks prior to the introduction of crops to allow for the transformation of cyanamide to nitrogen (Tremblay *et al*., 2005). Cyazomafid, a fungicide developed in the early 2000's has been shown to reduce severity of disease on Chinese cabbage and Shanghai pak choy (Mitani *et al*., 2003; Ohshima *et al*., 2004; Gossen *et al*., 2012a).

Boron has been shown to be effective at controlling *P. brassicae*, with indications it may alter host cell structure and in combination with pH through liming can control the pathogen (Donald & Porter, 2009; Deora *et al*., 2012). Calcium, used commonly in the form of lime to raise pH to above 7 (Kowata-Dresch & Mio, 2012), is known to affect resting spore viability at high concentrations, it is also known to change cell host structure (Myers & Campbell, 1985). There are also a number of biocontrol agents that have been tested for their ability to suppress

P. brassicae infection including *Bacillus subtilis* and *Gliocladium catenulatum* (Peng *et al*., 2011).

Temperature, although not easily controlled on field sites, can play an important role in the ability of *P. brassicae* to infect a host. Below a temperature of 15˚C Sharma *et al*., (2011) observed a reduction in infection sites on a susceptible host. McDonald and Westerveld (2008) were able to control disease caused by *P. brassicae* in two Asian brassica vegetables when they were grown in a low temperature soil. Gossen *et al*., (2012b) again highlighted the importance of temperature for a successful infection by *P. brassicae* with confirmation that the optimal temperature range for infection is between 18-24˚C.

A number of brassica lines have been selected for breeding on the basis of their resistance to *P. brassicae*. The three common species are *B. napus*, *B. oleracea* and *B. rapa*, which have had clubroot resistant (CR) genes introduced into their genomes. Two *B. napus* cultivars have been released for commercial use, cv. Mendel by NPZ-Lembke and cv. Tosca by Svalöv-Weibull (Donald & Porter, 2009; Piao *et al*., 2009). There are some problems with this approach; an ever changing *P. brassicae* genetic diversity means that many cultivars bred for resistance become susceptible again. A second problem occurs when working with highly susceptible cultivars with introduced CR genes; often the resistant traits are unable to provide an adequate resistance response (Piao *et al*., 2009).

1.3 Brassicaceae

1.3.1 General Background

The Brassicaceae family commonly referred to as Cruciferae or mustard family, is one of the most important plant families on earth. This family comprises a large variety of species; roughly 3700 distributed worldwide, among the most important are the crop plants, such as *Brassica oleracea*, *Brassica rapa* and *Brassica napas* (Al-Shehbaz et al., 2006; Lihova et al., 2006). *Brassica oleracea* encompasses such crop species as broccoli, cabbage, cauliflower and Brussels sprouts. *Brassica rapa* includes turnips and mustards, while *Brassica napus* includes rape, oilseed rape and canola.

Although crop plants form a major branch of the Brassicaceae family, one of the best studied and arguably the most important flowering plants to science, *Arabidopsis thaliana* (L.) Heynh is a member of the family (Hoffmann, 2002). Although it is considered a weed, its size, growth rate and seed turn around make it an extremely valuable tool in the field of agricultural research. In this thesis Arabidopsis is used to explore the role of SA in an interaction with *P. brassicae*, by utilising a number of SA pathway mutants.

1.3.2 *Arabidopsis thaliana*

Arabidopsis thaliana is a small weed which is thought to have originated in Europe, making its way to South America and Asia a few hundred years ago (Sharbel et al., 2000; Vander Zwan et al., 2000). Although there are a number of ecotypes associated with *A. thaliana* there is very little genetic variation between them. Important factors that allowed *A. thaliana* to become one of the most

widely used and successful scientific models are its quick growth rate from seeding, large production of seed sets in a relatively short period of time and its size (Meinke *et al*., 1998).

One of the most important scientific breakthroughs was completed by The *Arabidopsis* Genome (TAG) Initiative (2000). The *Arabidopsis* genome was fully sequenced, providing researchers a valuable tool in botany research. With this tool, researchers were able to gain an insight into the plant's defence, growth and homeostatic and general life cycle responses. Researchers have since been able to produce large numbers of mutant ecotypes with which to further understand the importance of particular genes all of which are stored and accessed on 'The *Arabidopsis* Information Resource' (TAIR) website. Mutants currently available may have important genes inhibited, knocked out or over-expressed, in particular those responsible for; growth, defence, seed production and flowering.

Commonly used ecotypes in research include; Columbia (Col-0), Landsberg erecta (Ler-0) and Wassilewskija (Ws-0). A number of mutant phenotypes have been derived from these and other ecotypes. Critical defence pathways have been manipulated to provide information on important roles of certain chemical signals such as salicylic acid and jasmonic acid.

1.3.3 Salicylic Acid enhanced/deficient mutants

The ability to knock-out and silence genes in *Arabidopsis* has led to the production of *Arabidopsis* with enhanced or deficient signalling pathways (Table

1. 2). Salicylic acid and many of the critical expression genes are known to be important signalling molecules in defence against biotrophic pathogens and may possibly lead to SAR. *Arabidopsis* mutants such as, *NahG*, *npr1*, SAI, EDS and SARD are deficient in important signalling molecules, such as SA and proteins, such as PR, which allow for defence against a number of biotrophic pathogens. Alternatively mutants such as, *cpr1*, EDR and *dnd1* are known to posses enhanced levels of SA or over expression of PR genes leading to enhanced resistance against normally virulent pathogens. Studying the effect of these missing or enhanced components allows for researchers to single out important pathways and expressing proteins in defence.

1.3.4 *Brassica oleracea*

Brassica oleracea comprises a number of agriculturaly important crops, including Kale, cauliflower, Brussels sprouts and broccoli. Broccoli (*Brassica oleracea* var. *italica)* is one of many vital economic crops found throughout Australasia, the U.S. and parts of Europe. It is of particular importance to Victoria with the majority of Australia's broccoli produced in this state. However, pathogens such as *P. brassicae* the cause of clubroot and *Albugo candida* the cause of white blister are threatening the productivity of broccoli and many other brassica crops (Donald *et al*., 2006; Petkowski *et al*., 2010).

As of 2009 it had been estimated the gross value of broccoli in Australia was $101.2 million, with it being the 10th largest vegetable crop produced. Victoria as of 2009 accounted for 50% of the national production. With an increase in floods and other extreme weather events and with the added problem of

clubroot disease, the production of broccoli and other brassica vegetables has likely dropped, leading to increased consumer prices (Australian Bearue of Statistics, 2011; AusVeg, 2012).

1.4 Plant Defence

1.4.1 General background

Plants come into contact with different pathogens on a day-to-day basis; often these don't create a problem as physical barriers and other defence mechanisms prevent any infection (Dangl & Jones, 2001). However in some situations a plant will be infected by a pathogen; there are usually 2 results, either the plant will be able to stop the invading pathogen and thereby prevent infection, or the pathogen will infect and the plant will succumb to infection.

In the event a pathogen is able to penetrate into the cells of a healthy plant, the infected plant may overcome the pathogen by means of systemic acquired resistance (SAR), which involves transport of signals. In most cases this involves the up-regulation of defence genes and proteins, along with a number of molecules needed for SAR to take place (Durrant & Dong, 2004; Vlot et al., 2008). It has been well documented that Pathogenesis-related (PR) genes play a vital role in whether a plant will eliminate an invading pathogen.

Table 1. 2: *Arabidopsis* mutant lines, their full and abbreviated names and respective phenotypes as described in previous literature.

Abbreviation	Full name	Mutation phenotype	Reference
ADR	Activated disease resistance	Has elevated levels of SA and reaction oxygen species, confers resistance to microbial pathogens.	Grant *et al*., 2003
CDR	Constitutive disease resistance	Over-expresser of SA-mediated defence genes and expression of *PR-1* and PR-2.	Xia *et al*., 2004
cpr1	Constitutive expresser of PR genes	Continual expression of PR genes.	Clarke *et al*., 2000
dnd1	Defence no death	Has elevated levels of SA and constitutive expression of PR genes, does not produce a hypersensitive response.	Clough *et al*., 2000
Jar	Jasmonate resistant	Plants produce markedly lower levels of JA than wild-type plants.	Wang et *al*., 2007
NahG	Salicylate hydroxylase	Salicylate hydroxlase converts SA to catechol, *NahG* line is deficient in the accumulation of SA.	van Wees *et al*., 2003
npr1	Non-expresser of PR genes	This mutation prevents expression of PR genes following exposure to SA.	Lin *et al*., 2004

1.4.2 Susceptibility

In the case of a susceptible interaction between *P. brassicae* and a host plant, the pathogen will initiate a response within the plant that allows for the pathogen to undergo its life cycle and ultimately leads to stunted growth or in severe interactions a host's death (Dixon, 2009a; Ludwig-Muller, 2009). Susceptible interaction can be seen in almost all brassica species, of particular note are *B. oleracea*, *B. rapa* and *B. napus* (Donald *et al*., 2006).

1.4.3 Tolerance

A tolerant response can be either active or passive; in an active tolerance response the pathogen is able to cause some disease symptoms however the plant is able to compensate this by altering its own metabolic activity i.e. generating new leaves, or lateral root development after initial pathogen infection. In a passive tolerance response the pathogen is able to develop within the plant however it causes no disease symptoms (Mauricio et al., 1997) (Prell & Day, 2001). Hansen (1989) identified tolerant traits in cabbage through genetic *P. brassicae* crosses, with some hosts able to generate new healthy roots.

1.4.4 Resistance

Resistance of a plant to a pathogen is characterized by the plants ability to defend itself against the invading pathogen and prevent colonization (Prell & Day, 2001). One possible defence system against an invading pathogen comes from a Hypersensitive Response (HR), which is a form of cell death. The development of HR lesions is an important mechanism for the plant to survive. The cells surrounding the pathogen which die, effectively strangle the pathogen

from obtaining any further nutrients, eventually leading to its elimination. HR is dependent upon a number of chemical and signal pathways which include: ion fluxes, protein synthesis and salicylic acid a key molecule in HR (Heath, 2000). Bennett et al., (2000) were able to clearly show HR lesions which occurred in lettuce cells in response to penetration of *Bremia lactucae*; this response is typical of an incompatible response between the *R* and *Avr* genes of plant and pathogen respectively. A recent review by Gossen *et al*., (2013) has noted that most resistance to *P. brassicae* occurs as a result of a pathotype-specific interaction, and although a number of clubroot-resistant cultivars of Brassica crops have been released, their resistance traits are often broken down.

1.4.5 Innate Immunity

Innate resistance is separated into two forms, 'non-specific' or 'general resistance' in which the plant is able to defend itself against numerous pathogens by a number of biochemical and mechanical mechanisms which include HR, regulation of reactive oxygen species and thickening of cell walls (Edreva, 2004; Kiraly et al., 2007). The second form of innate resistance is 'specific resistance' in which a plant is able to specifically defend itself against a certain strain of pathogen which typically involves gene-for-gene interactions that ultimately leads to HR. Recently gene silencing was found to be involved in innate resistance (Kiraly et al., 2007).

1.4.6 Systemic Acquired resistance (SAR)

Systemic Acquired Resistance (SAR) is a biological defence mechanism that all plants are able to initiate against certain exogenous stimuli, that is, biotic and

abiotic factors that are present and perceived as a threat (Jeun, 2000). However in certain cases a pathogen may not be detected in time leading to infection (Hardham, 2005). *Phytophthora cinnamomi* for example can infect the roots of susceptible plants within 20-30 minutes and within 2-3 days sporangia have been seen to appear on the root surface (Hardham, 2005). SAR and induced resistance IR against *Plasmodiophora brassicae* as yet has not been clearly shown in relevant *Brassica* crops; it is unclear as to the mechanism of resistance against the pathogen.

Important factors for SAR to initialise are Pathogenesis-Related (PR) genes; it is known that PR genes play an important role in various degrees of pathogen resistance. Expression of PR genes often correlates with activation of SAR (Kinkema, 2000). *PR-1*a is known to play an important role in the tolerance of infection in tobacco plants to *Peronospora tabicina* and *Phytophthora parasitica* var. *nicotianae* (Alexander et al., 1993). High levels of *PR-1*a have been shown to correlate with the onset of SAR. SA can aid in the induction of SAR at certain intracellular concentrations, however it is believed that methyl salicylate and not SA is the translocated signal (Ryals et al., 1995; Hayat *et al*., 2010).

Salicylic acid-binding protein 2 (SABP2) which had been isolated from tobacco, has shown a high affinity for SA, it was reasoned that this complex is a major component in SAR induction (Du & Klessig, 1997; Forouhar et al., 2005). It is now known that SABP2 transforms inactive MeSA into acitve SA (Fu & Dong, 2013). Silencing of SABP2 in tobacco plants revealed two important findings: when tobacco plants with silenced SABP2 proteins were inoculated with tobacco

mosaic virus, infection was more prevalent compared to tobacco plants with a functioning SABP2. Also *PR-1* activity in response to SA induction was noted to be lower than the controls as a response to the silencing of SABP2 in tobacco plants (Kumar & Klessig, 2003).

Although SA plays a vital role in defence, another hormone, jasmonic acid (JA), has a role in defence by accumulating within the phloem of leaves in less than 12 hours post inoculation with *Pseudomonas syringae*. This fast accumulation is believed to be the initial trigger of SAR (Truman et al., 2007). However, it is known that JA and SA are antagonistic and in some circumstances a biotrophic pathogen will release an elicitor to fool the plant into producing a high level of JA in order to suppress SA and its effects. Such as is in the case of *P. syringae* and its production of coronatine which mimics JA and is known to reduce SA accumulation by the plant (Cui *et al*., 2005).

1.4.7 Induced Resistance by Chemicals

A number of chemicals have the ability to mimic the effects of SA and help to induce IR and SAR whilst not accumulating SA. Acibenzolar-S-methyl (BTH) has a very similar structure to SA and it's through this similarity that BTH is able to induce a resistance in a number of plants (Friedrich et al., 1996). Another analogue of SA, 2, 6-dichloroisonicotinic acid has also been shown to provide an IR response; again the similar structure to SA aids IR (Ward et al., 1991).

1.5 Salicylic Acid Pathway and Formation

There are two major pathways that a plant may utilise to increase intracellular SA to protect against an invading pathogen; the shikimate pathway and the phenylalanine pathway (Figure 1. 6). In both cases the pathways are complex in their nature and increases in SA lead to the production of many proteins and chemicals that are utilised against invading pathogens.

The shikimate pathway is highly regulated during a pathogen attack and occurs following the glycolysis pathway (Maeda & Dudareva, 2012). Phosphoenolpyruvic acid, a final product of the glycolysis cycle allows for the production of 3-dehydroshikimate, which in turn is converted to shikimate. Shikimate is converted to chorismate, isochorismate synthase converts chorismate into isochorismate and finally pyruvate lyase converts isochorismate into salicylic acid and pyruvate (Herrmann & Weaver, 1999; Vlot et al., 2009; Maeda & Dudareva, 2012).

The phenylalanine pathway is known to produce a small amount of salicylic acid (Wildermuth *et al*., 2001); however there is little evidence to determine if this pathway is directly associated with the production of SA required for defence. The conversion of phenylalanine into *trans*-cinnamate occurs by the action of the enzyme, phenylalanine ammonia-lyase (PAL). Coenzyme A converts *trans*-cinnamate (*trans*-cinnamate may also be converted to *p*-coumarate, which in turn can be converted into salicylic acid) into cinnamoyl-CoA, an addition of water forms hydroxycinnamoyl-CoA (Maeda & Dudareva, 2012; Plant Metabolic Network, 2012).

The reduction of hydroxycinnamoyl-CoA through the addition of $NADP^+$ forms oxocinnamoyl-CoA, the addition of coenzyme A then forms benzoyl-CoA and acetyl-CoA. Benzoyl-CoA is either converted to benzoate by the addition of water, or salicyloyl-CoA. The addition of NADPH allows for the conversion of benzoate into SA, whilst the addition of water allows for the conversion of salicyloyl-CoA into SA (Yalpani *et al*., 1993; Métraux, 2002; Plant Metabolic Network, 2012; Maeda & Dudareva, 2012).

1.5.1 Salicylic Acid (SA)

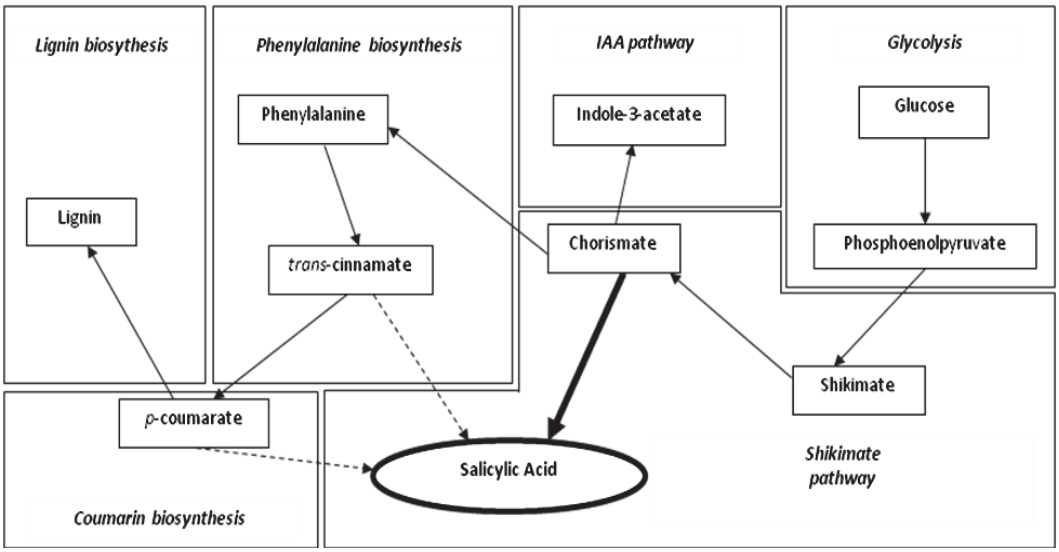
Although salicylic acid (SA) is known to be involved in defence, it is only recently that a large amount of research has been centred on SA and its role in plant-pathogen defence. SA is a major component of defence in plants and is usually associated with the protection against a number of biotrophic pathogens (Catinot *et al*., 2008). Recent research has indicated a possible role of SA derivatives in defence, including SAG (SA *O*-*β*-glucoside), SGE (salicyloyl glucose ester) and MeSA (methyl salicylate) (Figure 1. 7) (Vlot *et al*., 2009 & Rivas-San Vicente & Plasencia, 2011).

While the role of SA in defence has become clear, little research has been undertaken on the application of exogenous SA to promote plant defence. Most investigations into induced-resistance that involve the SA pathway have utilised a number of chemical inducers which are conjugates of SA and include benzothiadiazole and 2, 6-dichloroisonicotinic acid. Systemic acquired resistance (SAR), induced resistance (IR) and the hypersensitive response (HR) are known to be regulated by increases in endogenous SA.

Figure 1. 6: Salicylic acid biosynthesis

The final stage of glycolysis gives rise to the shikimate pathway, through which chorismate is formed. Chorismate can then be used in the IAA pathway to produce indole-3-acetate, the shikimate pathway to produce salicylic acid or the phenylalanine biosynthesis pathway to produce phenylalanine. Within the phenylalanine pathway *trans*-cinnamate can synthesise salicylic acid or be used in the coumarin biosynthesis pathway to produce *p*-coumarate which can synthesise lignin or salicylic acid.

Salicylic acid is known to be produced through 3 pathways: Shikimate pathway, Phenylalanine biosynthesis pathway and coumarin biosynthesis pathway. However SA associated with a defence response is believed to be produced only via the shikimate pathway through chorismate synthesis. Thick arrow indicates main source of synthesised SA, while dashed arrows indicate SA synthesised but not associated with defence (Schematic modified from Vlot *et al*., 2009 Maeda & Dudareva, 2012).


Lignin biosythesis
Phenylalanine biosynthesis
IAA pathway
Glycolysis
Phenylalanine
Indole-3-acetate
Glucose
Lignin
trans-cinnamate
Chorismate
Phosphoenolpyruvate
Shikimate
p-coumarate
Salicylic Acid
Shikimate pathway
Coumarin biosynthesis

Salicylic acid and its conjugates have been reported to suppress the auxin signalling pathway (Wang et al., 2007), which is important in the susceptibility of host plants to *P. brassicae* (Ludwig-Muller & Schuller; 2008). *Arabidopsis* exposed to low levels of benzothiadiazole were found to have reduced levels of auxin compared to controls (Wang et al., 2007).

Interestingly a key biochemical molecule in the production of SA, chorismate, has been found to play an important role in the production of auxin (Bartel, 1997). It is therefore possible that in an interaction between *P. brassicae* and host, the pathogen is able to trick the plant into producing excess auxin, thus allowing for the pathogen to complete its life-cycle.

In a number of plant species, basal levels of SA are known to be relatively high compared with *Arabidopsis* and tobacco plants. Potato (*Solanum tuberosum* L. cv. Désirée) has a naturally high basal level of SA which may be the source of resistance against the soil-borne pathogen *Phytophthora infestans* (Yu, *et al*., 1997). *NahG* potato plants, unable to produce SA, were infected with *P. infestans*, and although in almost all cases there was no significant increase in lesion size, real-time PCR revealed a significant increase in pathogen growth on *NahG* potato plants (Yu, *et al*., 1997; Halim *et al*., 2007). Rice (*Oryzae sativa* L.) like potato appears to have high levels of basal SA. When infected with blast fungus (*Magnaporthe grisea* (T. T. Herbert)) healthy rice plants are able to control pathogen spread, in contrast, transgenic rice deficient in SA appear to be unable to control the disease (Yang *et al*., 2004).

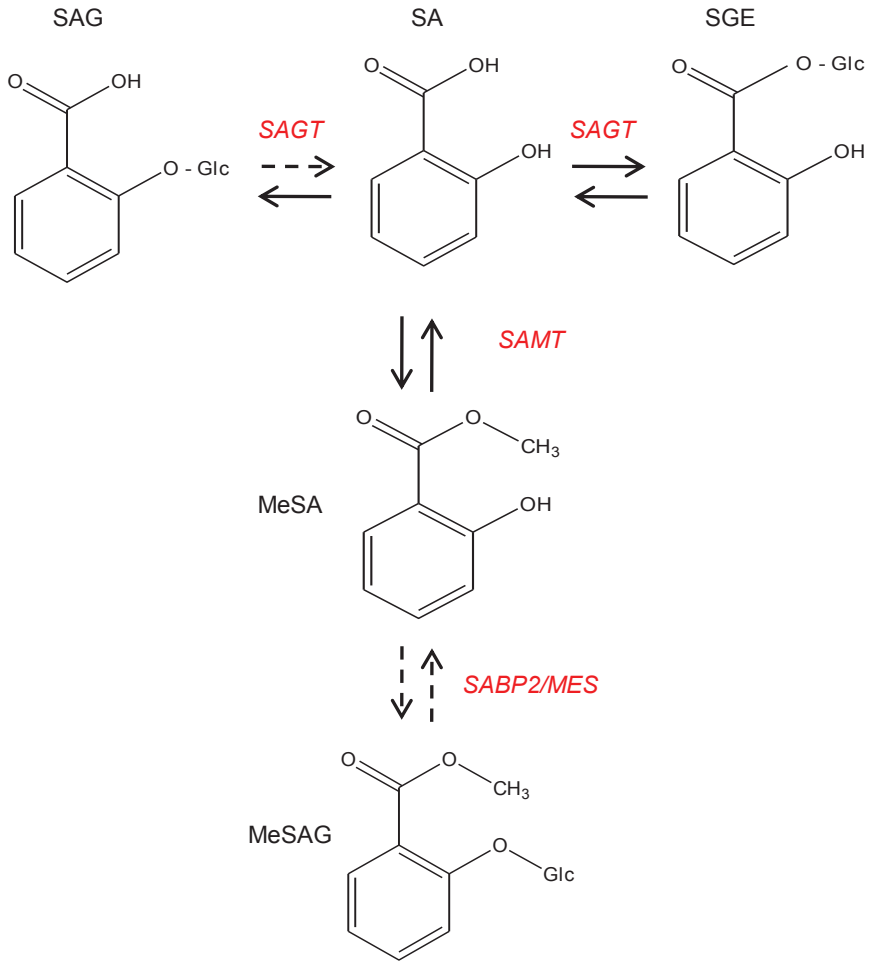


Figure 1. 7: Structure of SA and its derivatives

Structure of salicylic acid and its derivatives SAG and SGE, which have been associated with pathogen defence and mobile signals associated with SA defence, MeSA and MeSAG. Abbreviations of structure names (black); SA, salicylic acid; SAG; SA *O*-*β*-glucoside; SGE, salicyloyl glucose ester; MeSA, methyl salicylate; MeSAG, methyl salicylate *O*-*β*-glucoside. Abbreviations of possible enzymes (red); SAGT, SA glucosyltransferase; SAMT, SA methyltransferase; SABP2, SA-binding protein 2; MES, methyl ester. Dashed lines indicate possible transformation, diagram modified from Vlot *et al.*, (2009) and Rivas – San Vicente & Plasencia (2011).

1.5 Aim of the study

The aim of the research conducted was to demonstrate and better understand the role of salicylic acid in the interaction between *Plasmodiophora brassicae* and an economically important crop *Brassica oleracea*. To aid the study of this interaction, wild type *Arabidopsis thaliana* and a number of salicylic acid pathway mutants were used.

This thesis aimed to do the following:

1. Determine the importance of salicylic acid in the plant – pathogen interaction using *Arabidopsis thaliana* wild types and mutants with differing levels of SA and comparing disease severity (Chapter 2).

2. Determine the physiological, morphological and biochemical effects of salicylic acid on the economically important crop, *Brassica oleracea* (Chapter 3).

3. Determine the effect salicylic acid has on disease severity on *Brassica oleracea* associated with the soil – borne pathogen *Plasmodiophora brassicae* in both glass house and field trials (Chapter 4).

Chapter 2: Analysis of SA-dependent pathways and the influence of SA on interactions of *Arabidopsis thaliana* with *Plasmodiophora brassicae*

2.1 Abstract

Arabidopsis ecotypes and defence pathway mutants were analysed for their susceptibility to an Australian isolate of *P. brassicae*. Defence against the pathogen is thought to occur via the salicylic acid pathway; however, the application of SA to susceptible *Arabidopsis* ecotypes, Col-0, Tsu-0 and Tul-0, did not provide a resistance response. Arabidopsis pathway mutants over-expressing in the SA pathway, *cpr1* and *dnd1* both showed control of the pathogen, the addition of SA increased this control in *dnd1*. *NahG*, an SA deficient mutant, was found to be susceptible to an Australian isolate, the addition of SA did not change this interaction. The use of the single spore isolate 'e3' has highlighted the importance of understanding the virulence of an isolate in a population. This work has helped us better understand the interaction between plant and pathogen and the role SA plays.

2.2 Introduction

The soil-borne obligate biotroph *Plasmodiophora brassicae* is a devastating plant pathogen which affects agricultural crops of the Brassicaceae family including, broccoli, cabbage and cauliflower and is the causal agent of clubroot (Dixon, 2009a; Donald *et al,* 2006; Ludwig-Müller & Schuller, 2008). *P. brassicae*

has been isolated on almost every continent and is particularly damaging to brassica crops in parts of Europe. It was first isolated in Australia in 1891 and believed to have been accidentally imported on contaminated feed stock from Europe (Donald *et al,* 2006).

An internationally recognized classification system for *P. brassicae*, the European Clubroot Differential (ECD), has been used to identify up to 128 different populations, with indications of a wide range of pathogenicity between individual pathotypes (Kuginuki *et al,* 1999). Within Australia one of the most common isolates found has the ECD code 16/2/31 and is known to infect a wide range of crops in the *Brassica oleracea* species (Donald *et al,* 2006).

Complications arise when studying Australian isolates of *P. brassicae* as different populations differ in their host ranges. This occurs due to the large diversity in populations, with estimates of up to 15 different pathotypes in a single isolate, each potentially varying in pathogenicity. Over 40 isolates have been collected nationally with ECD coding that suggests that 3 populations are found nationwide, 16/3/31, 16/3/12 and 16/2/31 (Donald *et al*., 2006). Within Victoria 10 different isolates have been catalogued, with a host range including, *Brassica rapa*, *B. napus* and *B. oleracea*.

Arabidopsis thaliana has been used extensively in the study of plant-pathogen interactions and is of critical importance in the study of *P. brassicae* as it belongs to the same family as many of the economically important crops affected by the pathogen (Siemens *et al*., 2002; Agarwal *et al*., 2009). There are indications that

within the *Arabidopsis* genus, different ecotypes show varied levels of susceptibility to isolates of *P. brassicae*. Ecotypes such as Col-0 and Ler are both known to be highly susceptible to most *P. brassicae* isolates including, most Australian isolates and isolate 'e', a *P. brassicae* isolate discovered in Germany and used for the production of single spore isolates, such as 'e3'. Tsu-0 and Ta-0 on the other hand are both resistant to isolate 'e' but not to the Australian isolates tested (Agarwal *et al*., 2009; Siemens *et al*., 2002). The background for the variability in susceptibility is not clear, nor has the role that SA may play, been investigated in this interaction.

Arabidopsis mutants have offered researchers a powerful tool for studying specific defence pathways in interactions between plants and pathogens. Siemens *et al*., (2002) studied the interaction between a number of mutant lines, including defence over-expressers, pathway deficient and hairless root lines, and their susceptibility to an isolate of *P. brassicae*, isolate 'e', and surprisingly found that almost all were susceptible to infection. Those that did show resistance were the root hair defective mutant, *rhd 3-1*; two secondary metabolite mutants, tu3 and tu8 and the hormone and signal transduction mutants *det 2-1* and *det 1-1*.

Most plant-pathogen interactions utilizing SA *Arabidopsis* mutants, has been undertaken primarily with leaf pathogens. The *Arabidopsis* defence mutant, *cpr1*, is known to be resistant to the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* (van Wees *et al*., 2000). *Cpr1* has elevated pathogenesis related gene 1 expression, which is thought to increase its resistance to some

biotrophic pathogens. Love *et al*., (2007) found the Arabidopsis mutants *cpr1-1* and *cpr5-2* were able to control cauliflower mosaic virus (CaMV) more successfully than the wild-type Col-0. There are also indications that a reduction in SA levels do not result in a higher level of susceptibility to CaMV as *NahG* and *npr1* mutants showed no increase to susceptibility compared to Col-0.

Su'ud *et al* (2011) tested a number of SA mutants for their susceptibility to two strains of *Alternia brassicicola*, Ab42464 which has a compatible interaction with Col-0 and Ab40857 which has an incompatible interaction with Col-0. The *Arabidopsis* mutant *dnd1-1*, shows resistance to both strains of *A. brassicicola*, whilst *NahG* and *npr1* show compatible and incompatible interactions as Col-0 does. NPR1 is responsible for the regulation of defence genes in a defence response against an invading pathogen (Gust & Nürnberger, 2012). *NPR1* itself is regulated by salicylic acid and requires NPR3 and NPR4 in order to initiate a defence response (Moreau *et al*., 2012; Zheng *et al*., 2012).

Defence-related mutants may prove to be valuable in recognition of important components of the SA pathway, for this reason two key over-expressing lines and 2 key SA deficient lines have been selected to study their susceptibility to an Australian isolate of *P. brassicae*. Col-0, Ler and Tsu-0 all appear to be susceptible to the pathogen with a reduction in plant biomass noted and severe galling. Tul-0, noted to be resistant to a number of *P. brassicae* isolates, was susceptible to the pathogen; however disease severity was lower when compared to the other ecotypes. Mutant lines *cpr1* and *dnd1*, both of which are over-expressers of the SA pathway, show control of disease as indicated by the

formation of small galls compared to wild-type ecotypes. These results suggest an important role for SA in defence against *P. brassicae*.

2.3 Materials and Methods

2.3.1 Treatment of plants with SA and *P. brassicae*

2.3.1.1 *Arabidopsis* growth and germination

Mutant *Arabidopsis* lines were obtained from the Arabidopsis Biological Resource Center (1060 Carmack Road, Columbus, OH, USA) and were grown in a physical containment level 2 (PC2) room at 21.5°C under fluorescent light. *A. thaliana* (Col-0, Ler-0, Tsu-0, Tul-0, *cpr1*, *dnd1*, *NahG*, *npr1* and *jar* (Table 2. 1) seeds were placed into separate 1.5mL microcentrifuge tubes with 450µl dH2O, 500µl Ethanol (AJAX Finechem, Seven Hills, NSW) and 50µl Hydrogen Peroxide (MERCK Pty Ltd. Kilsyth, Vic) and the tube was lightly agitated for 5 minutes. The tube was spun down for 30 seconds and the supernatant removed, seeds were washed in 1mL sterile sdH_2O and sdH_2O removed and the wash repeated twice. Sterilised seeds were then placed onto pre-made Murashige and Skoog media (MS) (MS media (4.4g), sucrose (16g) and bacteriological agar (8g) dissolved in 1L H_20 and pH adjusted to 5.8) and placed at 4°C for 4 days, before being placed into a growth chamber for 14 days at 21°C with a day/night cycle of 12hr/12hr. Once grown *A. thaliana* were carefully extracted from the media and potted into propagation mix (Debco Pty, Ltd., Tyabb, Victoria, Australia).

Table 2. 1: *Arabidopsis* ecotypes and mutants and their respective abbreviations and common names used in this research.

***Arabidopsis* used in this research**			
Wild-type		**Mutant**	
Col-0	**Columbia (Columbia, USA)**	***cpr1***	**Constitutive expression of PR genes**
Ler	**Landsberg erecta (Germany)**	***dnd1***	**Defence, no death**
Tsu-0	**Tsu (Tsushima, Japan)**	***jar***	**Jasmonate resistant**
Tul-0	**Turk Lake (Turk Lake, USA)**	***NahG***	**Salicylate hydroxylase**
		npr1	**Non-expresser of PR genes**

2.3.1.2 Salicylic acid Treatment

Arabidopsis ecotypes Col-0, Ler, Tsu-0 and Tul-0 were tested for their sensitivity to SA at concentrations of 0, 1, 5 and 10 mM. A stock solution of 100 mM SA was made by diluting SA powder in (v/v50:50) MeOH/H_20 and diluted in water to get to desired concentrations. Plant health including reductions in weight and chlorosis was observed 3 and 5 day's post-treatment.

Arabidopsis wild-type and pathway mutants were root treated with SA 24 hr prior to *P. brassicae* inoculation. In order to make a 1 mM solution, salicylic acid (SA) (99% plant extract, Sigma Aldrich) was dissolved in (v/v1:20) MeOH/H_2O and pH adjusted to 7 using 1M dipotassium hydrogen orthophosphonate (K_2HPO_4). Treatment of *Arabidopsis* occurred by pipetting 1mL of a SA directly onto the soil surface surrounding the base of the stem. Once treated plants were not watered for 24 hr, inoculation with *P. brassicae* was performed 24 hr post – SA – treatment.

2.3.1.3 *Plasmodiophora brassicae* inoculation and gall scoring

Australian *Plasmodiophora brassicae* isolates were obtained from infected cabbage from Lindenow, Vic (Department of Primary Industries; ECD code 16/2/31). Single spore isolate 'e3' as described by Siemens *et al*., (2002) was donated to the Department of Primary Industries by Professor Jutta Ludwig-Müller (University of Dresden, Germany). Inoculum was prepared by grinding galled roots in a mortar and pestle with water and the contents filtered through 8 layers of muslin, spore dilutions were performed from the stock suspension. Inoculation of *Arabidopsis* occurred by pipetting 125ul of a 10^6 spores ml^{-1}

suspension into close proximity of the roots via pipetting onto the soil surface at the base of the stem. Once roots were inoculated the plants were watered. Root galling was assessed 4 weeks post-treatment, 20 plants were assessed with one repeat. Gall scoring was modified from Agarwal *et al*., (2009); A score of 0 = No galling; 1 = Small gall on lateral root, with no adverse effect to plant health; 2 = More than 1 small gall on lateral root with no adverse effect on plant health; 3 = Galling on tap root, small galls on laterals, plant appears to be stressed; 4 = Large galling on taproot and lateral roots, plant health is severely compromised.

2.3.1.4 Measurement of shoot weight and storage of shoots and roots

Shoot weight was assessed at either 15 days and 25 days or 4 weeks post-treatment with *P. brassicae*. Shoots were cut from the roots and placed onto top-loading scales and weights recorded. Shoots were then placed into 1.5ml microcentrifuge tubes, frozen in liquid nitrogen and stored at -80°C until use. Roots were carefully removed from the soil and gently rinsed with water to remove excess soil; once cleaned roots were stored in a fixative (FAA) until use.

2.3.2 RNA Isolation and cDNA Synthesis

Samples were collected and frozen in liquid nitrogen for RNA isolation at 15 and 25 days post treatment with *P. brassicae*. Total RNA was isolated from 100mg of *Arabidopsis* shoot material using TRI REAGENT© (Molecular Research Center, Inc. Cincinnati, USA). Tissue was placed into microcentrifuge tubes, frozen in liquid nitrogen and homogenized to a fine powder. For every 100 mg of tissue, 1 ml of TRIzol reagent was added to the tissue and vortexed for 30 sec and incubated for 5 min at room temperature. For every 1ml of TRIzol reagent

added, 0.2 ml of chloroform was added and tubes were vortexed for 15 sec before being centrifuged at 12,000 x g for 30 min at 4°C. The aqueous phase (top layer) was then transferred to an RNase-free 1.5 ml microcentrifuge tube and 0.5 ml of isopropyl alcohol was added followed by inversion and finally incubating at room temperature for 10 min. The mixture was then centrifuged at 12,000 x g for 10 min at which point an RNA pellet formed at the bottom of the tube. Supernatant was removed and an additional spin performed and residual liquid carefully removed with a pipette. The pellet was washed in 0.5 ml cold RNase-free ethanol (75%) and centrifuged at 12,000 x g for 10 min. The supernatant was discarded and an additional spin performed, the microcentrifuge tube was inverted onto Kimwipe's (Kimberly-Clarke, Milsons Point, NSW, Australia) for 10-15 min, to allow for residual liquid to dry off. To the pellet was added 20-30 µl of RNase-free water and pellet was resuspended by pipetting 20-30 times, samples were stored at -80°C. To assess RNA quantity, RNA was checked on a Nanodrop (NanoDrop Technologies, Inc. Wilmington, DE USA)). To remove excess DNA contamination, Deoxyribonuclease I, Amplification Grade (Invitrogen) was used as per manual, while cDNA synthesis was undertaken using Superscript III™ (Invitrogen) as per manual.

2.3.2.1 PCR Primers and Conditions

The following primers were used for gene-expression analysis; *PR-1* (Pathogenesis related gene 1) 5'-AAGAGGCAACTGCAGACTCA-3' and 5'-TCTCGCTAACCCACATGTTC-3'; *PDF 1.2* (Plant defensin gene 1.2) 5'-

TTGCTGCTTTCGACGCA-3' and 5'-TGTCCCACTTGGCTTCTCG-3'; Actin (reference gene) 5'-ACGTGGACATCAGGAAGGAC-3' and 5'-GAACCACCGATCCAGACACT-5'.

The following reagents were added to a 0.2ml microcentrifuge tube (Axygen, Inc. Union City, CA USA); 12.5µl GoTaq© (Promega), 1.5 µl F-Primer, 1.5 µl R-Primer, 8.5 µl sdH20 and 1 µl of appropriate cDNA. PCR-cycling conditions comprised an initial polymerase activation step at 95°C for 3 min, followed by 28 cycles for *PDF 1.2* and *PR-1* and 35 cycles for actin, of 95°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec. PCR products were visualised under UV after running on a 2% agarose gel at 80 volts for 40 min and staining with EtBr. Five repeats were performed for gel visualisation and quantification. Gel quantification for relative gene expression was performed using Image J (Image J v1.43; National Institutes of Health, USA), where background material was removed and band intensity from target gene was calculated against reference gene.

2.3.3 Aniline blue staining of *Arabidopsis* roots

Arabidopsis roots were cut from their shoots and placed in formalin acetic acid (FAA) (Formaline 6%; 95% Ethanol 50%; Acetic acid 4%; H_2O 40%) and stored indefinitely. Once ready for microscopy, roots were rinsed in water and placed into an Aniline Blue solution (0.05%) for 1 minute and then rinsed in water before being mounted on a slide with water and a cover slip. Roots were viewed using a microscope under bright light (Axioskop 2 mot plus, Zeiss, Göttingen, 37030, Germany) and photographed using a camera mounted to the microscope (SPOT RT Slider, Diagnostics Instruments Inc.).

2.3.4 Statistical analysis

Statistical analysis was performed using SPSS (PASW 18.0) (IBM, Armonk, New York, USA) and a statistics package (Microsoft Excel 2010). In all experiments, One-Way ANOVAs, specifically Tukey's test and Fisher's least significant difference (LSD) test with a P value = 0.05, were used to analyse significant differences between each treatment group.

2.4 Results

2.4.1 Virulence of two different isolates *P. brassicae* on *Arabidopsis*

A single spore isolate, 'e3', was tested for its virulence to *Arabidopsis* ecotypes Col-0, Ler, Tsu-0 and Tul-0 and *Arabidopsis* mutants *cpr1*, *dnd1*, *jar*, *NahG* and *npr1* and compared to a wide spread Australian isolate (16/2/31) (Figure 2. 1). *Arabidopsis* ecotypes Col-0 and Ler were found to be susceptible to the single spore isolate, with the production of a small gall 25 days post-inoculation. There was no gall formation on *Arabidopsis* ecotypes Tsu-0 and Tul-0 25 days post-inoculation with 'e3'. The Australian isolate of *P. brassicae* was highly virulent in all ecotypes resulting in the production of large root galls.

Mutant lines, *cpr1* and *dnd1* were both found to be tolerant to the Australian isolate of *P. brassicae*, with small gall production, while *npr1* was slightly more susceptible (Figure 2. 2). Lines, *jar* and *NahG* were both highly susceptible to the Australian isolate, with the formation of large root galls. The 'e3' isolate did not cause galling in *cpr1*, *dnd1*, *NahG* and *npr1*; however there was very small galling in *jar* (Figure 2. 3).

Figure 2. 1: Interaction between *Arabidopsis* ecotypes and isolates of *P. brassicae.*

(**A**) Col-0 and (**B**) Ler are susceptible to both an Australian isolate and the single spore isolate 'e3' of *P.brassicae* as noted by the characteristic galling of the roots 25 days post inoculation.

(**C**) Tsu-0 and (**D**) Tul-0 are susceptible to an Australian isolate of *P. brassicae* but are not susceptible to infection from the single spore isolate 'e3' (scale bar = 1cm).


P. brassicae isolate (Australian)
Control Uninfected
P. brassicae isolate ('e3')
A
B
C
D

Figure 2. 2: Interaction between *cpr1*, *dnd1* and *npr1* to isolates of *P. brassicae*

(**A**) *cpr1*, (**B**) *dnd1* and (**C**) *npr1* all show symptoms of infection caused by an Australian *P. brassicae* with root galling observed on all *Arabidopsis* 25 days post inoculation. Infection does however appear to be limited to only a couple of lateral roots, indicating a possible partial resistance response. The single spore isolate 'e3' is unable to infect the *Arabidopsis* mutants (scale bar = 1cm).

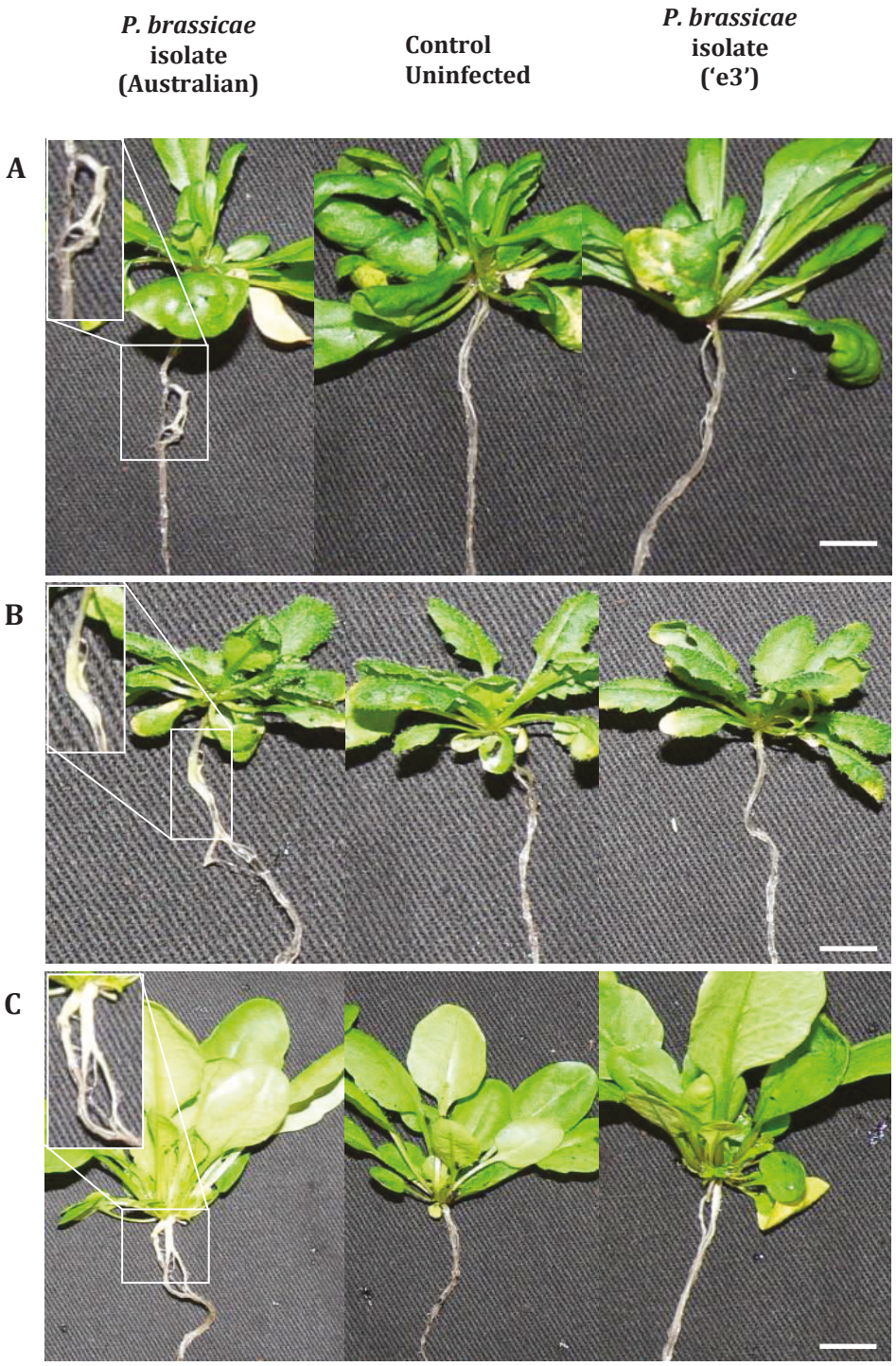

P. brassicae isolate (Australian)
Control Uninfected
P. brassicae isolate ('e3')
A
B
C

Figure 2. 3: Interaction between *NahG* and *jar* and isolates of *P. brassicae*

(**A**) *NahG* and (**B**) *jar* are both highly susceptible to an Australian isolate of *P. brassicae* with large galling observed 25 days post-treatment. The single spore spore isolate 'e3' causes minimal galling in *jar*. (scale bar = 1cm).

2.4.1.2 Effect of two different clubroot isolates on *Arabidopsis* biomass

Reduction in plant weight was only assessed in *Arabidopsis* wild-type plants. The reduction in plant weight on *Arabidopsis* ecotypes, Col-0, Ler, Tsu-0 and Tul-0, caused by both clubroot isolates was assessed 25 days post-inoculation (Figure 2. 4). Clubroot isolate 'e3' did not cause any significant change in plant weight in any of the 4 ecotypes tested. The Australian isolate did however significantly reduce plant weight in all ecotypes when compared to the respective control.

2.4.2 Susceptibility of *Arabidopsis* ecotypes to *P. brassicae*

2.4.2.1 Effect of SA on *Arabidopsis* ecotypes

Prior to treating *Arabidopsis* plants with the pathogen, SA was tested for its toxicity on *Arabidopsis* ecotypes at different concentrations. *Arabidopsis* were treated with 0 (control), 1, 5 and 50 mM SA and assessed 3 and 5 days' post treatment (Figure 2. 5). Plants treated with 50 mM all showed signs of heavy chlorosis to the leaves at 3 and 5 days post-treatment. There were no signs of chlorosis or growth effects on plants treated with 1 mM at 3 and 5 days; however plants treated with 5 mM showed the initial signs of impaired growth after 5 days, there were no signs of chlorosis; for this reason 1 mM SA was used in further experiments.

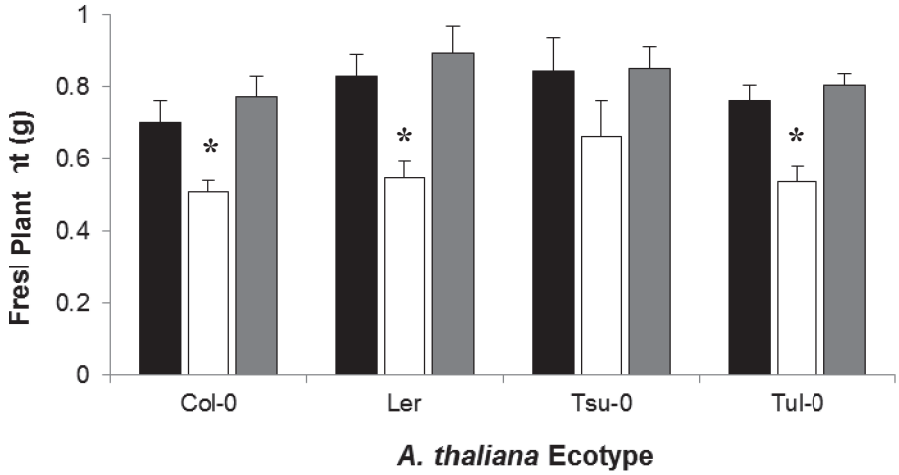


Figure 2. 4: Effect of *P. brassicae* isolates on Arabidopsis plant weight.

Arabidopsis ecotypes Col-0, Ler, Tsu-0 and Tul-0 have reduced shoot mass following inoculation with an Australian isolate of *P. brassicae* (white bars) and no adverse effects on growth to inoculation with the single spore isolate 'e3' (grey bars) when compared to the controls (black bars). Symbols represent significant difference to respective controls (n=15) (p = 0.05).

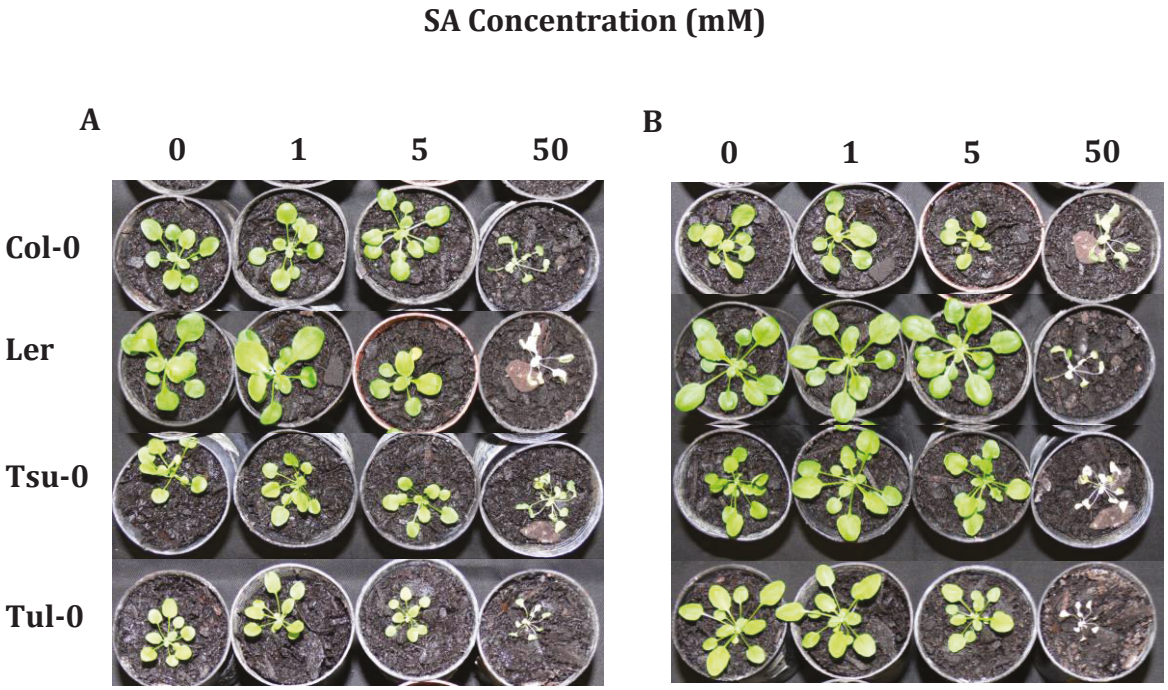


Figure 2. 5: Phytotoxic response of *Arabidopsis* to SA

Arabidopsis ecotypes Col-0, Ler, Tsu-0 and Tul-0 show a phytotoxic response to SA at 50 mM at 3 (**A**) and 5 (**B**) days post-treatment with plant health severily compromised. There does not appear to be an effect of SA on *Arabidopsis* at concentrations of 0, 1 and 5 mM.

2.4.2.2 Reduction in fresh weight of *Arabidopsis* following infection by *P. brassicae*

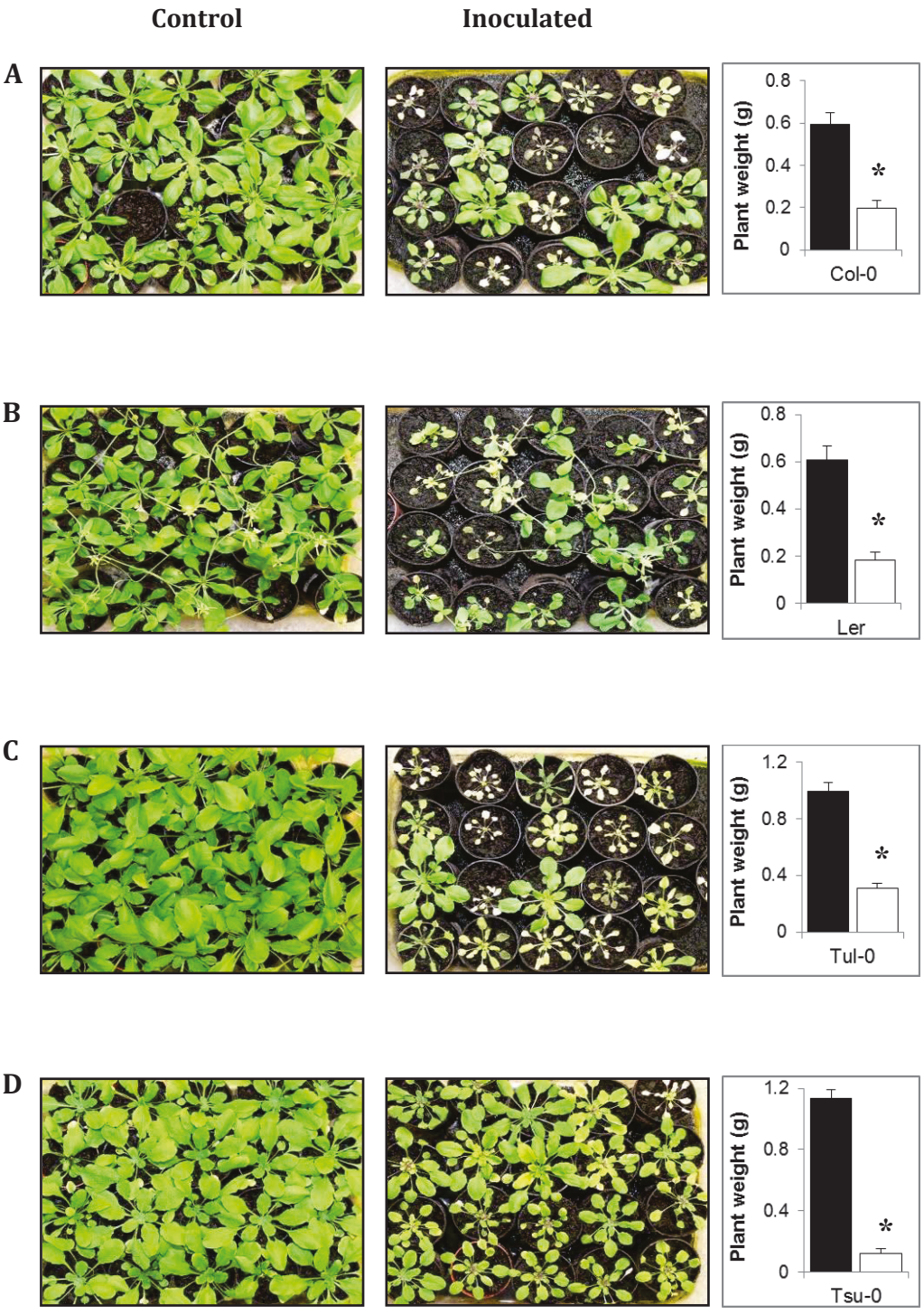
Arabidopsis ecotypes Tul-0, Ler, Tsu-0 and Col-0 were tested for their susceptibility to an Australian isolate of *P. brassicae* (Figure 2. 6). All ecotypes showed a high level of susceptibility to the pathogen 25 days post-inoculation with a drop of over 60% mean plant weight in infected plants compared to the control.

2.4.2.3 Effect of SA on disease associated with *P. brassicae* in *Arabidopsis* ecotypes

Arabidopsis ecotypes Col-0, Tsu-0 and Tul-0 were assessed for their susceptibility to the pathogen and in combination with 1 mM SA, 24 h post-treatment with the phytohormone. Fifteen days post-treatment with the pathogen, ecotypes Col-0 and Tsu-0 showed a reduction in shoot weight when treated with both SA and *P. brassicae*, but not *P. brassicae* by itself. Ecotype Tul-0 however, had reduced shoot weight in both treatment groups (Figure 2. 7). Twenty Five days post-treatment with the pathogen all three ecotypes showed a significant reduction in shoot weight in both *P. brassicae* and SA and *P. brassicae* treated groups (Figure 2. 7).

Figure 2. 6: Response of *Arabidopsis* ecotypes to *P. brassicae*

(**A**) Col-0, (**B**) Ler, (**C**) Tsu-0 (**D**) and Tul-0 all show severe signs of infection casued by *P. brassicae* 25 days post inoculation. Plant healthy is compromised with a large reduction in shoot weight (g) noted in all ecotypes when compared to the control. Symbols indicate significant difference to respective control (n = 40) (p = 0.05) (black bars = control; white bars = inoculated).


Control
Inoculated
A
Plant weight (g)
0.8
0.6
0.4
0.2
0
*
Col-0
B
Plant weight (g)
0.8
0.6
0.4
0.2
0
*
Ler
C
Plant weight (g)
1.2
0.8
0.4
0
*
Tul-0
D
Plant weight (g)
1.2
0.8
0.4
0
*
Tsu-0

2.4.2.4 Root galling severity in SA treated *Arabidopsis* ecotypes following *P. brassicae* infection

In order to determine if there was any effect of SA on the pathogen's ability to infect, root galling was assessed 25 day's post-treatment with the pathogen (Figure 2. 8). *Arabidopsis* ecotypes Col-0 and Tsu-0 were the most susceptible to the pathogen with an average root gall score of 3.5. Tul-0 appeared to be less susceptible to the pathogen with an average gall score of 2.5. There was no significant difference in disease severity following treatment with SA in each of the ecotypes tested.

Figure 2. 7: Response of *Arabidopsis* to *P. brassicae* following SA treatment.

(**A**) At 15 day's post treatment with P. brassicae there is an observed reduction in plant weight (g) in Tul-0 only, the addition of SA prior to *P. brassicae* infection causes a reduction in plant weight in Col-0, Tsu-0 and Tul-0, when compared to the controls.

(**B**) At 25 days post treatment there is a large reduction in plant weight (g) in Col-0, Tsu-0 and Tul-0 in both *P. brassicae* and SA + *P. brassicae* treated groups when compared to the untreated controls. Symbols represent significant difference to untreated controls (n = 40) (P = 0.05) (black bars = control; white bars = *P. brassicae*; grey bars = *P. brassicae* + SA).

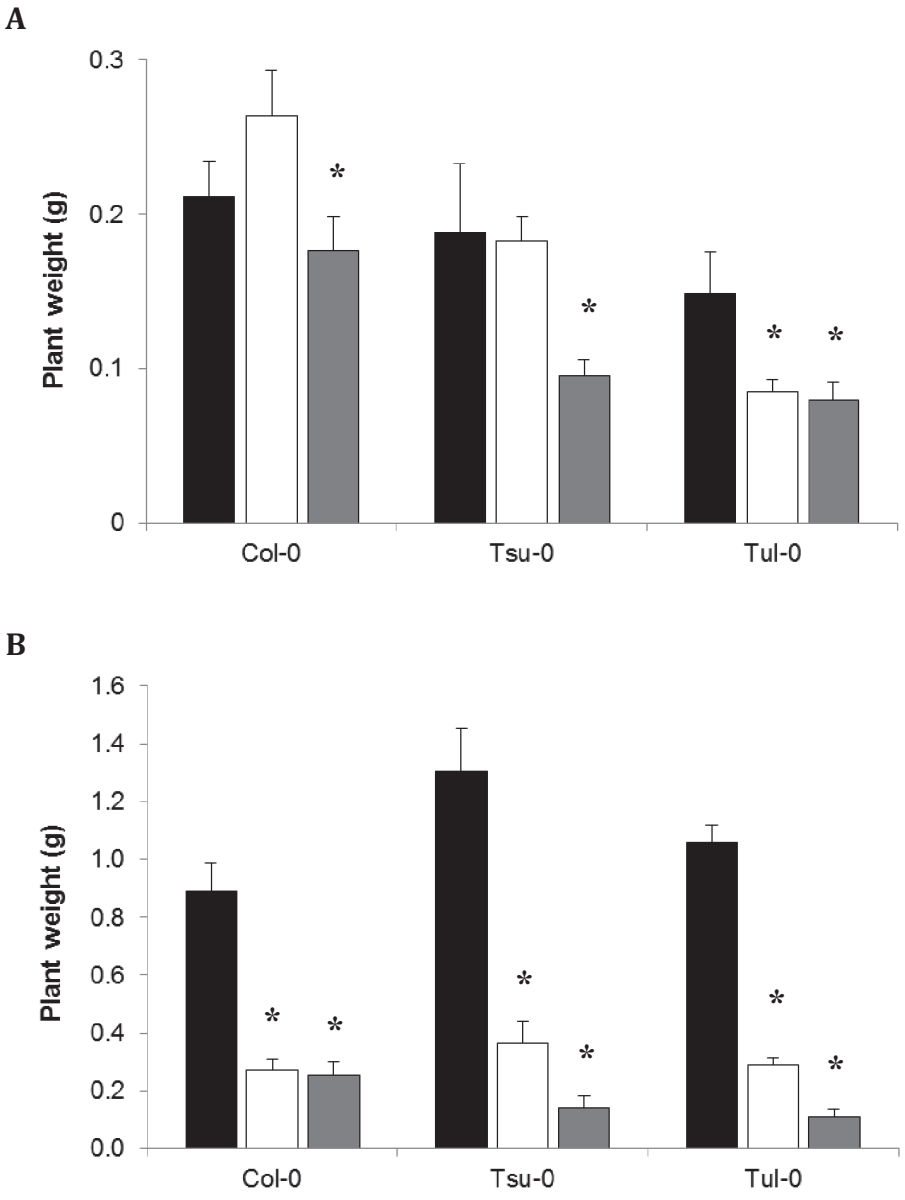
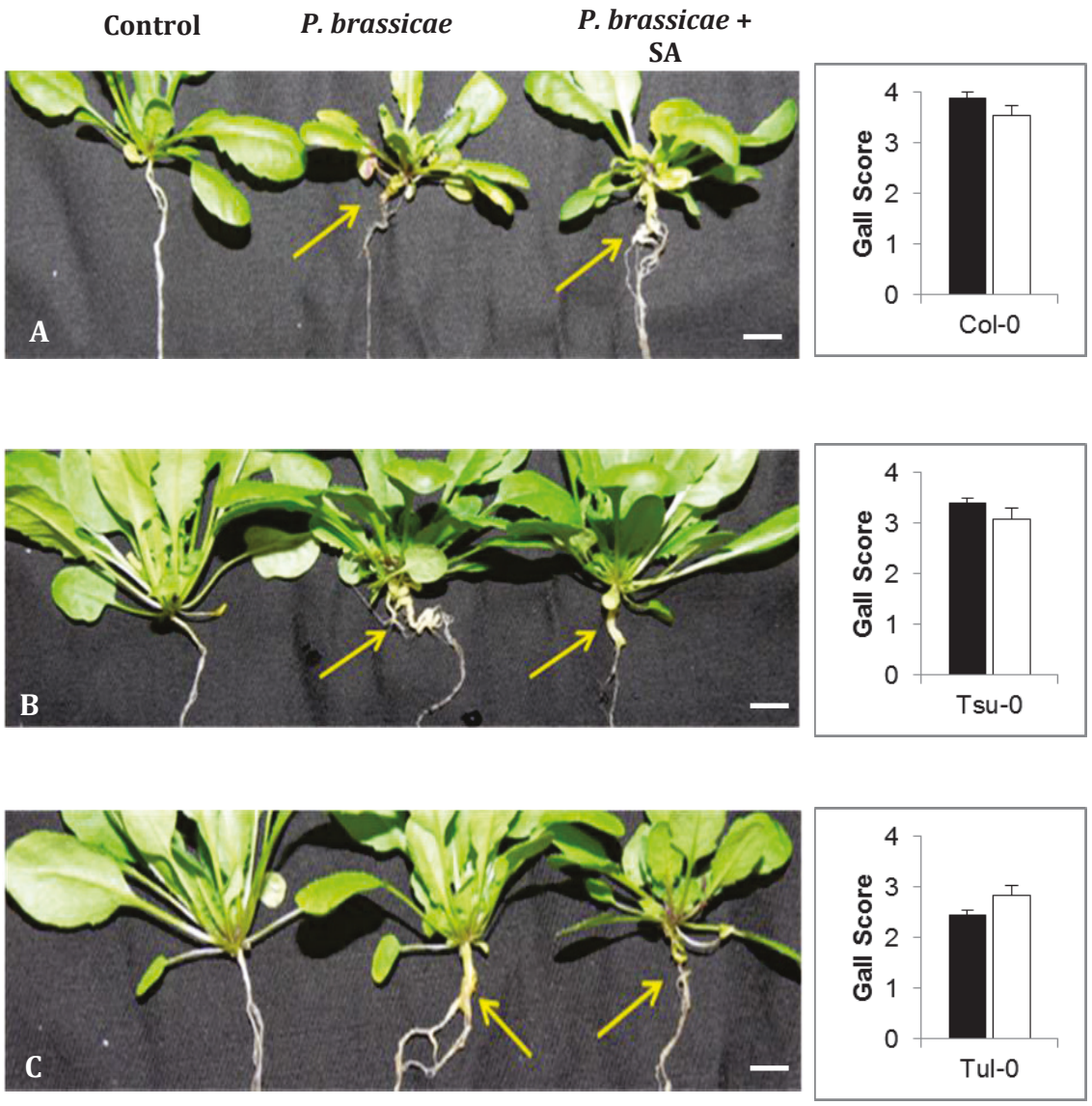

A
Plant weight (g)
0.3
0.2
0.1
0
Col-0
Tsu-0
Tul-0
B
Plant weight (g)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Col-0
Tsu-0
Tul-0

Figure 2. 8: Root galling and gall scores of *Arabidopsis* ecotypes

(**A**) Col-0 and (**B**) Tsu-0 are both highly susceptible to P. brassicae 25 days post-inoculation, with gall scores of 4 and 3.5 respectively. The addition of SA prior to inoculation does not significantly reduce the galling associated with *P. brassicae*

(**C**) Tul-0 is less susceptible to the pathogen with a noted gall socre of 2.5, 25 days post-inoculation. The addition of SA however, does not reduce the disease severity associated with *P. brassicae* (n = 40) (P = 0.05) (black bars = P. brassicae; white bars = P. brassicae + SA).


Control
P. brassicae
P. brassicae + SA
A
B
C
Gall Score
0
1
2
3
4
Col-0
Tsu-0
Tul-0

2.4.2.5 Microscopy of *P. brassicae* infected *Arabidopsis* roots

Arabidopsis roots were stained with 0.15% Aniline blue and viewed under light microscopy (Figure 2. 9). *Arabidopsis* plants treated with either *P. brassicae* or *P. brassicae* and SA, both showed similar infection rates and infection development. Root hairs had developed zoosporangia and the initial stages of formation of secondary plasmodia were visible in in the root cortex.

2.4.2.6 Induction of defence genes in *Arabidopsis* following *P. brassicae* infection

The expression of the defence gene *PR-1* and the expression of *PDF 1.2*, a potential susceptible marker to *P. brassicae* as it is involved in the jasmonic acid pathway, where assessed 15 and 25 days post inoculation with either *P. brassicae* or *P. brassicae* and SA.

Col-0 showed an increase in *PR-1* expression at both 15 and 25 days post-treatment with *P. brassicae* and in combination with SA. *PDF 1.2* expression was slightly up 15 days following treatment with *P. brassicae*, but highly expressed when in combination with SA. Twenty five days post-treatment however, *PDF 1.2* expression was high in plants treated with *P. brassicae* only, there was no expression of *PDF 1.2* in plants treated in combination with SA. Relative gene expression confirmed the expression of PR-1 and PDF 1.2 (Figure 2. 10).

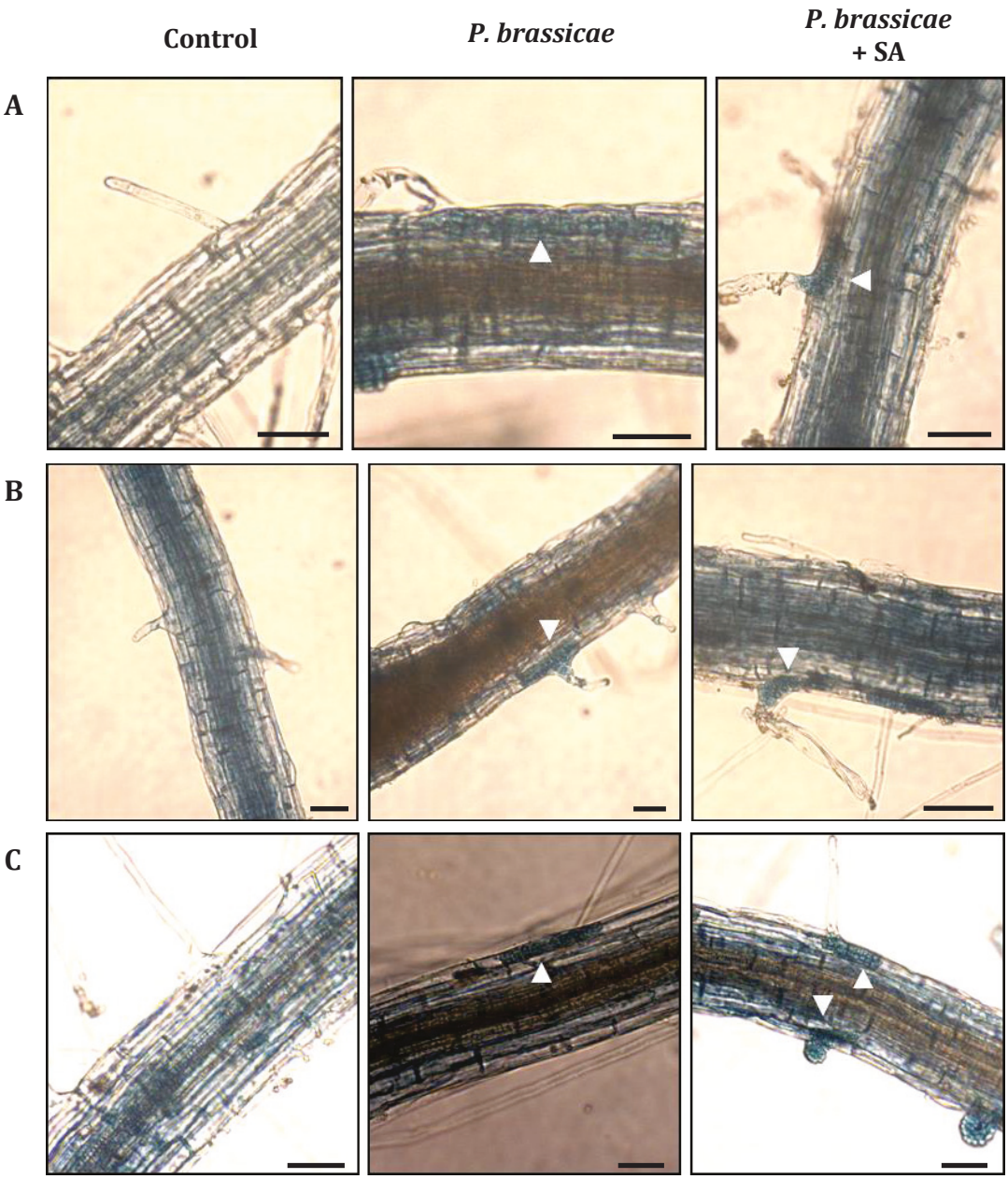


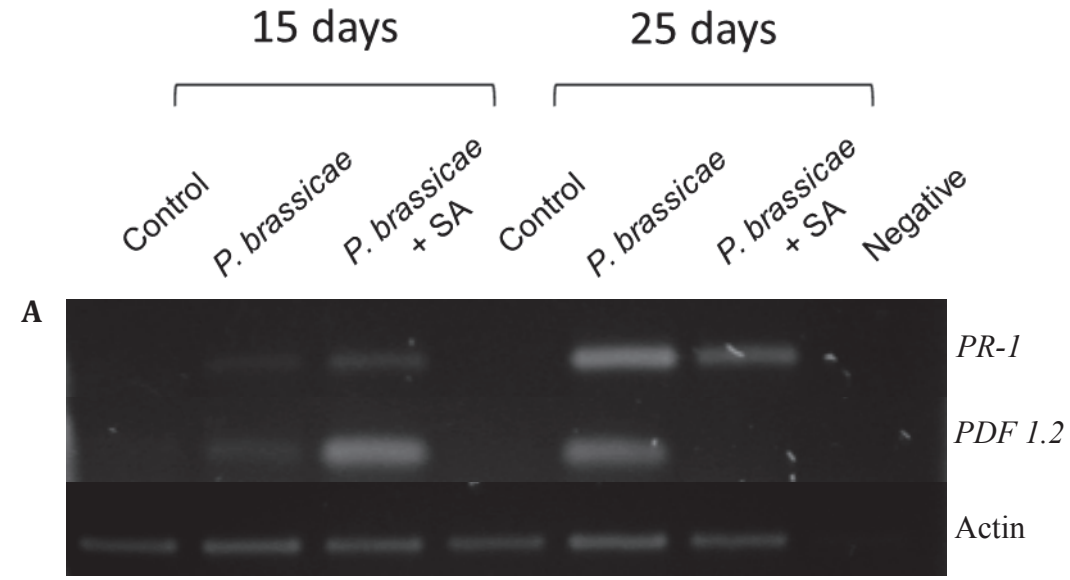
Figure 2. 9: Microscopy of *Arabidopsis* roots following *P. brassicae* infection

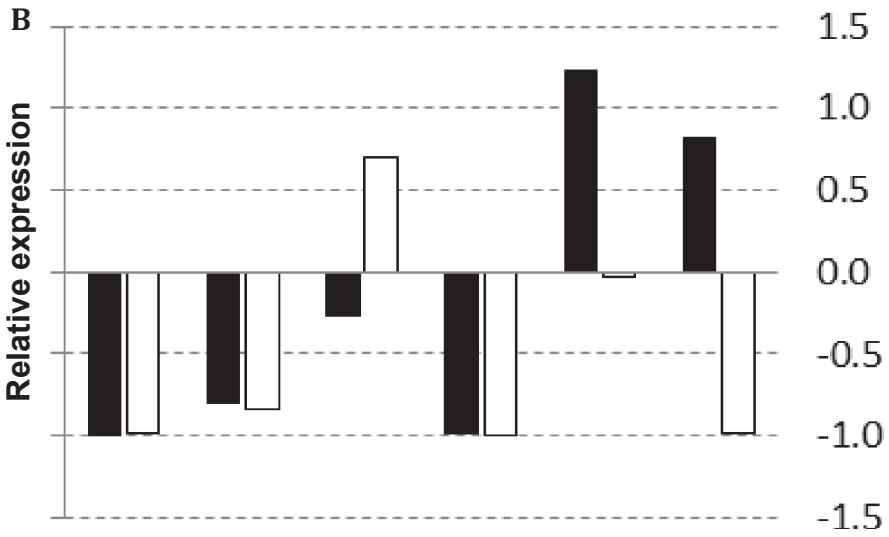
Roots of (**A**) Col-0, (**B**) Tsu-0 and (**C**) Tul-0 infected with *P. brassicae* under light microscopy after staining with aniline blue. White arrows indicate pathogen infection sites, scale bar = 50µm.

Figure 2. 10: Defence gene expression in Col-0 following *P. brassicae* infection.

(**A**) *PR-1* and *PDF 1.2* expression in Col-0 plants 15 and 25 days post treatment with *P. brassicae* or in combination with SA. *PR-1* is highly expressed 25 days post treatment after *P. brassicae* treatment and in combination with SA. Actin was probed for as an experimental control.

(**B**) Relative expression of *PR-1* (black bars) and *PDF 1.2* (white bars) from gel electrophoresis using actin as the control confirms that *PR-1* is highly expressed 25 days post treatment. Figures are representative only.


15 days
25 days
Control
P. brassicae
P. brassicae + SA
Control
P. brassicae
P. brassicae + SA
Negative
A
PR-1
PDF 1.2
Actin


B
Relative expression
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5

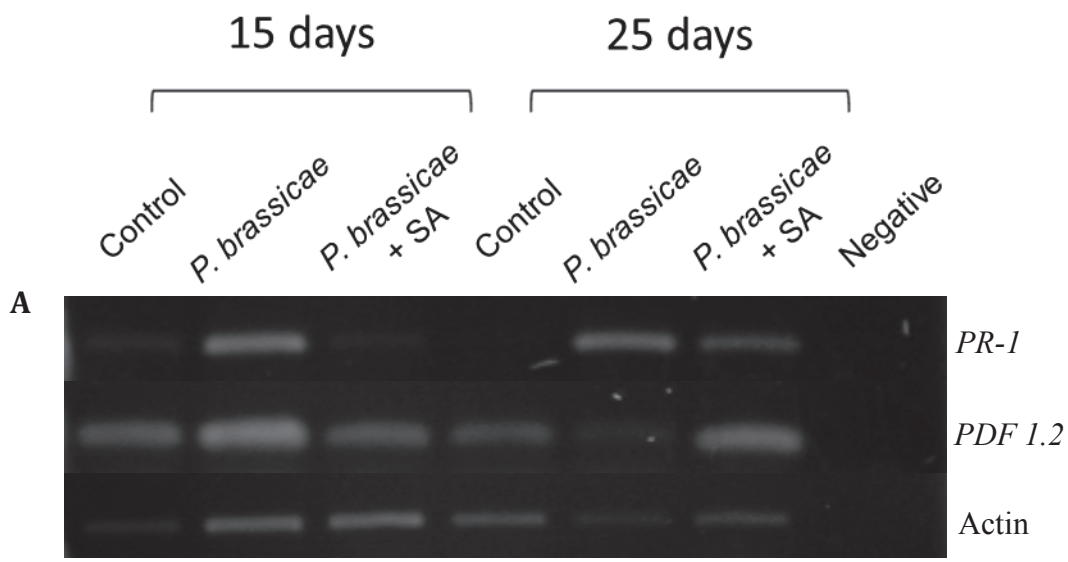
Tsu-0 showed an increase in *PR-1* expression 15 days post-treatment with *P. brassicae,* but no increase in combination with SA was observed. Twenty five days post-treatment, Tsu-0 plants treated with *P. brassicae* and in combination with SA showed an increase in *PR-1* expression. *PDF 1.2* expression in Tsu-0 was observed to be slightly up 15 days post-treatment with *P. brassicae* but not in combination with SA. At 25 days post-treatment, a combination of SA and *P. brassicae* showed an increase in *PDF 1.2* expression, there appeared to be no increase in plants treated with only *P. brassicae*. Relative gene expression confirmed the expression of *PR-1* and *PDF 1.2* (Figure 2. 11).

Tul-0 plants appear to have a high level basal expression of *PR-1* as all treatment groups including the controls at both 15 and 25 days showed *PR-1* expression. There was no expression of *PDF 1.2* 15 days post-treatment with a combination of *P. brassicae* and SA, however 25 days post-treatment there was reduced expression in plants when treated with *P. brassicae* only, but high expression when treated in combination with SA. Relative gene expression confirmed the expression of *PR-1* and *PDF 1.2* (Figure 2. 12).

Figure 2. 11: Gene expression in Tsu-0 following *P. brassicae* infection.

(**A**) *PR-1* and *PDF 1.2* expression in Tsu-0 plants 15 and 25 days post treatment with *P. brassicae* or in combination with SA. *PR-1* is highly expressed 15 and 25 days post treatment after *P. brassicae* treatment and in combination with SA. PDF 1.2 was highly expressed 15 days post inoculation with *P. brassicae* only. Actin was probed for as an experimental control.

(**B**) Relative expression of *PR-1* (black bars) and *PDF 1.2* (white bars) from gel electrophoresis using actin as the control confirms that *PR-1* is highly expressed 15 and 25 days post treatment, while PDF 1.2 is also expressed 15 days post-inoculation with *P. brassicae*. Figures are representative only.


15 days
25 days
Control
P. brassicae
P. brassicae + SA
Control
P. brassicae
P. brassicae + SA
Negative
A
PR-1
PDF 1.2
Actin

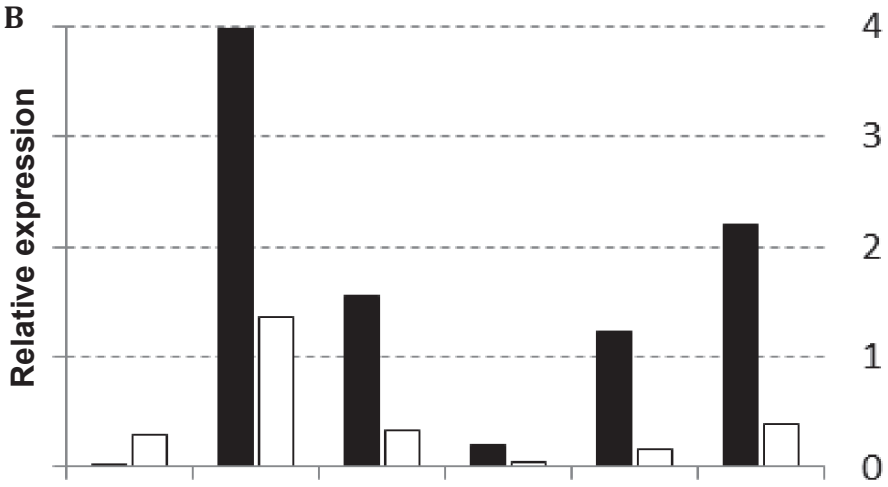
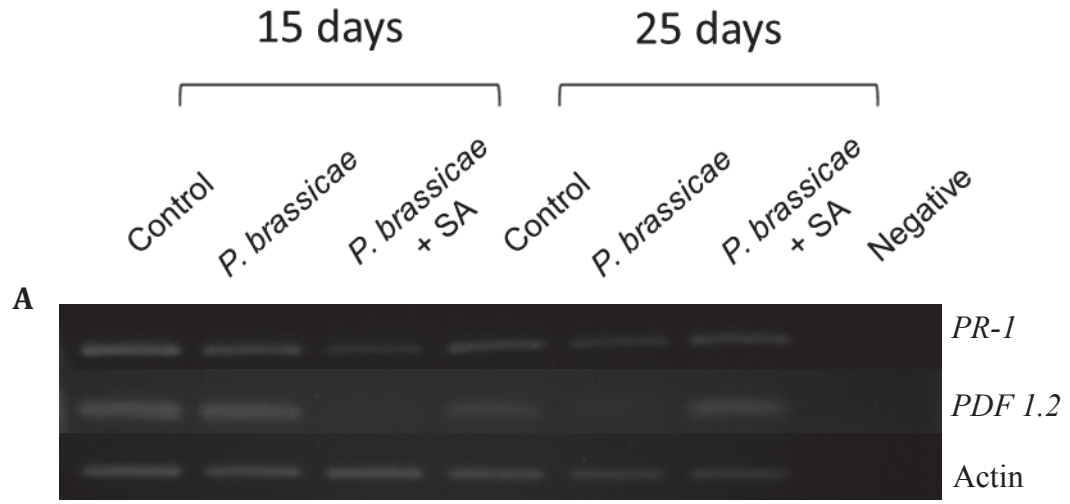
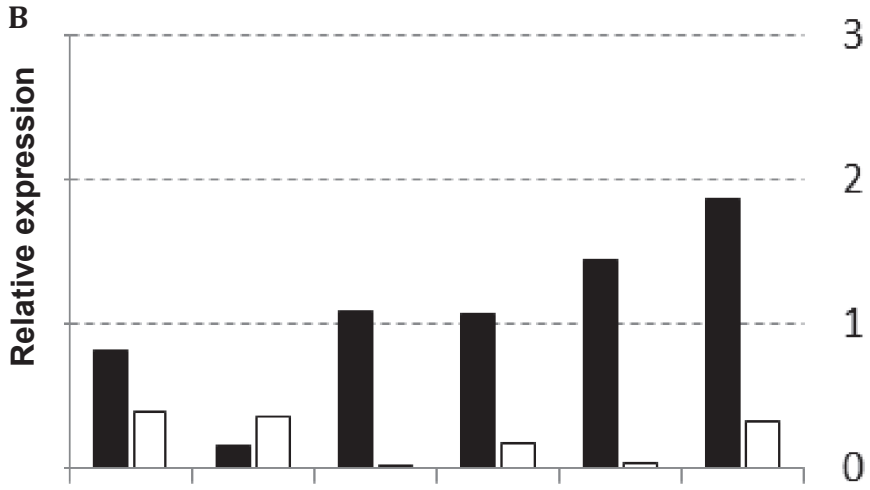

B
Relative expression
4
3
2
1
0

Figure 2. 12: Gene expression in Tul-0 plants following P. brassicae infection.

(**A**) *PR-1* and *PDF 1.2* expression in Tul-0 plants 15 and 25 days post treatment with *P. brassicae* or in combination with SA. *PR-1* is highly expressed in Tul-0 15 days post treatment after *P. brassicae* and SA treatment and in the control. At 25 days, *PR-1* is highly expressed in all treatment groups including the control. Actin was probed for as an experimental control.

(**B**) Relative expression of *PR-1* (black bars) and *PDF 1.2* (white bars) from gel electrophoresis using actin as the control confirms that *PR-1* is highly expressed 15 and 25 days post treatment *P. brassicae* and in combination of SA. Figures are representative only.


15 days
25 days
Control
P. brassicae
P. brassicae + SA
Control
P. brassicae
P. brassicae + SA
Negative
A
PR-1
PDF 1.2
Actin


B
Relative expression
3
2
1
0

2.4.3 Interaction between *Arabidopsis* pathway mutants and *P. brassicae*

2.4.3.1 Reduction in weight in pathway mutants of *Arabidopsis* following inoculation with *P. brassicae*

Arabidopsis pathway mutants were tested for their susceptibility to *P. brassicae* to further understand the role of SA. The susceptibility was determined by observing the impact of the disease on the plant weight of the *Arabidopsis* lines (Figure 2. 13). The lines used in this study were; *cpr1*, *dnd1*, *jar*, *NahG* and *npr1*. The *Arabidopsis* line *cpr1* and *dnd1* which are over-expressers of the SA pathway were found to have the least reduction in plant weight associated with the pathogen. *Cpr1* showed no significant decrease in plant weight compared to the control, while there was a significant difference in *dnd1*; the reduction was less than 40%. *Arabidopsis* lines *NahG* and *npr1* which both are deficient in an aspect of the SA pathway, were observed to be highly susceptible with a significant reduction in plant weight associated with the disease. A reduction of more than 60% total plant weight was observed for these two lines. The Jasmonic acid insensitive line, *jar*, was found to have a significant decrease in plant mass, with a reduction in over 60% observed.

2.4.3.2 Effect of *P. brassicae* on *A. thaliana* mutant shoot weights following SA treatment

Arabidopsis lines, *cpr1*, *dnd1*, *NahG* and *npr1* were assessed for their susceptibility to *P. brassicae* and in combination with 1 mM SA, 24 h post-treatment with the phytohormone. Fifteen days post-treatment with the pathogen, lines *cpr1* and *dnd1* showed no significant reduction in shoot weight when treated with the pathogen or in combination with SA. Lines *NahG* and

npr1 both showed a significant reduction in plant weight when treated with the pathogen or in combination with SA (Figure 2. 14a).

Twenty Five days post-treatment with the pathogen *cpr1* showed no significant decrease in plant weight in either treatment group. *Dnd1*, *NahG* and *npr1* all showed a significant reduction in shoot weight when treated with *P. brassicae* and in combination with SA (Figure 2. 14b).

2.4.3.3 Enhanced gall suppression in SA over-expressing mutants

Root galling was assessed 25 day's post treatment in the *Arabidopsis* mutant lines, to determine if the pathogen growth was being controlled. *Arabidopsis* over-expresser lines of SA, *cpr1* and *dnd1* (Figure 2. 15), both appeared to control the growth of the pathogen as the gall scores were 0.8 and 1.2 respectively. The addition of SA did not significantly reduce the galling in *cpr1*, however, *dnd1* showed a significant reduction in galling.

The *Arabidopsis* SA deficient line, *NahG*, was very susceptible to the pathogen, with an average score of 3 for root galling. The addition of SA did not significantly reduce disease severity (Figure 2. 15). *Npr1*, a non-expresser of *PR-1*, appeared to be tolerant to the pathogen, with an average root gall score of 1.8. The addition of SA did not significantly reduce the severity of disease associated with *P. brassicae* (Figure 2. 15).

Figure 2. 13: *Arabidopsis* pathway mutants 25 days post treatment with *P. brassicae.*

(**A**) *dnd1*, (**C**) *jar*, (**D**) *NahG* and (**E**) *npr1* inoculation with *P. brassicae* show a significant reduction in plant weight (g) compared to their respective controls. However, (**B**) *Cpr1* inoculated with *P. brassicae* shows no significant reduction in plant weight (g) compared to the control. Symbols indicate significant difference to respective control (n = 40) (p = 0.05) (black bars = control; white bars = inoculated).

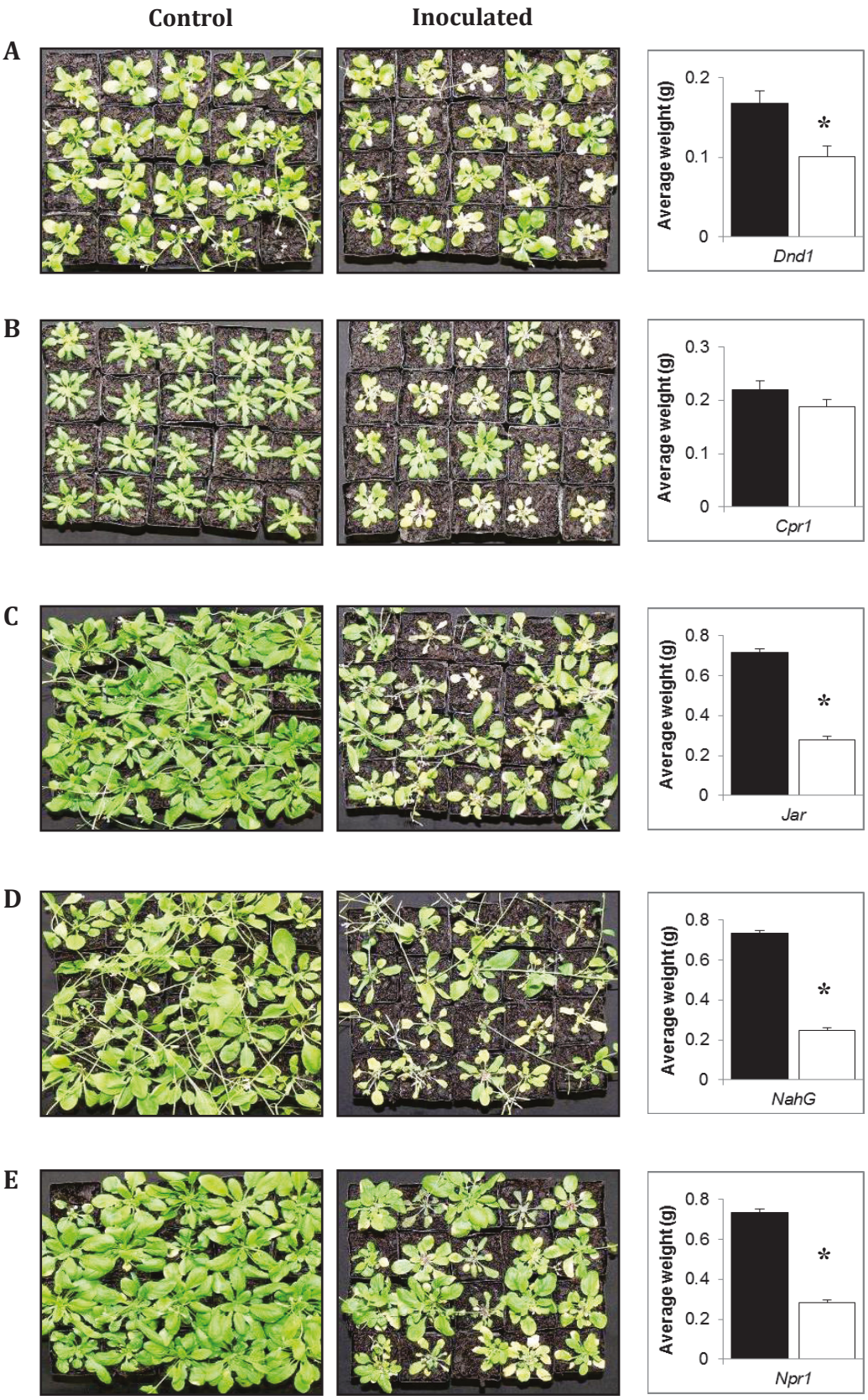

Control
Inoculated
A
Average weight (g)
0.2
0.1
0
*
Dnd1
B
Average weight (g)
0.3
0.2
0.1
0
Cpr1
C
Average weight (g)
0.8
0.6
0.4
0.2
0
*
Jar
D
Average weight (g)
0.8
0.6
0.4
0.2
0
*
NahG
E
Average weight (g)
0.8
0.6
0.4
0.2
0
*
Npr1

Figure 2. 14: Response of *Arabidopsis* mutants to *P. brassicae*

(**A**) At 15 day's post treatment with *P. brassicae* there is an observed reduction in plant weight (g) in *NahG* and *npr1*, the addition of SA prior to *P. brassicae* inoculation does not reduce the loss in plant weight in *NahG* and *npr1*, when compared to the controls.

(**B**) At 25 days post treatment there is a large reduction in plant weight (g) in dnd1, NahG and npr1 in both *P. brassicae* and SA + *P. brassicae* treated groups when compared to the controls. *Cpr1* is not affected at 15 or 25 days after P. brassicae inoculation. Symbols represent significant difference to respective controls (n = 40) (P = 0.05) (black bars = control; white bars = *P. brassicae*; grey bars = *P. brassicae* + SA).

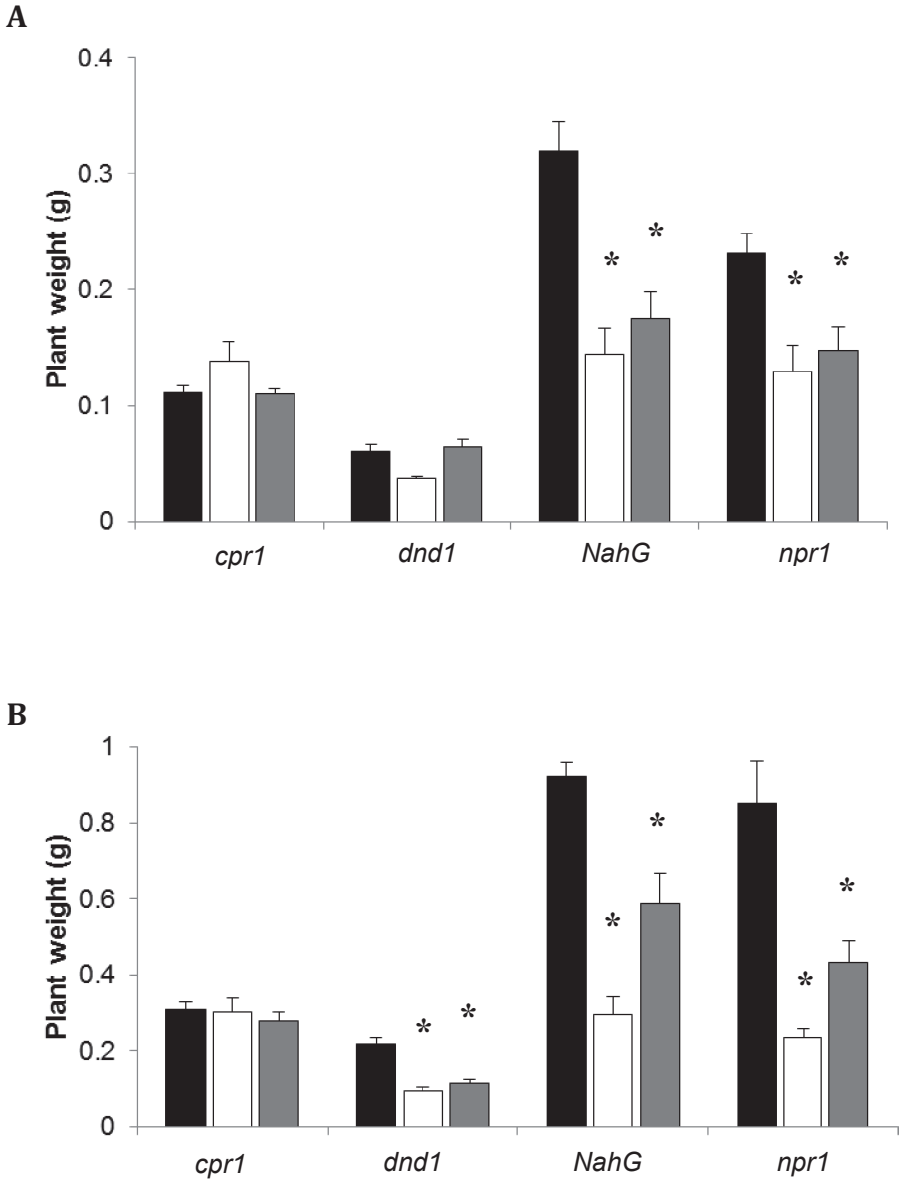
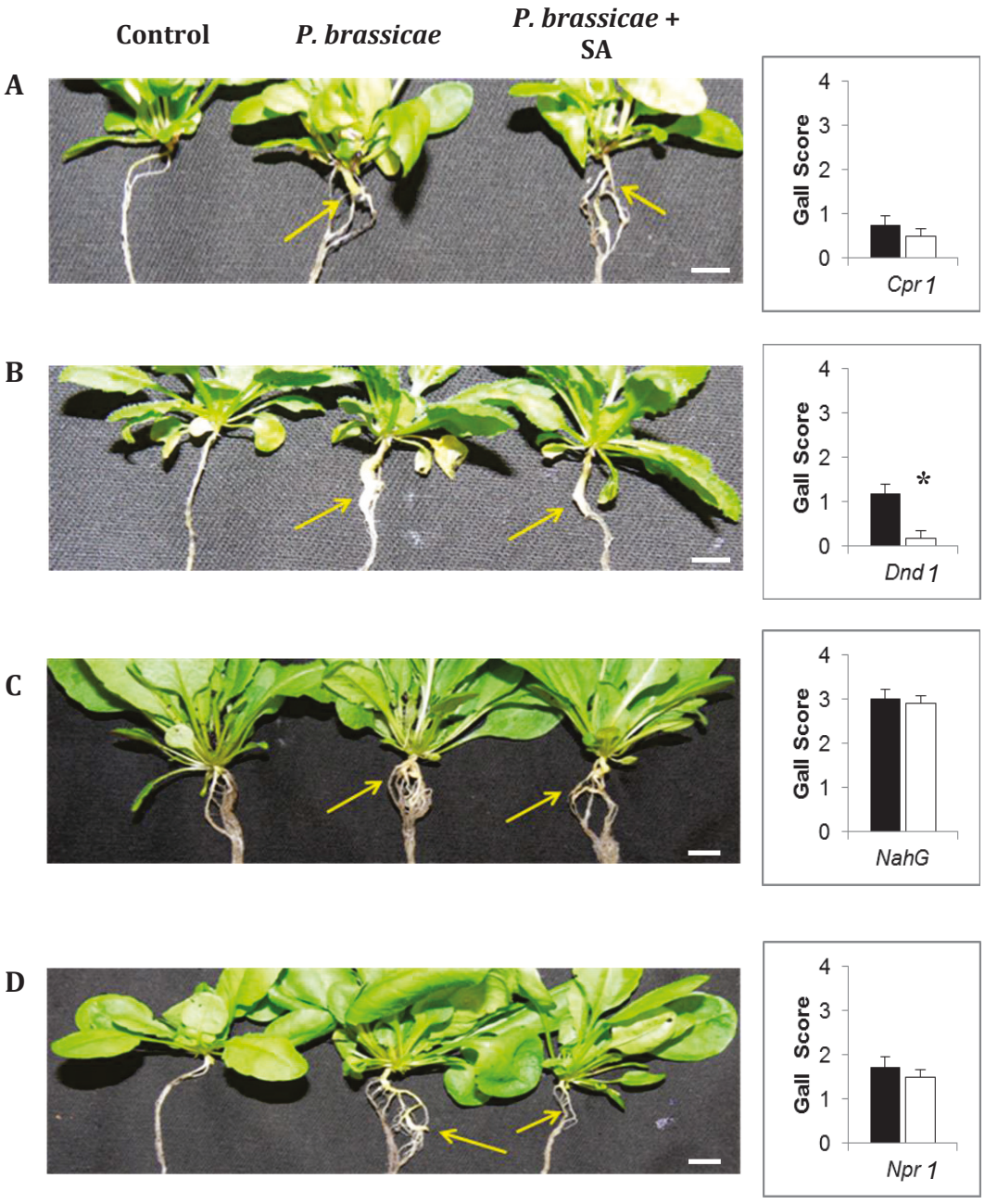

A
Plant weight (g)
0.4
0.3
0.2
0.1
0
cpr1
dnd1
NahG
npr1
B
Plant weight (g)
1
0.8
0.6
0.4
0.2
0
cpr1
dnd1
NahG
npr1

Figure 2. 15: Root galling and gall scores in *Arabidopsis* mutants

(**A**) *cpr1* and (**B**) *dnd1* both show a level of resistance to *P. brassicae* 25 days post-inoculation, with gall scores of 0.8 and 1.2 respectively. The addition of SA prior to inoculation not significantly reduce the galling associated with *P. brassicae* in *cpr1*, however SA added to *dnd1* significantly reduces galling.

(**C**) *NahG* is highly susceptible to the pathogen with a noted gall socre of 3, 25 days post-inoculation. (**D**) *npr1* although unable to fully express *PR-1* does show reduction in galling compared to *NahG* with a score of 1.8. The addition of SA however, does not reduce the disease severity associated with *P. brassicae* in *NahG* and *npr1*. Symbol indicates significant difference (n = 40) (P = 0.05) (black bars = *P. brassicae*; white bars = *P. brassicae* + SA).


Control
P. brassicae
P. brassicae + SA
A
B
C
D
Gall Score
0
1
2
3
4
Cpr 1
*
Dnd 1
NahG
Npr 1

2.4.3.4 Induction of defence genes in SA pathway mutants following *P. brassicae* infection

The expression of the defence gene *PR-1* and the expression of *PDF 1.2* were assessed 15 and 25 days post inoculation with *P. brassicae* or in combination with SA. The over-expresser SA lines of *cpr1* and *dnd1* (Figure 2. 16 & Figure 2. 17) both had heightened *PR-1* expression in all treatment groups, including the controls, 15 and 25 days post-treatment. *PDF 1.2* was not observed to be switched on in *cpr1* plants 15 days post treatment, however, 25 days post treatment; there was a slight increase when treated with only the pathogen. *PDF 1.2* expression in *dnd1* was seen to be up-regulated 15 days post-treatment with *P. brassicae* only and slightly up in combination with SA. Twenty five days post treatment, *PDF 1.2* expression was up-regulated in plants treated with the pathogen only. Relative gene expression confirmed the expression of *PR-1* and *PDF 1.2*.

Arabidopsis line *NahG*, deficient in SA production, did not show any signs of *PR-1* expression in any plant material tested. There was a slight increase in *PDF 1.2* 15 days post-treatment with *P. brassicae only*, while 25 days post-treatment; there was a slight increase in *PDF 1.2* when in combination with SA (Figure 2. 18). The *Arabidopsis* line, *npr1*, which has reduced PR expression, did not appear to express *PR-1* 15 days post treatment in either of the treatment groups, there was however a slight increase in combination with SA 25 days post-treatment. *PDF 1.2* expression was not apparent at either 15 or 25 days post-treatment with *P. brassicae* or in combination with SA. Relative gene expression confirmed the expression of *PR-1* and *PDF 1.2* (Figure 2. 19).

Figure 2. 16: Gene expression in *cpr1* following *P. brassicae* inoculation

(**A**) *PR-1* and *PDF 1.2* expression in *cpr1* plants 15 and 25 days post treatment with *P. brassicae* or in combination with SA. *PR-1* is highly expressed in all tissue 15 and 25 days post treatment after *P. brassicae* and in combination with SA treatment, and in the control. *PDF* 1.2 is not considered to be highly expressed in any of the treatments.

(**B**) Relative expression of *PR-1* (black bars) and *PDF 1.2* (white bars) from gel electrophoresis using actin as the control confirms that *PR-1* is highly expressed 15 and 25 days post treatment *P. brassicae* and in combination of SA and in the controls. Figures are representative only.

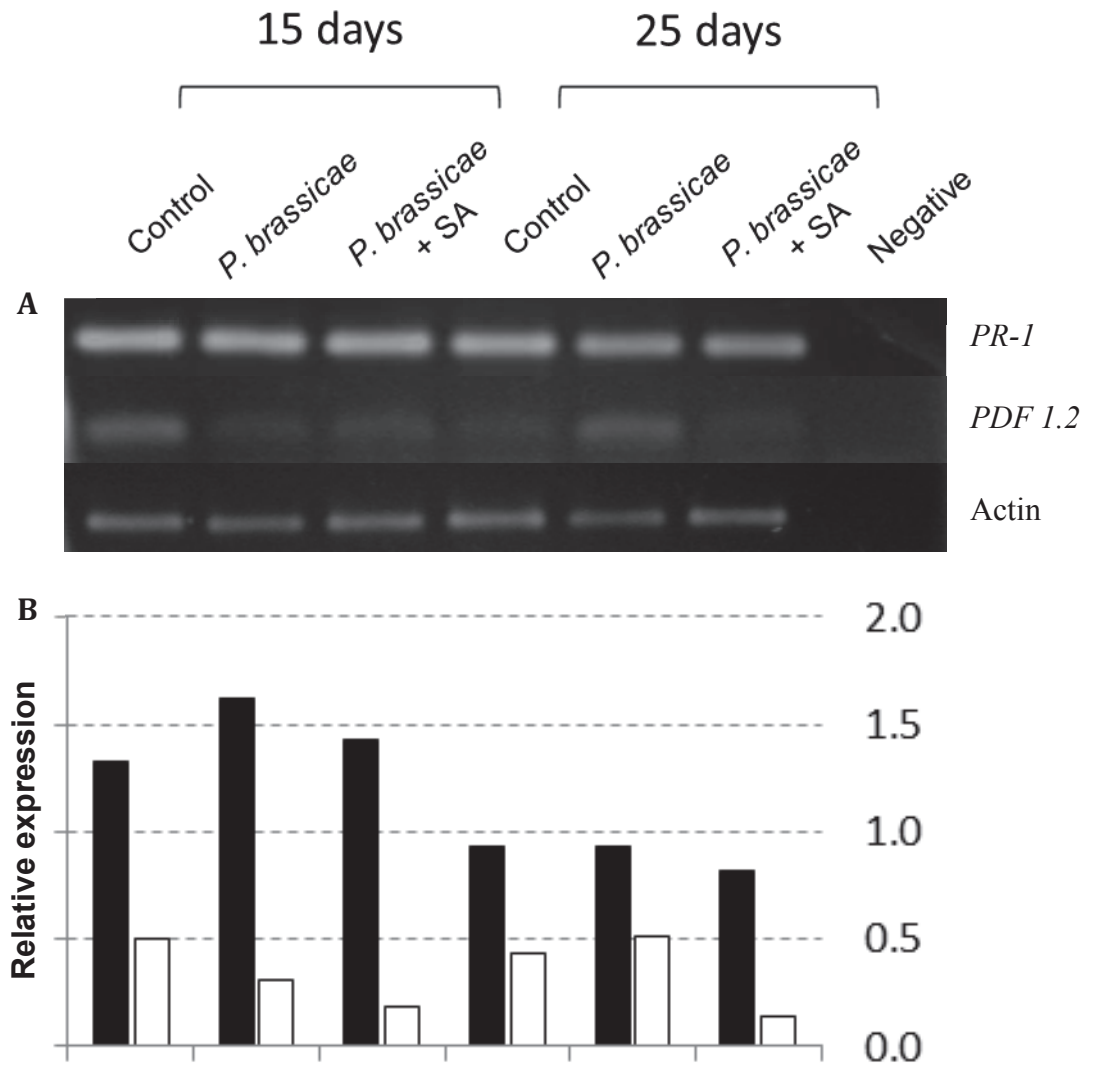

15 days
25 days
Control
P. brassicae
P. brassicae + SA
Control
P. brassicae
P. brassicae + SA
Negative
A
PR-1
PDF 1.2
Actin
B
Relative expression
2.0
1.5
1.0
0.5
0.0

Figure 2. 17: Gene expression in *dnd1* following *P. brassicae* inoculation

(**A**) *PR-1* and *PDF 1.2* expression in *dnd1* plants 15 and 25 days post treatment with *P. brassicae* or in combination with SA. *PR-1* is highly expressed in dnd1 15 and 25 days post treatment after *P. brassicae* inoculation and in combination with SA treatment, and in the control. PDF 1.2 is highly expressed 15 days post inoculation with *P. brassicae*

(**B**) Relative expression of *PR-1* (black bars) and *PDF 1.2* (white bars) from gel electrophoresis using actin as the control confirms that *PR-1* is highly expressed 15 and 25 days post treatment *P. brassicae* and in combination of SA and in the control. Figures are representative only.

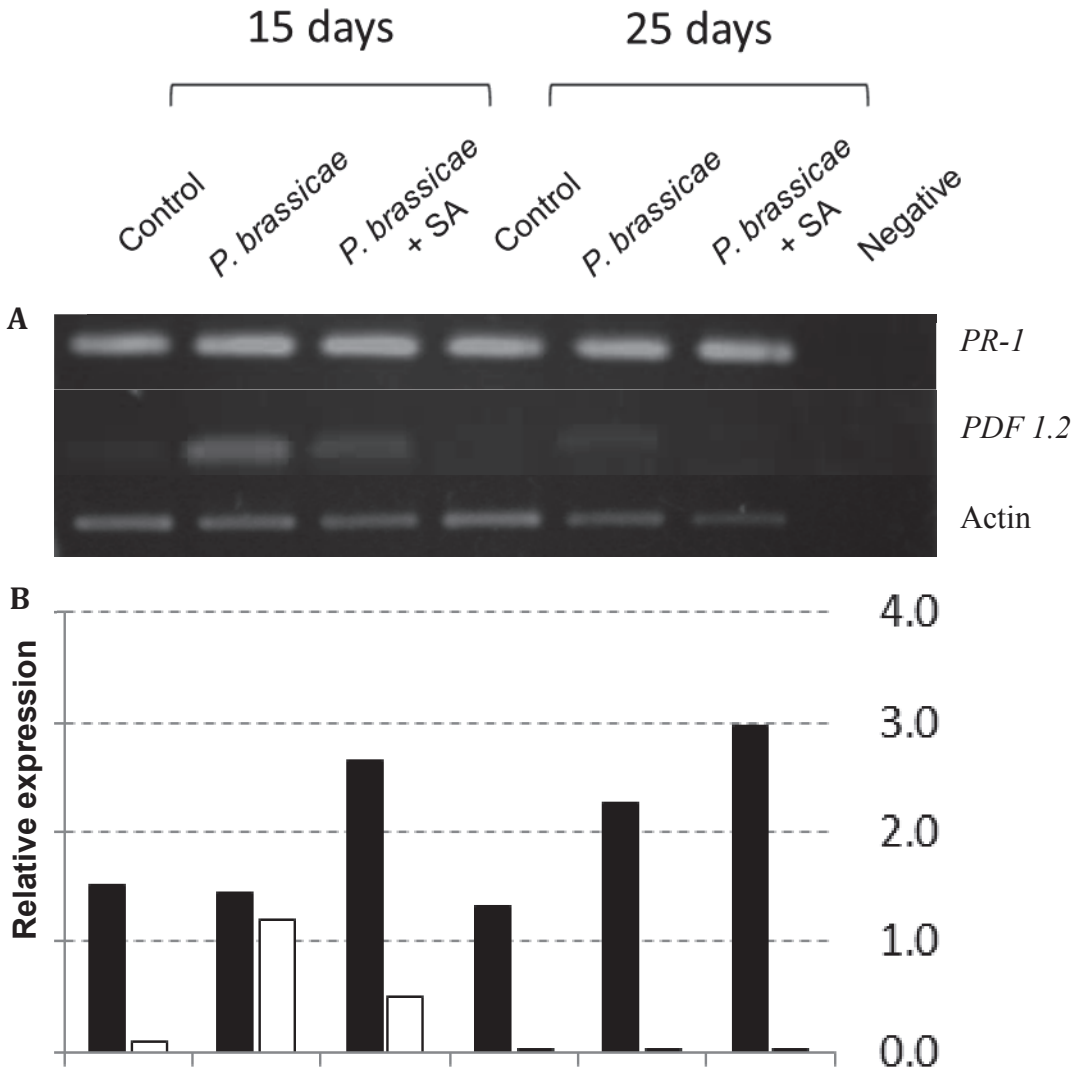

15 days
25 days
Control
P. brassicae
P. brassicae + SA
Control
P. brassicae
P. brassicae + SA
Negative
A
PR-1
PDF 1.2
Actin
B
Relative expression
4.0
3.0
2.0
1.0
0.0

Figure 2. 18: Gene expression in *NahG* following *P. brassicae* inoculation

(**A**) *PR-1* and *PDF 1.2* expression in *NahG* plants 15 and 25 days post treatment with *P. brassicae* or in combination with SA. As expected, *PR-1* is not expressed in NahG 15 and 25 days post treatment after *P. brassicae* inoculation and in combination with SA treatment, and in the control. *PDF 1.2* is also not expressed in any of the treatments.

(**B**) Relative expression of *PR-1* (black bars) and *PDF 1.2* (white bars) from gel electrophoresis using actin as the control confirms that *PR-1* and *PDF 1.2* are not expressed 15 and 25 days post treatment *P. brassicae* and in combination of SA and in the controls. Figures are representative only.

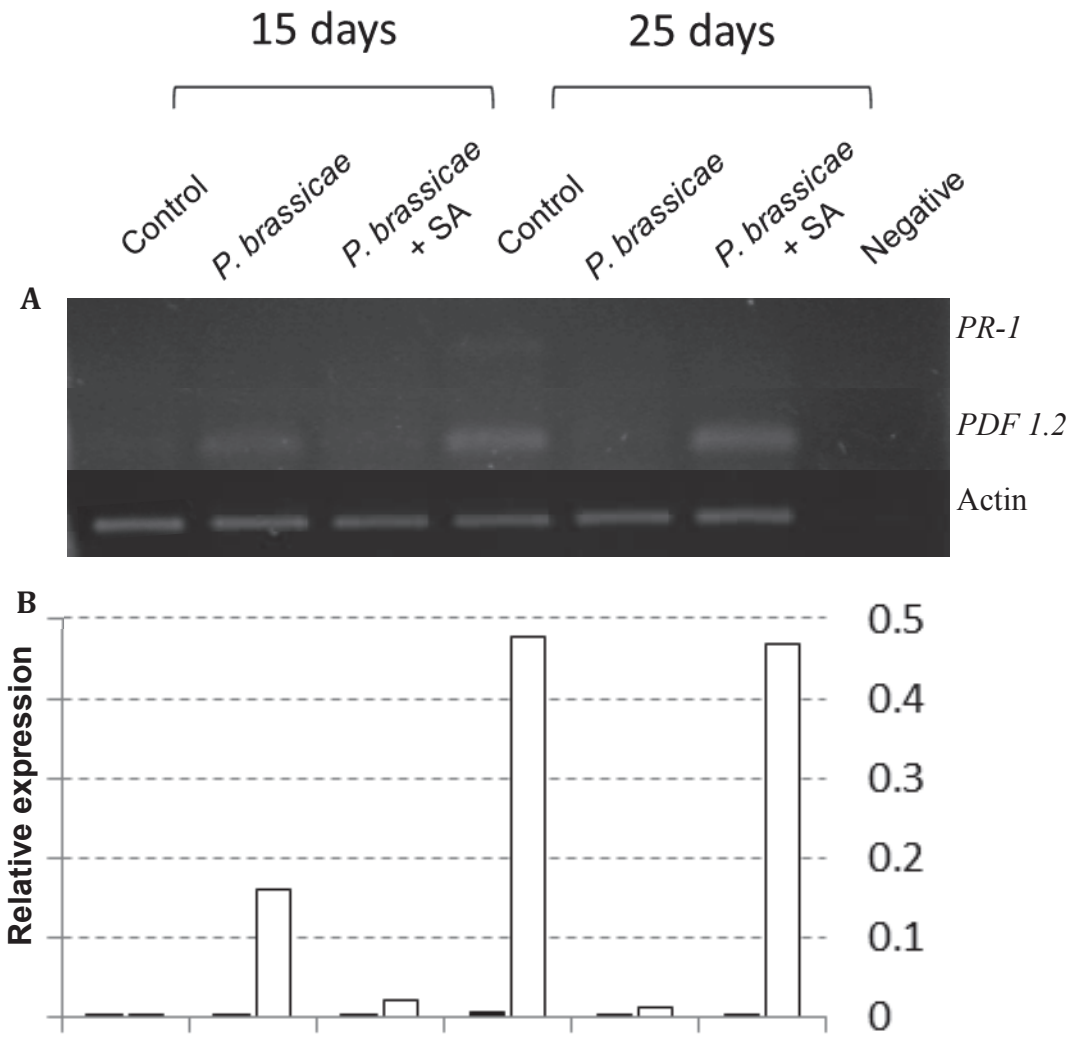
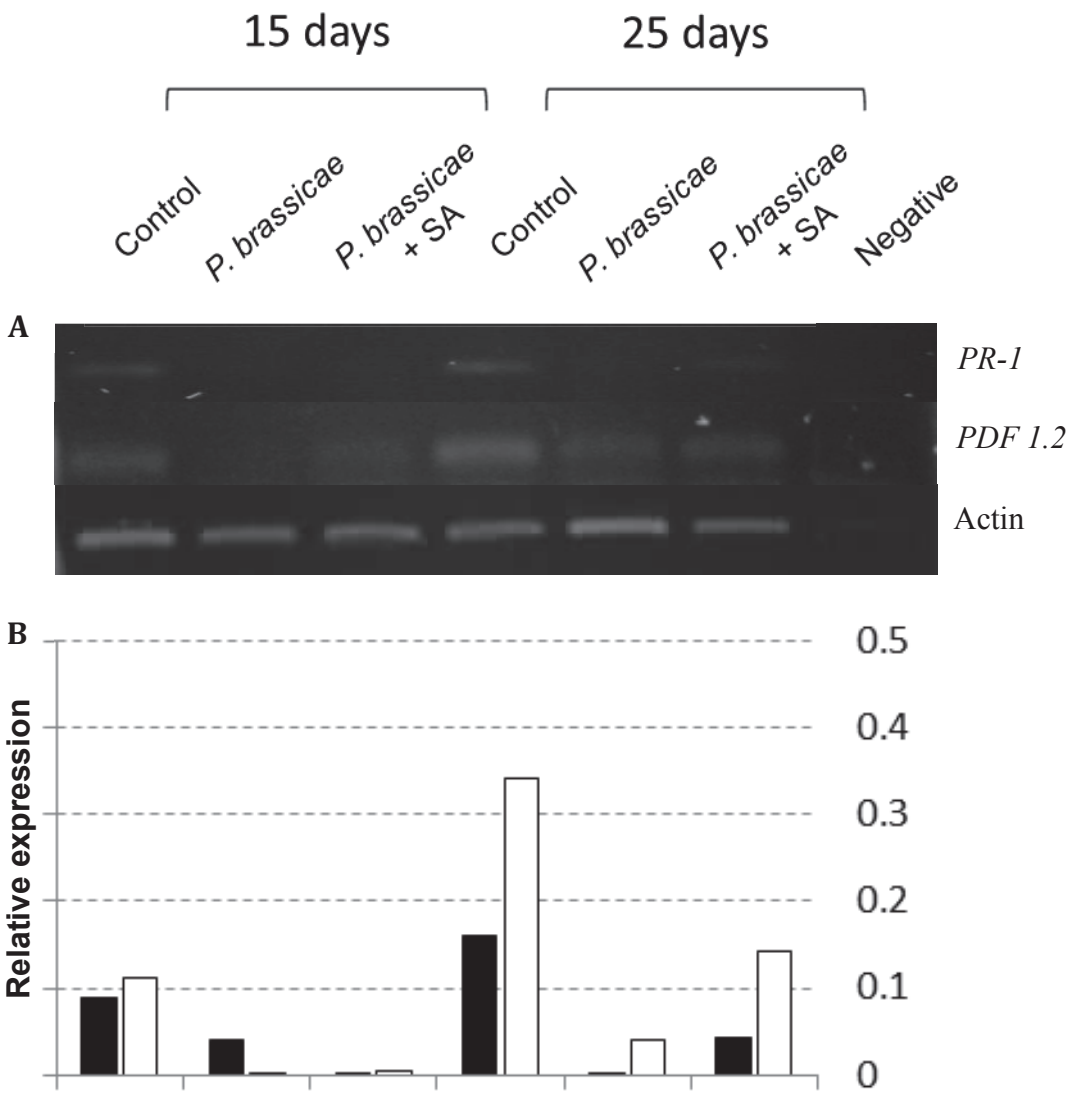

15 days
25 days
Control
P. brassicae
P. brassicae + SA
Control
P. brassicae
P. brassicae + SA
Negative
A
PR-1
PDF 1.2
Actin
B
Relative expression
0.5
0.4
0.3
0.2
0.1
0

Figure 2. 19: Gene expression in *npr1* following *P. brassicae* inoculation

(**A**) *PR-1* and *PDF 1.2* expression in *npr1* plants 15 and 25 days post treatment with *P. brassicae* or in combination with SA. As expected *PR-1* is not expressed in *npr1* 15 and 25 days post treatment after *P. brassicae* inoculation and in combination with SA treatment, and in the controls. PDF 1.2 is also not highly expressed in any of the treatments.

(**B**) Relative expression of *PR-1* (black bars) and *PDF 1.2* (white bars) from gel electrophoresis using actin as the control confirms that *PR-1* is not expressed 15 and 25 days post treatment *P. brassicae* and in combination of SA and in the controls. Figures are representative only.


15 days
25 days
Control
P. brassicae
P. brassicae + SA
Control
P. brassicae
P. brassicae + SA
Negative
A
PR-1
PDF 1.2
Actin
B
Relative expression
0.5
0.4
0.3
0.2
0.1
0

2.5 Discussion

The aim of this research was to determine the importance of the phytohormone salicylic acid in the interaction between *Arabidopsis thaliana* and *Plasmodiophora brassicae*. *Arabidopsis* ecotypes and a number of mutant lines enhanced or deficient in the SA pathway were used to determine their susceptibility with and without the application of SA. The use of SA at 1mM although regarded as an unrealistic endogenous concentration, it is not detrimental to plant health, provides the required defence response and has been briefly studied previously by Agarwal *et al*., (2011).

It had been suggested following this study that the 'e3' isolate obtained by DPI is no longer a single spore isolate, which may explain differences in infection to wild-type *A. thaliana* observed in this study compared to Siemens *et al*., 2002. A *P. brassicae* isolate from Australia and a single spore isolate from Germany were tested for their pathogenicity to 4 *Arabidopsis* ecoptyes and 5 mutant lines. The Australian isolate was able to successfully infect all *Arabidopsis* ecotypes and lines, while the single spore isolate, 'e3', was only able to mildly infect ecotypes Col-0 and Ler and the mutant line *jar*.

These results may suggest the variation in pathogenicity between clubroot populations within a single root gall, which has been suggested in a number of key studies. Klewer *et al*., (2001) first recognised this pattern, as they found different clubroot isolates in Germany located in close proximity to each other which had different host ranges. Donald et al., (2006) set out to determine if Australian isolates of *P. brassicae* also showed a large variation in host ranges.

The results of this study suggested that within Australia, at least 23 populations with differing pathotypes are present.

Agarwal *et al*., (2009) used a different Australian *P. brassicae* isolate and observed its interaction with a number of *Arabidopsis* ecotypes, including Col-0 and Tsu-0 and both of these ecotypes were found to be highly susceptible. In a 2011 study, Agarwal *et al*., observed a reduction in disease symptoms associated with *P. brassicae* in *Arabidopsis* following treatment with SA, the authors clearly showed a role for SA in resistance. Using a similarly virulent isolate, ECD 16/2/31, and with the addition of SA, there was no reported decrease in disease in *Arabidopsis* ecotypes Col-0, Tsu-0 and Tul-0, suggesting a highly susceptible interaction occurred.

The *Arabidopsis* lines used in this study are essential tools in understanding the critical components of the salicylic acid pathway in a defence response. *Cpr1* which has a constant expression of pathogenesis related (PR) genes, and *dnd1*, which does not produce a hypersensitive response but has elevated SA levels and constant *PR* expression, and have minimal symptom development from inoculation with *P. brassicae*. To date, both these lines had not previously been assessed for their susceptibility to any Australian isolate or the 'e' isolate and any of the single spore isolates generated from it.

The *dnd1* mutant has been used primarily to study foliar pathogens such as *Pseudomonas syringae*. Yu *et al*., (1998) compared disease severity in Col-0 and *dnd1* following *P. syringae* inoculation and discovered the lack of a HR response in *dnd1* did not affect the overall defence response to the pathogen, whilst Col-0 was severely infected. *PR-1* expression in *dnd1* was consistently high in each of the tissue samples tested including the control. This follows previous findings, that *PR-1* is constantly expressed in plant tissue. Yu *et al*., (1998) probed for *PR-1* using an RNA blot and determined the expression level of *PR-1* in *dnd1* was almost 10 times greater than Col-0 in control tissue.

Previous research into the *Arabidopsis* – *P. brassicae* interaction using the PR deficient line *npr1*, has been studied, however only with use of the 'e' isolate (Siemens *et al*., 2002). *Npr1* has been shown to be susceptible to the Australian isolate and is also known to be susceptible to the 'e' isolate, however not to the single spore 'e3' isolate. This again suggests a possible difference in pathogenicity within populations. The SA deficient line, *NahG*, had not been assessed for its susceptibility to any of the Australian isolates or isolate 'e'. Our results show a susceptible interaction to an Australian isolate, which is not a surprising result as SA appears to be of high importance for a successful resistance interaction.

Although the *jar* mutant was not exstensively studied in this research, Agarwal *et al*., 2011 noted the *jar1* mutant was highly susceptible to an Australian isolate of *P. brassicae*, while Siemens *et al*., 2002 noted its susceptibility to the single spore isolate 'e3'. These results conflicit with reports that suggest jasmonic acid

may be involved in gall development (Ludwig-Müller & Schuller, 2008) and that jasmonic acid may act as an antagonist to SA (Kunkel & Brooks, 2002).

SA enhanced *Arabidopsis* mutants have provided evidence of the importance of SA in an interaction with *P. brassicae*; however the addition of SA to SA deficient mutants failed to reduce disease severity. In subsequent studies with *Brassica oleracea*, SA has been quantified to ensure exogenously applied SA increases intracellular SA.

Chapter 3: Influence of SA on growth of broccoli (*Brassica oleracea* var. *italica*) and evidence for a role in systemic signalling

3.1 Abstract

Salicylic acid is an important plant phytohormone, which is required for defence against biotic and abiotic stresses. SA plays an important role in systemic acquired resistance (SAR) and induced resistance (IR) with the up-regulation of specific defence genes. However like many hormones and chemicals, SA at high levels is detrimental to plant health, with a reduction in both shoot and root weight occurring when applied exogenously. Concentrations of SA above 20 mM were found to be the most detrimental to plant health with significant reduction in both shoot and root weight and high phytotoxicity. Salicylic acid was however found to be transported systemically from the root to the shoots through analysis using high performance liquid chromatography. An application of SA does promote an SAR response, as root drenched broccoli plants exhibit high *PR-1* levels in leaves. The research presented here highlights the critical nature SA plays in defence, through its accumulation and up-regulation of *PR-1*.

3.2 Introduction

Phytohormones, of which SA is one, regulate all aspects of plant development and are also involved in the activation of defence against potential pathogens and in the response of plants to abiotic stress (Horváth *et al*., 2007). SA has roles

not only in defence against pathogens, but in normal growth and development, via the regulation of fundamental processes such as photosynthesis, flower induction and uptake of essential minerals and ions (Hayat *et al*., 2007). There is good evidence that SA is synthesised in the chloroplast (Fragniére *et al*., 2011). The involvement of SA in plant responses to abiotic stresses such as chilling was shown by Janda *et al*., (1999) who found that treating maize (*Zea mays* L.) with 0.5 mM of SA, increased tolerance. Exogenous applications of SA also prevented phytotoxicity caused by metals such as copper in cucumber, tobacco and sunflower (Strobel & Kuć, 1995; El-Tayeb *et al*., 2006) and is protective against salt stress (Poór *et al*., 2011). Transient increases in SA and its glucose conjugates were found in wounded leaves of *Arabidopsis* (Ogawa *et al*., 2010).

Salicylic acid is synthesised primarily through the shikimate pathway, but it is known the phenylalanine pathway can produce a small amount however this is believed to be not associated with a defence response (Wildermuth *et al*., 2001). Intracellular levels of SA vary among different species of plants with rice, potato and tomato known to have elevated levels compared to many other plants, often leading to increased resistance to pathogens (Yu *et al*., 1997; Yang *et al*, 2004). Levels of salicylic acid can be analysed through PCR and RT-qPCR by analysing gene expression and through direct analysis of the hormone by state-of-the art methods including high performance liquid chromatography (HPLC) with appropriate internal standards.

Systemic acquired resistance (SAR) and the hypersensitive response (HR) are regulated by increases in endogenous SA (Lee *et al*., 2006, Vlot *et al*., 2009). It

has been highlighted in a recent review by Spoel & Dong (2012) that methylsalicylic acid, azelaic acid and glycerol-3-phosphate may be the initial signalling molecules prior to SA accumulation in an IR and SAR response. An increased concentration of endogenous SA prior to and during IR and SAR has been correlated with a systemic increase in the production of pathogenesis-related (PR) proteins throughout the plant. PR proteins play an important role in various degrees of pathogen resistance and their activation is often correlated with IR and SAR induction (Dempsey *et al*., 1999; Kinkema, 2000). In tobacco plants, for example, *PR-1* has been shown to be up-regulated in leaves in the presence of SA (Niki *et al*., 1998: Yalpani *et al*., 1991) and the expression of a number of PR genes was stimulated in *Malus hupehensis* leaves, stems and roots upon treatment with SA (Ziadi *et al*., 2001; Zhang *et al*., 2010).

In the present study I investigate SA and its potential to stimulate defence in *Brassica oleracea* var. *italica* (Broccoli) and its ability to be taken up and distributed throughout the plant. Using high performance liquid chromatography SA levels will be analysed, while the defence genes PR-1 and PR-2 will be analysed for their up-regulation following SA treatment.

3.3 Materials and Methods

3.3.1 Germination of *B. oleracea* seeds on salicylic acid and jasmonic acid infused Murashige and Skoog Media

To examine the effects SA and JA on germination of *B. oleracea*, seeds were grown on Murashige and Skoog Basal Medium (MS) infused media. In a 2L conical flask MS media (4.4g), sucrose (15g) and bacteriological agar (8g) were

dissolved in dH_2O (1L) on a magnetic stirrer. The pH was adjusted to 5.8 using either 1M dipotassium hydrogen orthophosphonate (K_2HPO_4) to raise or 1M potassium dihydrogen orthophosphonate (KH_2PO_4) to lower the pH. The MS agar was dispensed into 4 conical flasks, to a total of 250ml of MS agar per conical flask. Salicylic acid is stable when autoclaved and readily dissolves into liquids during this process, JA was added following autoclaving. A known concentration of SA was added to the MS media and autoclaved at 121°C for 20 minutes, while JA was added after autoclaving, following this it was poured into sterile 9cm diameter petri dishes.

Broccoli (cv. Marathon) (*Brassica oleraceae* var *italica*) seeds, obtained from the Department of Primary Industries, Knoxfield, Vicoria, Australia, were sterilised in a 50ml beaker in a solution of sdH_20: ethanol: sodium hyperchloride (45:50:5) and mixed for 30 seconds. Seeds were then washed in sdH_20 twice and allowed to air dry on filter paper before being placed onto MS media in petri dishes. The plates were then transferred to a growth cabinet (Thrermoline Growth Cabinet, Australia) at 22°C with a 12hr light period (300µmol m^{-2} s^{-1}) which was provided by sodium lamps. Germination and seedling development was recorded 7 days post-sowing.

3.3.2 Plant growth and maintenance

Broccoli seeds were sown 1-2cm deep in autoclaved potting mix (Debco Pty Ltd. Tyabb, Victoria, Australia) in 48 cell plastic seedling trays (4cm x 3.5cm x 6cm) and grown for a period of three weeks in a growth cabinet (Thermoline, Australia) as described above. Nineteen days after sowing, seedlings were

transferred from the cells to individual plastic pots (7cm x 5cm) and allowed to adjust for two days prior to SA treatment. Following treatment, plants were returned to the above conditions, 10 plants were used in each treatment group with a total of 50 plants in each experiment, with 2 repeats.

3.3.3 Treatment of plants with salicylic acid

A stock solution of 100mM salicylic acid (SA) (99% plant extract, Sigma Aldrich) was prepared in MeOH/sdH_2O (v/v1:1) and dilutions made with sdH_2O. Three-week-old plants were treated with SA at five concentrations that ranged from 0.05 mM to 100 mM. Plant roots were exposed to SA by application directly to the soil. For this purpose 2L of each concentration of SA was prepared and twenty five mL was poured from a beaker onto the soil surface of each pot; the remaining 1.8L was placed into the holding tray containing the pots. The level of the solution in the holding tray was 2 cm from the pot base and therefore the soil was freely accessible to the SA solution through the perforations in the base of the pots. The SA solution in the holding tray was left in place for 30 min before being removed and pots allowed to drain freely. The control group of plants were treated with an equivalent volume of sdH_20 without added SA. Immediately after treatment the pH of the soil within pots ranged from 4.5 (100 mM SA treatment) to 5.0 - 6.5 (20 mM – 0.050 mM SA treatment; the pH of the soil in the water control was 7.0. Commencing 24 h after treatment and for each day thereafter, at the same time, plants were watered with H_20. Plant weight and root and shoot measurements (see 3.3.4) were taken at 0, 2, 4 and 8 days following treatment with SA that ranged from 0.05 mM to 0.25 mM and at 0, 1 and 3 days for treatments with SA that ranged from 0.8 mM to 100 mM. For the

latter measurements chlorophyll content was also examined (see 3.3.5). Total SA was quantified in all samples at 0, 1 and 3 days post-treatment (see 3.36).

3.3.4 Measurement of root weight and length of tap root and shoot height

At the designated time points following treatment, plant fresh weights were determined. Plants and soil were gently removed from the pots, the roots carefully washed in running tap water to remove soil and then blotted onto absorbent paper. Whole plants were then placed on a top loading balance and fresh weight recorded. The length of the taproot and shoot height, measured from the base of the shoot to the tip of the most recently formed true leaf, were also determined for each plant. Images of each plant were captured using a digital camera (Panasonic, Matsushita Electrical Industrial Co., Ltd., Japan) and the images downloaded onto a computer. Tap root lengths and shoot height were determined from each image using image analysis software (Image J v1.43; National Institutes of Health, USA) and data was exported to a spreadsheet (Microsoft Excel 2010) for further analysis.

3.3.5 Measurement of relative chlorophyll content of leaves

The relative chlorophyll content of leaves was determined at 0, 1 and 3 days after treatment with SA at concentrations of 0.8 mM – 100 mM using a Soil Plant Analysis Development (SPAD) meter (SPAD-502, Minolta, Co., Ltd, Macquarie Park, NSW) following the method of Allardyce *et al*, (2012). Two measurements were taken from a single leaf for each of the plants in each treatment group at each time point. The SPAD meter determined the optical density of light transmitted through each leaf and inbuilt software facilitated the determination

of the relative chlorophyll content in each leaf. The mean relative chlorophyll content was expressed as a value between 1 and 100.

3.3.6 Quantification of total SA in shoots

SA uptake and transport to shoots was measured using reverse phase-high performance liquid chromatography (RP-HPLC) to quantify SA in extracts that were obtained using a modified method based on that of Li *et al*, (1999) and Pan *et al*, (2010). Measurement of total SA content was undertaken on 2 and 3 week old seedlings that had been treated with the various concentrations of SA as previously described. Approximately 200 mg of shoot material from each of 4 plants per treatment was frozen in liquid nitrogen in a 1.5 mL microcentrifuge tube (Mirella Research Pty, Ltd. Brunswick, Victoria, Australia) and then homogenized with a small metal rod to a fine powder and then 600 µl of methanol (90%) added. The mixture was then vortexed for 30 sec, sonicated (Unisonics Pty, Ltd. Sydney, Australia) for 20 min and then centrifuged at 14,000 × g for 10 min. The supernatant was gently removed and placed into a fresh 1.5 mL centrifuge tube and the pellet was re-suspended in 400 µl of methanol (100%). The mixture was centrifuged at 14,000 × g for 10 min and the resulting supernatant extracted and combined with the original supernatant and air dried under nitrogen gas.

To the dried samples 500 µl trichloroacetic acid (5% in sdH_20) was added and samples vortexed for 30 sec, then sonicated for 5 min and centrifuged at 14,000 × g for 10 min. The supernatants were collected and extracted in 1:1 (v/v) ethyl acetate in water with vortexing for 10 min. The organic phase (top layer) was

removed and placed into a new 1.5 mL microcentrifuge tube, the aqueous phase was acidified with 500 µl concentrated (100%) HCl and heating at 80°C for 1 h. The released SA was then extracted by partitioning against 1:1 (v/v) ethyl acetate, combined with the previous extract and dried under nitrogen. The dried extract was re-suspended in 250µl of mobile phase (0.2M potassium acetate, 0.5 mM EDTA [pH 5]) by vortexing for 30 sec and sonication for 5 min. Samples were filtered by application to filtered 1.5 mL microcentrifuge tubes and then centrifuged at low speed.

RP-HPLC separations were performed on an Agilent Technologies 1200 series HPLC consisting of a solvent degasser, autosampler, and column heater diode array and fluorescence detector. All separations were performed with an Altima C_{18} column 250 × 4.6mm 5µm particle size column, (Alltech). A solvent gradient was applied starting at 0 min: H_20, 95%, acetonitrile, 5% and finishing at 20 min: H_20, 5%, acetonitrile, 95%, flow rate of 1ml/min. was applied throughout. Samples (10 µl) were applied to the column and SA in the eluate detected using a fluorescence detector (excitation wavelength of 295nm and emission wavelength of 405nm). The concentration of SA was then determined by reference to a standard curve from known SA concentrations.

3.3.7 RNA isolation and cDNA synthesis

Six shoot samples were collected from 2 and 6 week old plants immediately after treatment and at 24 and 72 h post – treatment (ie at the time they were transferred into pots) and frozen in liquid nitrogen. Total RNA was isolated from 100mg of leaves using a commercial kit (TRIzol ©, Molecular Research

Center, Inc. Cincinnati, USA) by first placing tissue into a 1.5 mL microcentrifuge tube, freezing the sample with liquid nitrogen and then grinding with a metal rod to a fine powder. For every 100 mg of tissue, 1 ml of reagent was added to the powdered tissue and the tube vortexed for 30 sec followed by incubation for 5 min at room temperature. For every 1ml of TRIzol reagent added, 0.2 ml of chloroform was added and tubes vortexed for 15 sec before being centrifuged at 12,000 × g for 30 min at 4°C. The aqueous phase (top layer) was then transferred to an RNase-free 1.5 ml microcentrifuge tube and 0.5 ml of isopropyl alcohol was added followed by inversion and finally incubation at room temperature for 10 min. The mixture was then centrifuged at 12,000 × g for 10 min to form an RNA pellet at the bottom of the tube. The supernatant was removed and following a further centrifugation (12,000 × g for 10 min) residual liquid was carefully removed with a pipette. The pellet was washed in 0.5 ml cold RNase-free ethanol (75%) and centrifuged at 12,000 ×g for 10 min. The supernatant was discarded followed by further centrifugation; the microcentrifuge tube was inverted onto absorbent material (Kimwipes, Kimberly-Clarke, Milsons Point, NSW, Australia) for 10-15 min, to allow for residual liquid to dry off. To the pellet was added 20-30 µl of RNase-free water and the pellet was resuspended by pipetting 20-30 times, the samples were then stored at -80°C. RNA quantity and quality was assessed using a Nanodrop (NanoDrop Technologies, Inc. Wilmington, DE USA). To remove DNA contamination, Deoxyribonuclease I, Amplification Grade (Invitrogen) was used as per the instruction manual. cDNA synthesis was undertaken using Superscript III™ (Invitrogen) as per the instruction manual.

3.3.8 PCR primers and conditions

The following forward and reverse primers were used for gene-expression analysis; *PR-1* (pathogenesis related gene 1) 5´-GCGACTGCAGACTCGTACAC-3´ and 5´- TCTCGTTGACCCAAAGGT TC-3´; PR-2 (pathogenesis related gene 2) 5´-ACATTCATGGGAGCCTTCAC-3´ and 5´- AGATCGCTCGCTTACCAAGA-3´ and Actin (reference gene) 5´-ACGTGGACATCAGGAAGGAC-3´ and 5´-GAACCACCGATCCAGACACT-3´.

To a 0.2 mL microcentrifuge tube (Axygen, Inc. Union City, CA USA) the following reagents were added; 12.5µl GoTaq© (Promega), 1.5 µl F-Primer, 1.5 µl R-Primer, 8.5 µl sdH20 and 1 µl of appropriate cDNA. PCR-cycling (GeneWorks Pty, Ltd. Hindmarsh, South Australia, Australia) conditions comprised an initial polymerase activation step at 95°C for 3 min, followed by 30 cycles for actin and *PR-1* and 38 cycles for PR-2, of 95°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec. PCR products were run on a 2% agarose gel at 80 V for 40 min and then stained with ethidium bromide and bands visualised under a UV light source. For RT-qPCR, the following reagents were added to 0.1ml tubes (Qiagen Pty, Ltd. Doncaster, Victoria, Australia); 12.5µl Master Mix, 2.5µl F-Primer, 2.5µl R-Primer, 5.5µl sdH20 and 2µl of appropriate cDNA. RT-qPCR-cycling was performed using a Rotor-Gene 3000 (Qiagen Pty, Ltd. Doncaster, Victoria, Australia) and conditions comprised an initial polymerase activation step at 95°C for 15 min, followed by, 40 cycles of 95°C for 40 sec, 55°C for 30 sec and 72°C for 30 sec, a melt (60-92°C) cycle was also performed with each unit of temperature held for 30 sec.

Mean fold change in gene expression of *PR-1* or *PR-2* (target genes) was calculated against Actin (reference gene (RG)) using the following equation as described by Livak and Schmittgen (2001): $\Delta\Delta C_T = (C_{T,\ \text{Target}} - C, \text{RG})\text{Time } x - (C_T, \text{Target} - C, \text{RG})\text{Time } 0$.

3.3.9 Statistical analysis

Statistical analysis was performed using SPSS (PASW 18.0) (IBM, Armonk, New York, USA) and a statistics package (Microsoft Excel 2010). In all experiments, One-Way ANOVAs, specifically Tukey's test and Fisher's least significant difference (LSD) test with a P value = 0.05, were used to analyse significant differences between each treatment group.

3.4 Results

3.4.1 Effect of phytohormones on germination of *B. oleracea* seeds

Salicylic acid and Jasmonic acid were tested for their effects on the germination of *B. oleracea* seeds, 7 days post-sowing on phytohormone infused MS plates. Although *B. oleracea* seeds were observed to germinate freely on MS plates infused with 0.25 and 0.5 and 1 mM SA, their development was slowed in comparison to the control. *Brassica oleracea* seeds grown on 0.25 mM JA were observed to germinate at a slow rate compared to the control 7 days post-sowing. Seeds grown on 0.5 and 1 mM did not fully germinate; seeds grown on a combination of 0.5 mM SA/JA also did not germinate (Figure 3. 1).

The observed germination and development of seeds on MS plates closely relates to the recorded shoot weights of *B. oleracea* (Figure 3.2). When grown on SA at 0, 0.25 and 0.5 mM there is no difference in weight compared to the control, with averages of 0.55g, 0.51g and 0.58g respectively. When the SA concentration were increased to 1 mM there was a significant reduction in shoot weight, with a decrease of 50% in shoot weight recorded. Jasmonic acid at 0.25 mM was shown to dramatically reduce shoot growth, while at 0.5 and 1 mM prevent seed germination. A similar response was observed when SA and JA were in combination at 0.5 mM.

3.4.2 Effect of SA on root and shoot growth in plants grown under controlled conditions

The effect of treatment on *B. oleracea* roots with SA on growth was measured by analysing length of the major root and height to newest leaf tip and root weight and total plant weight (Figure 3. 3). At the lower concentrations of SA used (0.05 mM, 0.1 mM and 0.25 mM) there was no difference in root weight to the control for all treatments at all time points (data not shown). There was also found to be no difference in shoot weight and plant length between groups (data not shown). However, at higher concentrations of SA (0.5 to 100 mM) the fresh weight of both shoots and roots was significantly lower 72 h post-treatment with 5 mM, 20 mM and 100 mM SA, a reduction of 25 – 50% was observed as SA concentration increased (Figure 3. 4a). There was however no effects of high SA concentrations on overall root length of broccoli plants 72 h post-treatment (Figure 3. 4b).

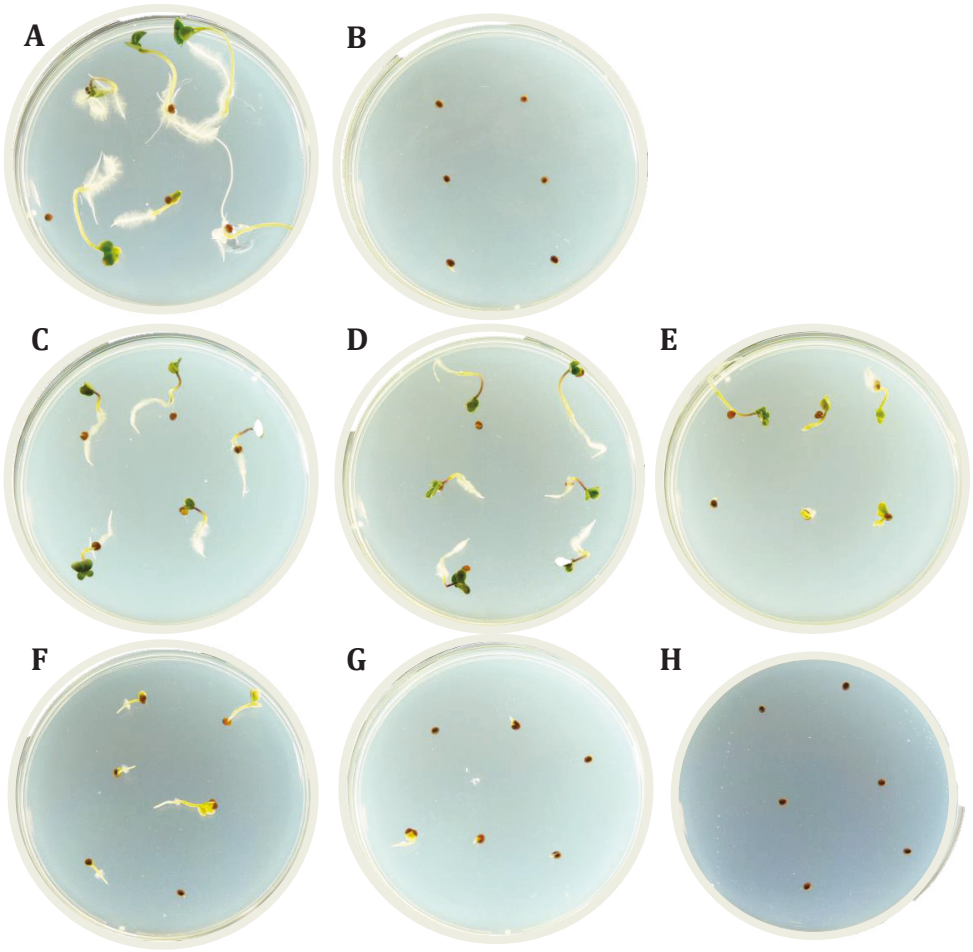


Figure 3. 1: Germination of *B. oleracea* seeds on SA/JA.

Germination of seeds on MS plates are not affected when grown on (**A**) 0 mM SA/JA (control), however there is no germination when grown in the presence of (**B**) 0.5 mM SA/JA. When MS plates are infused with SA there is growth at (**C**) 0.25 mM and (**D**) 0.5 mM, however there is reduced seedling growth at (**E**) 1 mM. When grown in the prescence of JA, there is reduced growth at (**F**) 0.25 mM and no germination at (**G**) 0.5 mM and (**H**) 1 mM.

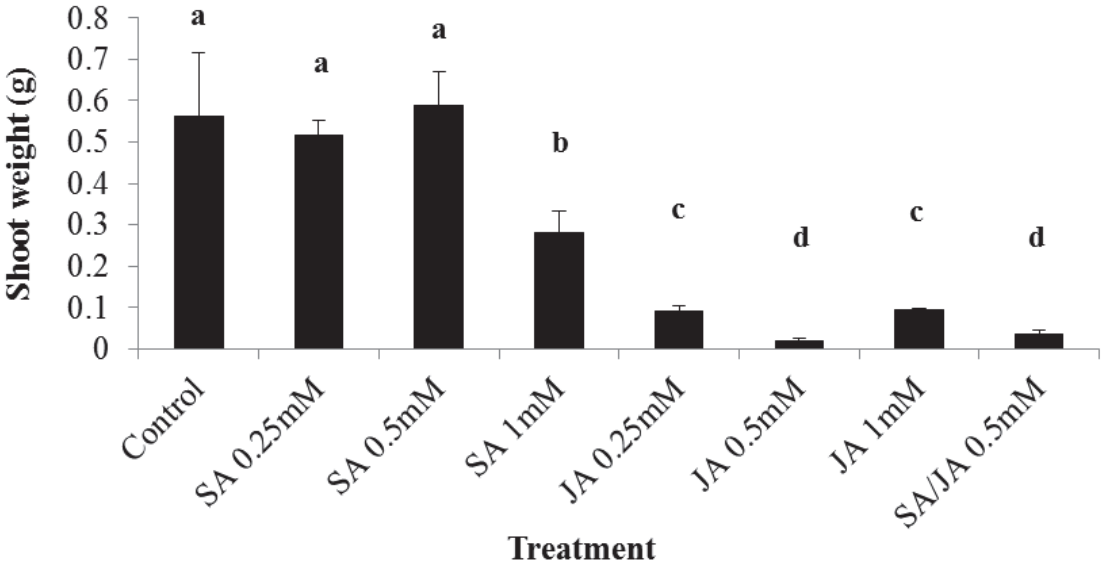


Figure 3. 2: Shoot weight of *B. oleracea* grown on hormone infused agar.

Brassica oleracea grown on SA infused MS agar at 0.25 and 0.5 mM show no significant difference in shoot weight compared to the control, while those grown on 1 mM SA have reduced shoot weight with a reduction of 50%. When grown on JA at 0.25, 0.5 and 1 mM or a combination of SA and JA at 0.5 mM, there are few or no seeds germinating, resulting in a significant reduction in shoot weight. Shoot weight was measured by minusing the combined shoot and seed weight from the average weight of 20 seeds. Letters indicate significant difference (n=18) (p=0.05).

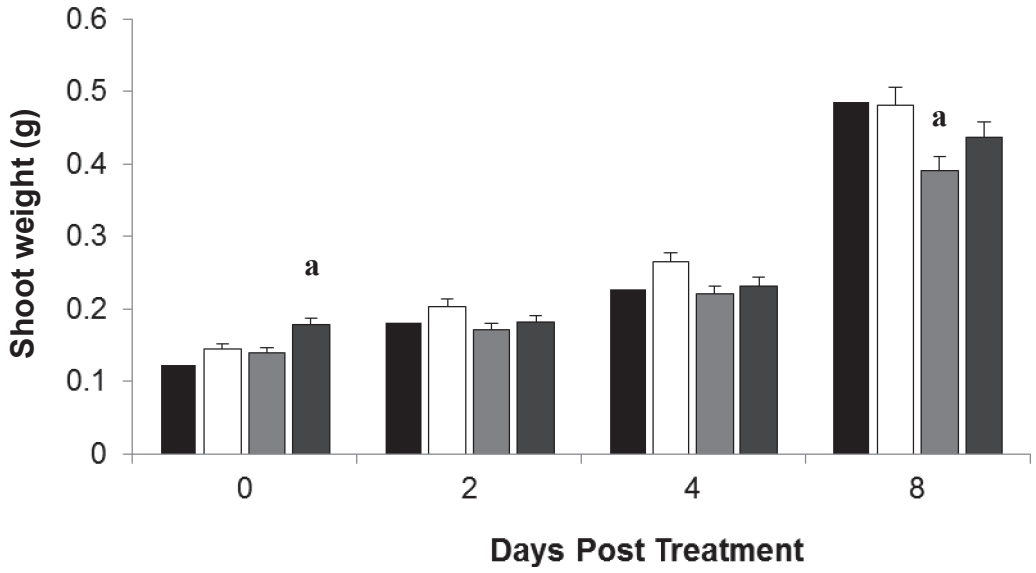


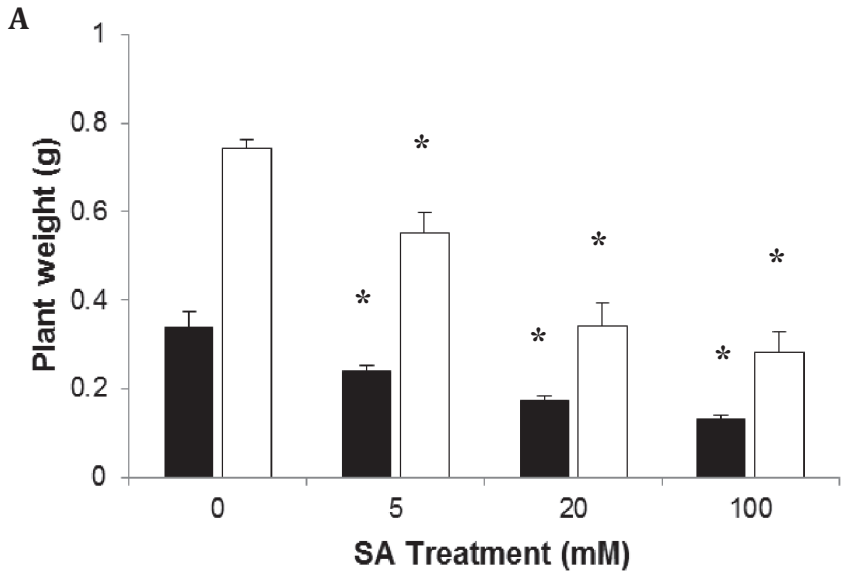
Figure 3. 3: Effect of low concentrations of SA on *B. oleracea* weight.

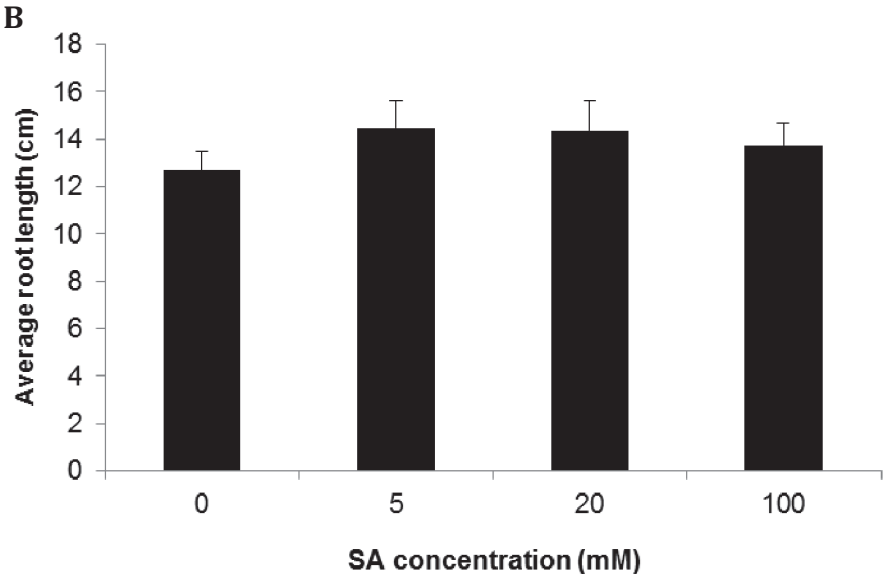
There is no signifant effect of SA on the growth of 2 week old *B. oleracea* plants at 2 and 4 days post-treatment. There is a significant reduction in *B. oleracea* weight 8 day's post-treatment with 0.1 mM SA. Letters indicate a significant difference to respective controls (black = control, white = 0.05 mM, grey = 0.1 mM, dark grey = 0.25 mM) (n=20) (p=0.005).

Figure 3. 4: Effect of high concentrations of SA on *B. oleracea* weight.

(**A**) A reduction in 3 week old *B. oleracea* shoot and root fresh weights were observed 3 days post-treatment with SA at levels above 5 mM and reduced in a dose dependent manner.

(**B**) There was no significant reduction in root length asoociated with SA concentration increase compared to the control. Error bars represent standard error of the mean, symbols represent significant difference to respective controls (n = 20) (p = 0.05) (Black bars: Root; White bars: Shoot).


A
Plant weight (g)
1
0.8
0.6
0.4
0.2
0
*
*
*
*
*
0
5
20
100
SA Treatment (mM)


B
Average root length (cm)
18
16
14
12
10
8
6
4
2
0
0
5
20
100
SA concentration (mM)

3.4.3 Effect of root-supplied SA on morphology of leaves and leaf chlorophyll content

There was no observable change in leaf morphology 72 h post-treatment of roots with SA at concentrations below 5 mM, whilst above 20 mM there was a change. Veinal and interveinal yellowing (Figure 3. 5) occurred in plants associated with SA treatment and there was pronounced shriveling and curling of cotyledons and leaves (Figure 3. 6). There was a noticeable reduction in root density as the concentration of SA gradually increased (Figure 3. 7).

To further investigate the effect of translocated SA on the leaves, plants were examined for chlorophyll loss at 24 and 72 h post-treatment. At 24 h post-treatment there was a significant ($P<0.05$) increase in chlorophyll content in plants treated with 100 mM. At 72 h post-treatment plants that were treated with SA at concentrations of 20 mM and 100 mM showed a significant ($P<0.05$) reduction in chlorophyll content compared with the controls (Table 3. 1).

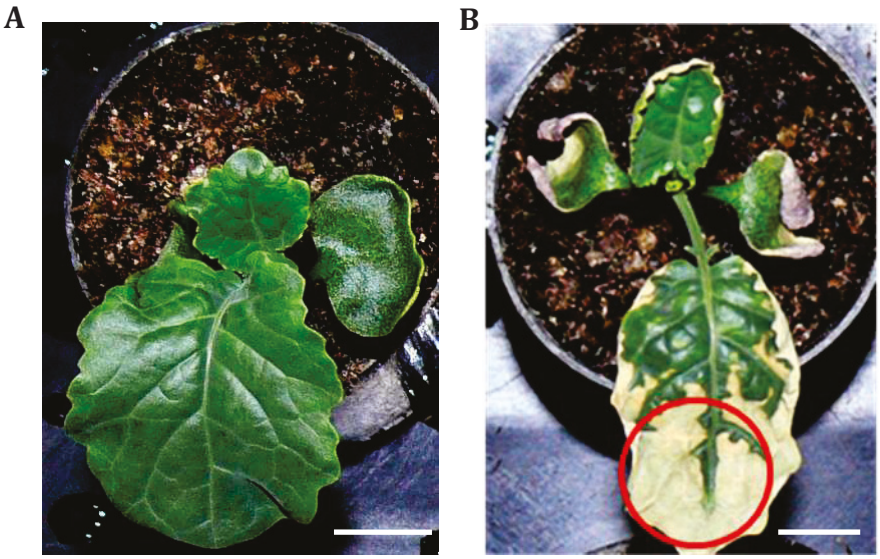


Figure 3. 5: Chlorosis due to SA on *B. oleracea* leaves

(**A**) A healthy 3 week old *B. oleracea* leaf in comparison to one showing signs of (**B**) chlorosis on the cotyledons and first pair of true leaves (red circle) 72 h post-treatment with 5 mM SA (Scale = 1cm).

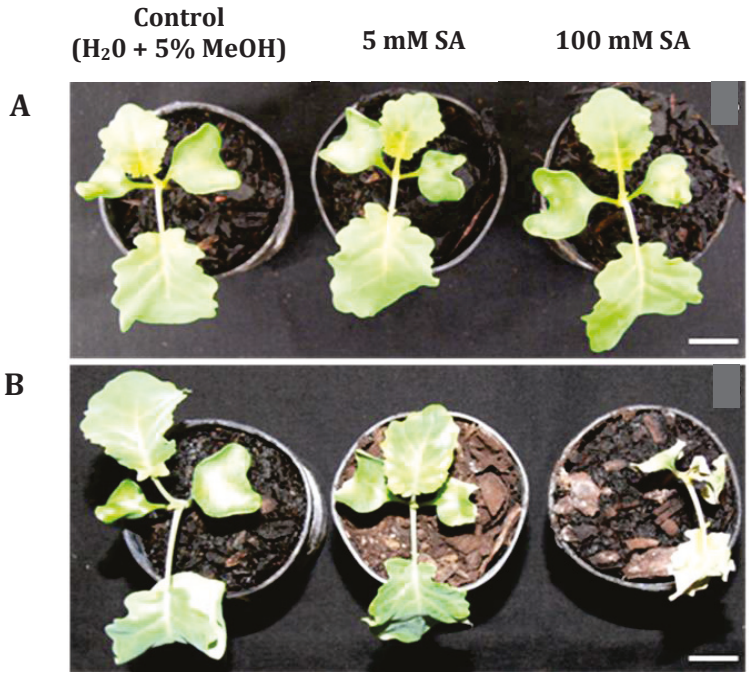


Figure 3. 6: Effect of SA on *B. oleracea* shoots

The effect of SA on 3 week old *B. oleracea* shoots at (**A**) 0 h and (**B**) 72 h post-treatment with concentrations of; 0 mM (Control: H_2O + 5% MeOH), 5 mM and 100 mM, reveal that 5 mM causes minimal damage, while 100 mM compromises plant health (Scale bars = 2.5cm).

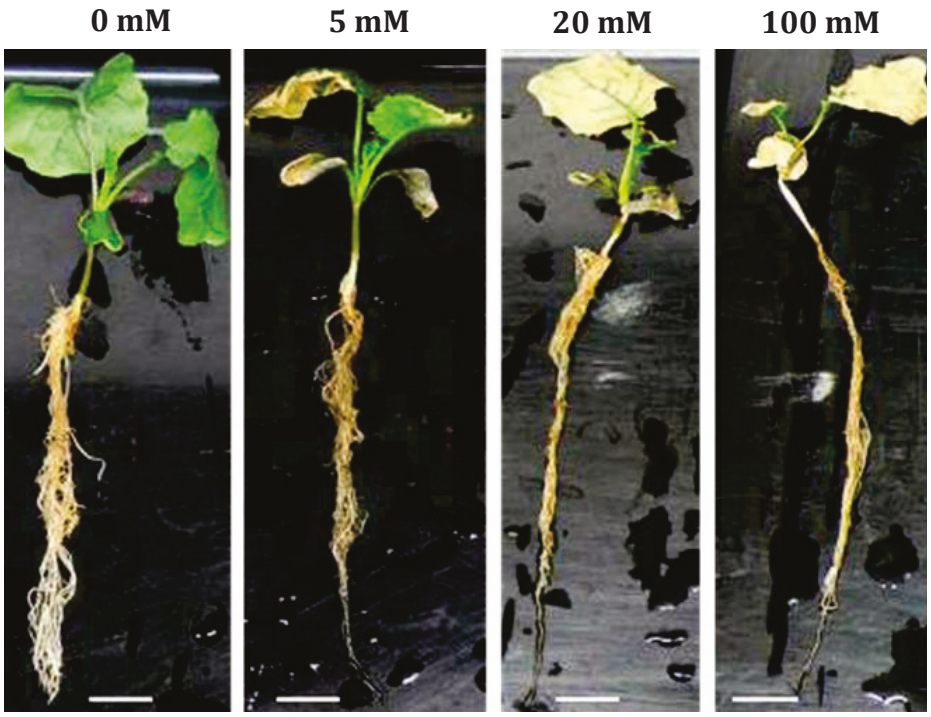


Figure 3. 7: Effect of SA on root and shoot morphology

Root and shoot morphology following SA treatment reveals SA at concentrations of 5 mM, 20 mM and 100 mM are all detrimental to the health of *B. olerace* with noted wilting, chlorosis and a decrease in root volume when compared to the control (0 mM) (Scale bars = 2.5cm).

Table 3. 1: Average relative chlorophyll content in 3 week old *B. oleracea* leaves following treatment with SA at 0, 24 and 72 hour's post-treatment, reveals a reduction at concentrations above 20 mM. Values in brackets represent the difference to the control (0 mM), symbol indicates a significant difference to the control (n = 20) (p = 0.05).

Treatment	**Hours Post-Treatment**		
mM	**0**	**24**	**72**
0	38.58	41.15	44.45
5	38.55 (-0.03)	41.65 (+0.5)	44.00 (-0.45)
20	39.45 (+0.87)	43.55 (+2.4)	38.85 (-5.6)*
100	39.05 (+0.47)	44.85 (+3.7)*	32.25 (-12.2)*

3.4.4 Development of a treatment regime to analyse salicylic acid through High performance liquid chromatography

Two different treatment regimes were conducted to determine the effectiveness by HPLC analysis, plants were tested for their SA accumulation at 0, 24, 48, 72 and 216 h post-treatment (Figure 3. 8).

A set of 6 week old *B. oleracea* plants were treated with 1 mM SA at 0 h and then analysed for SA accumulation at the mentioned time-points, this is regarded as a single dip. A second set of 6 week old *B. oleracea* plants were treated with 1 mM SA at 0, 24 and 48 h and analysed for SA accumulation at mentioned time-points, this is regarded as a triple dip (Figure 3. 9).

Single dip plants had a significantly higher intracellular SA level than the control at 24, 48 and 72 h, with a maximum of 0.71 mM at 72 h. As expected triple dip plants accumulated significantly more SA at 24, 48 and 72 h post-treatment than both the control and single dip, with a maximum of 1.4 mM recorded at 72 h. At 216 h there was no significant difference in the accumulation of SA, between single and triple dip, they were both however still significantly higher than the control.

Although the triple-dip method was seen to allow for a higher intracellular level of SA, there were signs of stress associated with phytotoxicty and stress and for this reason the single dip method was prefered.

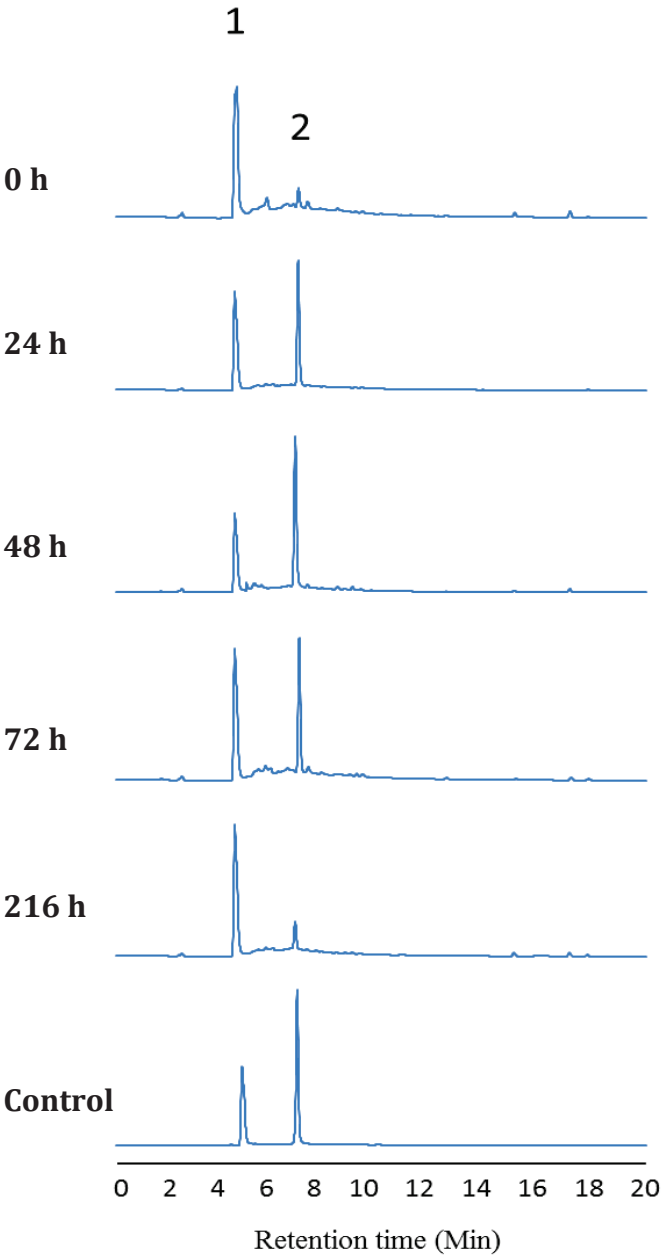


Figure 3. 8: HPLC chromatogram of SA separation

Chromatograms of an internal standard (caffeic acid (CA)) (peak 1) and the phytohormone SA (peak 2) separation through a C_{18} 250 × 4.6mm 5µm particle size column of *B. oleracea* leaf material following root treatment with 1 mM SA.

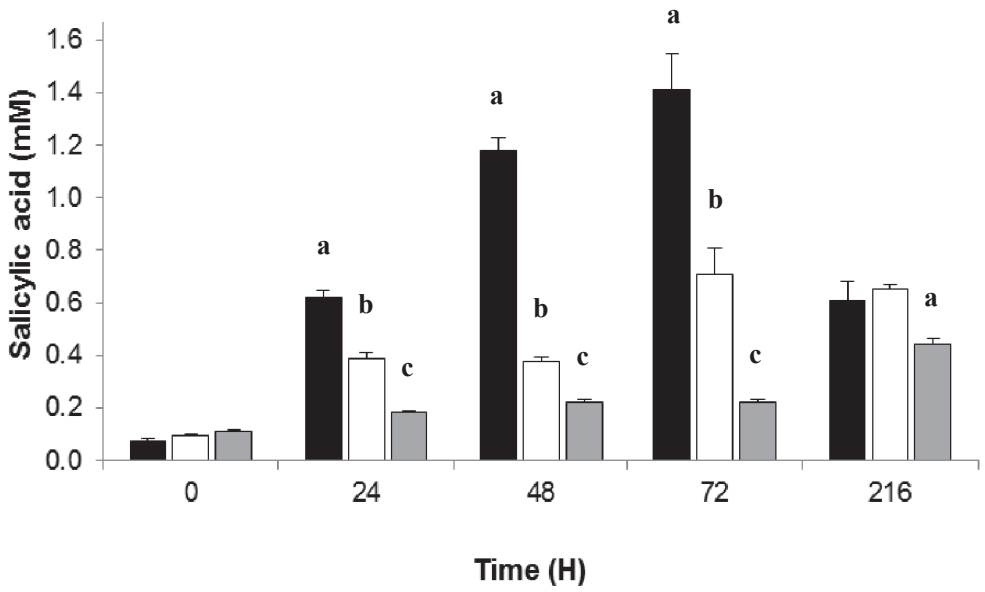


Figure 3. 9: Accumulation of SA in *B. oleracea*

Salicylic acid accumulation in 6 week old *B. oleracea* shoots following a triple dip (black bars) or single dip (white bars) root drench. There is a significant increase in intracellular SA in both the single and triple dip method at 24, 48, 72 and even as long as 216 h post treatment compared to the control (grey bars). Although the triple dip allowed for increased intracellular SA, this method was detrimental to plant health. Letters above indicate significant difference ($p = 0.05$) (n=8).

3.4.4 Quantitation of salicylic acid in leaves following uptake by the roots

Two week old *B. oleracea* plants grown under controlled conditions were analysed for their accumulation of SA following exogenous treatment at 24 and 72 h post treatment, the values calculated represent the percent increase compared to the control (0 mM). Reverse phase-high-performance liquid chromatography (RP-HPLC) analysis of SA pre-treated *B. oleracea* plants at concentrations of 0.05, 0.1 and 0.25 mM revealed that only 0.25 mM showed an increase compared to the control at both 24 and 72 h. When SA was increased to 0.5, 1 and 2.5 mM a significant difference for all concentrations 24 h post-treatment compared to the control. However, 72 h post-treatment only 2.5 mM showed a significant difference to the control. Increasing the concentration of exogenous SA to 5, 10 and 25 mM significantly increases the concentration of SA 24 and 72 h post-treatment compared to the control (Table 3. 2).

3.4.5 Effect of SA root treatments on the expression of *PR-1* and *PR-2* in leaves.

PR-1 and PR-2 induction was assessed using PCR in leaves of 2 week old *B. oleracea* seedlings following treatment of roots with SA. *PR-1* expression increased in a concentration dependent manner at both 24 and 72 h post treatment in the leaves (Figure 3. 10).

PR-2 was expressed in a concentration dependent manner at both 24 and 72 h after treatment with SA but at a much lower level compared with *PR-1*. For this reason RT-qPCR was only performed on *PR-1*.

Table 3. 2: Values represent the percent increase in total SA compared to the control following RP-HPLC analysis of 2 and 3† week old *B. oleracea* shoots at 24 and 72 h post-treatment. Symbols represents significant difference to the respective control, each value represents the mean of four replicates (n = 8) (p = 0.05).

SA Treatment **mM**	**Percent Increase in SA**	
	24 h	**72 h**
0	0	0
0.05 †	1.04	1.05
0.1 †	1.01	1.02
0.25 †	1.13 *	1.39 *
0.5	3.52 *	1.15
1	2.37 *	1.15
2.5	3.00 *	7.35 *
5	4.42*	8.75 *
10	17.25*	31.0 *
25	24 *	102.5*

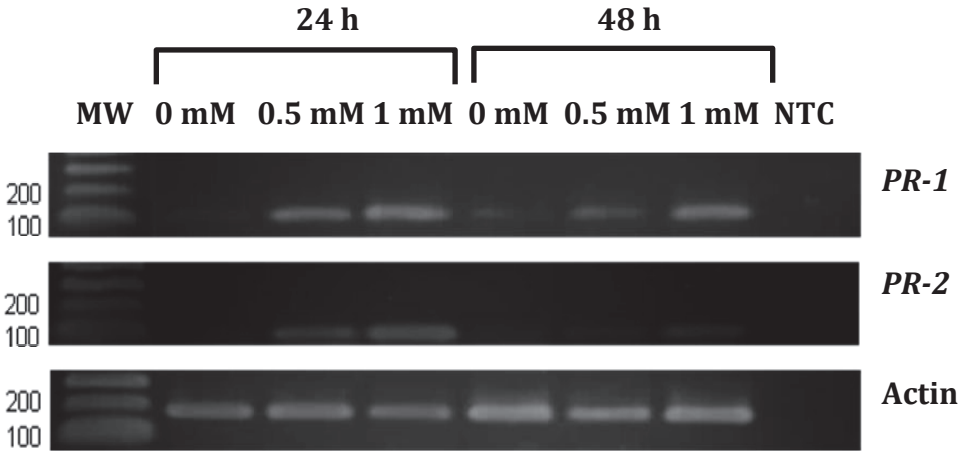


Figure 3. 10: Gene expression in 2 week old *B. oleracea* seedlings

post-treatment with SA at 0 mM, 0.5 mM and 1 mM at 24 and 72 h. *PR-1* and *PR-2* expression in 2 week old seedlings was found to be high at both 24 and 72 h time-points after treatment with SA at 0.5 mM and 1 mM. Actin was probed for as an expression control (MW = molecular marker, Bioline Hyperladder IV: NTC = No template control) (*PR-1* and Actin = 30 cylces, PR-2 = 38 cycles).

Real time-qualitative PCR (RT-qPCR) was used to quantify the expression of *PR-1* from leaves of both 2 (Table 3. 3) and 6 (Table 3. 4) week old seedlings at 24 and 72 h after treatment with SA. This analysis, revealed an elevated expression of *PR-1* in all treatment groups. Figure 3. 11 A & B show RT-qPCR cycling for 2 week old *B. oleracea* 24 & 72 h post-treatment.

Two week old seedlings treated with 0.5 mM SA had an estimated 6.1 and 4.0 fold increase in expression at 24 and 72 h post-treatment respectively. Whilst *PR-1* expression in 2 week old seedlings treated with 1 mM SA was increased 910.1 and 8.4 fold at 24 and 72 h post-treatment respectively. Six week old plants treated with 0.5 mM SA had a 46.9 and 1.2 fold increase in *PR-1* expression at 24 and 72 h post-treatment respectively (Table 3. 4). Those treated with 1 mM SA, had an increased expression of PR1 of 86.2 and 6.1 fold at 24 and 72 h post-treatment respectively. Figure 3. 12 A & B show RT-qPCR cycling for 6 week old *B. oleracea* 24 & 72 h post-treatment.

Table 3. 3: CT Values and estimated fold increase associated with 2 week old seedlings for *PR-1* and the reference gene Actin analysed 24 and 72 h post-treatment with 0 mM, 0.5 mM and 1 mM, reveals an increase in fold change associated with treatment of SA (N = 9).

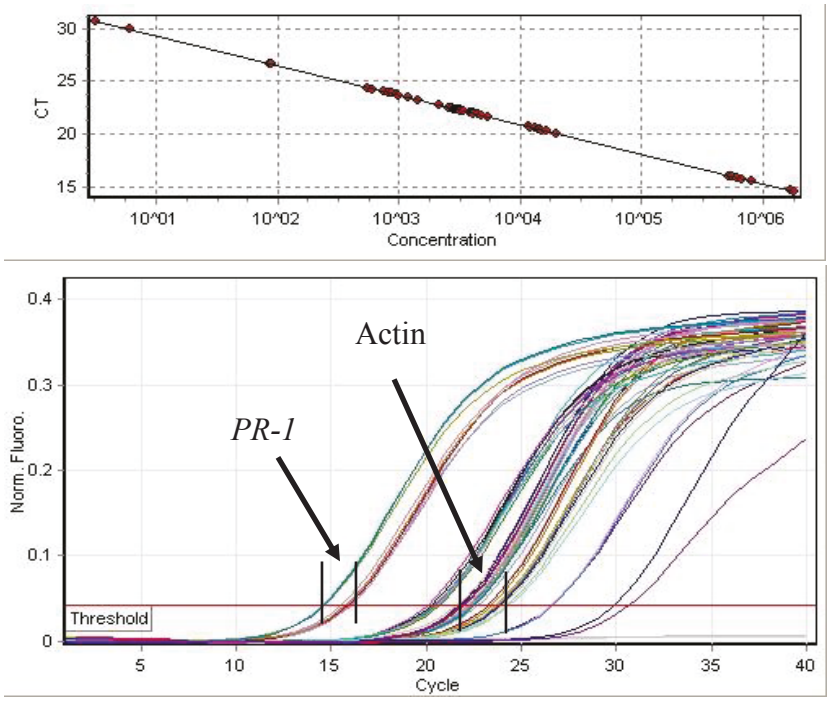
Treatment mM	**Time (h) after SA treatment**				**Fold Change**	
	24		**72**			
	Actin	**PR1**	**Actin**	**PR1**	**24 hour**	**72 hour**
0	22.04	24.71	21.6	23.31	1.0	1.0
0.5	21.59	21.64	21.88	21.58	6.1	4.0
1	22.55	15.39	22.6	21.23	910.1	8.4

Figure 3. 11: RT-qPCR cycling of 2 week old *B. oleracea*

Standard curve from *Ct* values of Actin and *PR-1* in 2 week old *B. oleracea* plants and cycling curve generated from RT-qPCR at (**A**) 24 and (**B**) 72 h post-treatment with salicylic acid.

RT-qPCR cycling confirms the expression of *PR-1* is elevated 24 h post treatment with 1 mM SA as *Ct* threshold values occur at around 15 cylces while actin threshold values occer between 23-24 cycles. While at 72 h post treatment with SA *Ct* threshold values for *PR-1* and actin occur between 22-24 cycles.

A


CT
Concentration
10^01
10^02
10^03
10^04
10^05
10^06
Norm. Fluoro.
Actin
PR-1
Threshold
Cycle

B

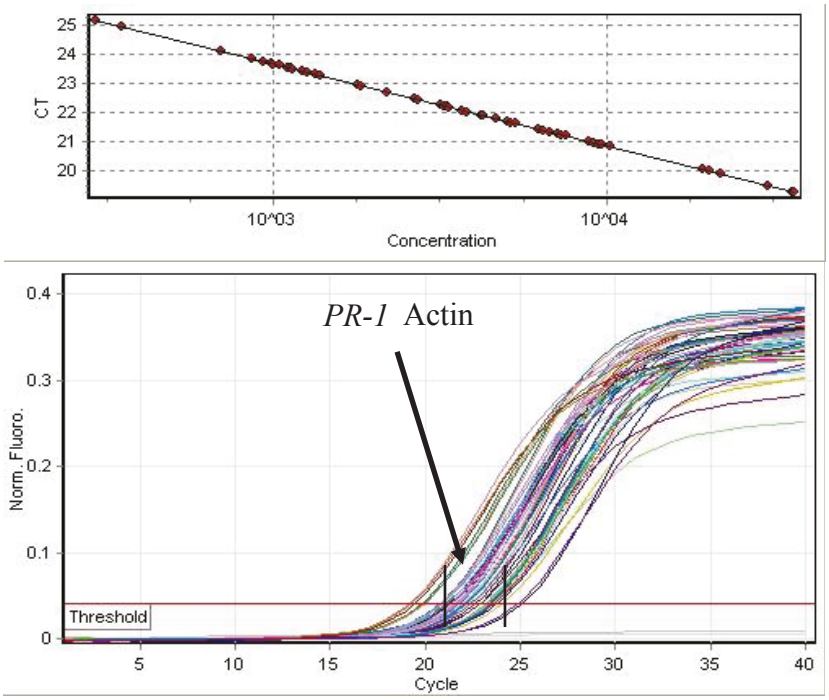

CT
Concentration
10^03
10^04
Norm. Fluoro.
PR-1 Actin
Threshold
Cycle

Table 3. 4: CT Values and estimated fold increase associated with 6 week old seedlings for *PR-1* and the reference gene Actin, analysed 24 and 72 h post-treatment with 0 mM, 0.5 mM and 1 mM, reveals an increase in fold change associated with treatment of SA (N = 9).

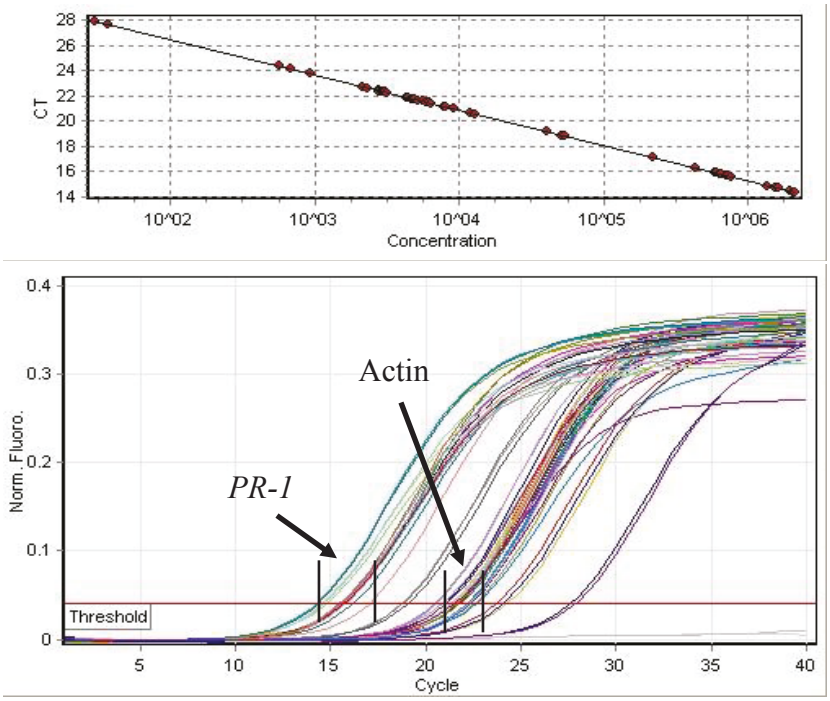
Treatment	**Time (h) after treatment**				**Fold Change**	
mM	**24**		**72**			
	Actin	***PR1***	**Actin**	***PR1***	**24 hour**	**72 hour**
0	21.94	21.82	22.15	22.01	1.0	1.0
0.5	21.32	15.65	21.66	21.06	46.8	1.1
1	21.8	15.25	21.62	18.64	86.2	6.1

Figure 3. 12: RT-qPCR cycling of 6 week old *B. oleracea*

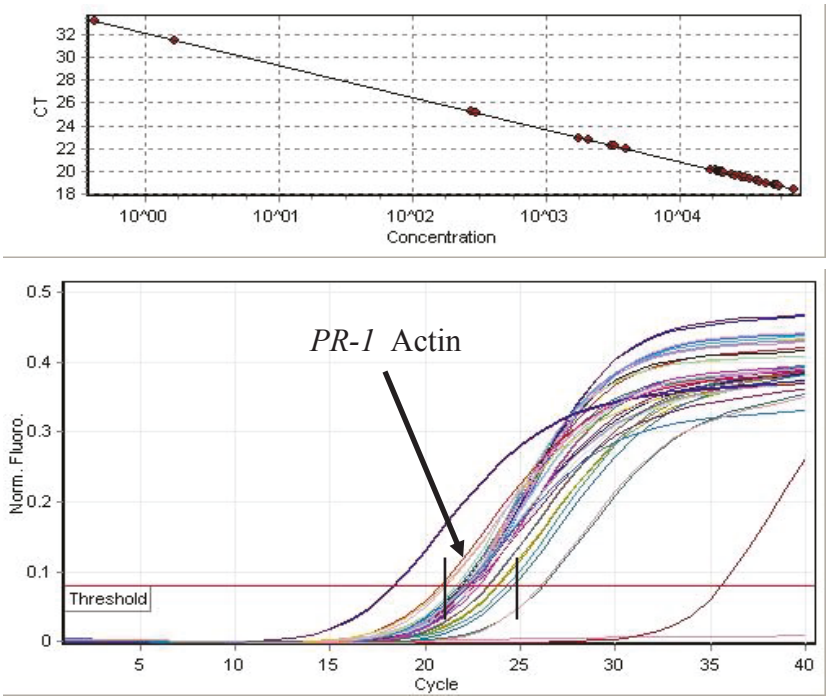
Standard curve from *Ct* values of Actin and *PR-1* in 2 week old *B. oleracea* plants and cycling curve generated from RT-qPCR at (**A**) 24 and (**B**) 72 h post-treatment with salicylic acid.

RT-qPCR cycling confirms the expression of *PR-1* is elevated 24 h post treatment with 0.5 & 1 mM SA as *Ct* threshold values occur between 15-16 cylces while actin threshold values occer between 21-23 cycles. While at 72 h post treatment with SA *Ct* threshold values for *PR-1* and actin occur between 21-24 cycles.

A


CT
Concentration
10^02
10^03
10^04
10^05
10^06
Norm. Fluoro.
Actin
PR-1
Threshold
Cycle

B


CT
Concentration
10^00
10^01
10^02
10^03
10^04
Norm. Fluoro.
PR-1 Actin
Threshold
Cycle

3.5 Discussion

Before embarking on a study of the interaction between plant, pathogen and SA it was important to determine the interaction between plant and the hormone. In developing this system it was critical to first examine and quantify any effect that SA may have had on the host. Experiments were carried out under controlled growth conditions in which, following application of SA to the roots, the concentration of SA in leaves was quantified to demonstrate both the uptake and distribution of the molecule throughout the plant. There are very few studies that have examined uptake of SA by roots, most have examined application to leaves and an analysis of the role of SA in resistance to leaf pathogens on model plants (Vlot *et al*., 2009) but as in the present study those of De Meyer *et al*., (1999) with bean and Poór *et al*., (2011) with tomato, for example, showed that when applied to roots, SA remains localised to the roots but is also transported systemically. In experiments described here when roots were exposed to SA at concentrations above 0.25 mM the low basal levels of SA in leaves were increased significantly. When applied to roots at concentrations above 5 mM SA caused detrimental effects including reduced growth (lower plant weight), impaired photosynthetic capacity (loss of chlorophyll) and leaf yellowing and necrosis. In subsequent glasshouse experiments and, in a field trial, detailed in Chapter 4, I have also shown that when used at concentrations above 5 mM there was an age-related and negative impact on seedling growth. As I have found with *B. oleracea*, when used at physiologically high concentrations SA has also been found by others to have inhibitory effects on photosynthesis and overall growth (Pancheva *et al*., 1996; Poór *et al*., 2011). For example, the dose-dependency of the effect of exogenous application of SA was

also shown for *Brassica juncea* (mustard) (Fariduddin *et al*., 2003) for which levels of SA applied at 0.01 mM had no effect on plant dry weight, however, when used at concentrations of 0.1 and 1 mM a reduction in dry weight and a reduction in photosynthetic capacity was observed. It must be noted that depending on the dosage and plant species being examined that the opposite i.e. increased photosynthetic capacity and growth, following SA application has also been reported (Rivas-San Vicente and Plasencia, 2011).

I examined the detectability of SA over time after SA root drenches and confirmed the movement of SA and distribution of it to leaves and found, perhaps not surprisingly, that exposure to SA above a concentration of 1 mM was detectable in the leaves. This result also showed that SA remains detectable in soluble form within the plant for an extended period and that it is unlikely to have been metabolised to a great degree into other forms such as SA *O*-β-glucoside, methyl salicylate or salicyloyl glucose ester (Vlot *et al*., 2009, Ogawa *et al*., 2010) although I did not specifically measure the amounts of any of these derivatives. The response of *B. oleracea* plants to SA is, therefore, primarily dependent on concentration applied, plant age and length of time after exposure. In chapter 4, I have thus attempted to take all these parameters into consideration in our experiments on the interaction of *B. oleracea* with *P. brassicae* and the impact of exogenously supplied SA.

The observed induction of *PR-1* is consistent with previous reports that the SA biosynthesis pathway is required for defence gene activation against a range of pathogens in a variety of hosts including strawberry, *Arabidopsis*, tobacco and

potato (Yalpani *et al*., 1991; Halim *et al*., 2007; Yoshioka *et al*., 2012; Grellet-Bournonville *et al*., 2012). Although *PR-1* expression was not assessed in root tissue, there is evidence to suggest PR expression does not occur in root tissue or that it occurs at very low levels. Edgar *et al*., (2006) found that treating *Arabidopsis* leaves with salicylic acid caused *PR-1* expression in leaf material but not root material.

The results presented in this chapter show that in *B. oleracea* plants treated with SA that SA is systemically transported from the roots to shoots and that when used at relatively low concentrations SA is not detrimental to plant growth, accumulates in the shoots and up-regulates defence-related genes such as *PR1*. This new understanding of the uptake, distribution and activity of SA may be useful in designing new ways to approach difficult to control horticultural diseases such as clubroot. One recent and novel example of this approach is in the use of an SA-inducible promoter derived from the figwort mosaic virus that responds to both increased endogenous SA and to exogenous application (Kumar *et al*., 2012). The SA-inducible promoter has enhanced root activity and could be used to drive the enhanced expression of defence genes.

Chapter 4: Salicylic acid suppression of disease caused by *Plasmodiophora brassicae* in broccoli (*Brassica oleracea* var. *italica*) under glasshouse and field trials

4.1 Abstract

The obligate biotroph *Plasmodiophora brassicae* is an economically important plant pathogen, responsible for millions of dollars of losses in revenue through destruction of a number of Brassicaceae crops, including broccoli. An Australian isolate of *P. brassicae* common to Victoria was tested for its virulence to broccoli following applications of salicylic acid, a key phytohormone in defence. The interaction between plant and pathogen was tested in glasshouse and field trials, glasshouse grown plants had significantly reduced disease symptoms associated with the pathogen following SA treatment above 5 mM. Field grown plants had significantly reduced galling following treatment with 2.5 mM SA. A combination of SA and jasmonic acid, a known antagonist of SA, was shown to provide a level of resistance with a reduction in gall scores compared to the control. This research provides key evidence of the importance of SA in the plant-pathogen interaction associated with *P. brassicae*.

4.2 Introduction

The obligate biotroph, *Plasmodiophora brassicae*, is an economically important plant-pathogen that includes as primary hosts a number of crop species in the Brassicaceae family for example, broccoli, cabbage and Brussels sprouts (Cao *et*

al., 2008; Osaki *et al*., 2008). Although there have been no recent surveys of the impact of the disease on crops in Australia, significant crop loss on individual farms is not infrequent and globally, the disease can lead to losses of over 10% (Dixon, 2009a).

Additionally, loss in market value occurs due to disease affecting the growth and development of host plants. The major characteristic of infection by *P. brassicae* is the formation of large swellings, 'galls', on roots, caused by both the production of spore masses in the cortex of infected tissues and unregulated cell division and hypertrophy (Ingram & Tommerup, 1972; Dixon, 2009b). Galling creates a sink that redirects sugars to the pathogen and limits the amount available for shoot development (Ludwig-Müller & Schuller, 2008; Siemens *et al*., 2011). Development of the disease in a susceptible host and its molecular basis has recently been examined using an *Arabidopsis* model (Agarwal *et al*., 2009; Agarwal *et al*., 2011). In the *Arabidopsis* system, following infection there was suppression of the activity of those genes normally found to be associated with resistance but treatment of plants with salicylic acid (SA) suppressed disease.

Current control measures are based on integrated application of cultural practices, lime, nutrients (calcium and boron) and a limited number of fungicides (Donald and Porter, 2009). Fluazinam, a fungicide which actively targets the zoospores of *P. brassicae*, has been shown to limit their ability to infect (Donald *et al*., 2001) and calcium cyanamide has been used for a number of years with some success and has some direct fungicidal activity against the pathogen (Donald *et al*., 2004). Host resistance to clubroot is much sought after by *Brassica* growers as a relatively cheap and reliable control measure to include

in an integrated control strategy. For example, resistant cabbage (cv. Maxfield) and cauliflower (cv. Highfield) were released commercially for the first time in Australia in 2007 and work is ongoing to more closely align the commercial characteristics of these varieties with market needs.

SA is now recognised as a central component of defence in plants against a number of biotrophic pathogens (Catinot *et al*., 2008, Vlot *et al*., 2009). Surprisingly, although SA is known to be involved in defence very little research has been undertaken on the effect of exogenous application of SA on plant-pathogen interactions and even less on application to control root diseases. As recently as 2008, a published review (Ludwig-Müller and Schuller, 2008) proposed a model for the involvement of hormones in the interaction of *P. brassicae* with *Arabidopsis*. However, the reviewers were unable to state if SA had a role in this interaction due to the limited experimental evidence available.

In contrast, there are a number of studies that have examined SA analogues including benzothiadiazole (BTH) and 2, 6-dichloroisonicotinic acid (INA) and their impact on various diseases. The application of BTH (commercial names: ASM, Bion® and Actigard®), has been found to reduce the severity of diseases caused by a variety of pathogens. For example, the severity of black root rot in cotton caused by the hemibiotroph *Thielaviopsis basicola* was reduced by applications of BTH at concentrations of 25 and 50 µg/mL (Mondal *et al*., 2005) and Ali *et al*., (2000) found that the infection of roots of *Pinus radiata* by *P. cinnamomi* was decreased in a dose-dependent manner by approximately 20% and 25% at application rates of 1 µg/L and 2.5 µg/L respectively. Powdery

mildew caused by *Sphaerotheca macularis* devastates strawberry crops but applications of BTH that increased soluble and cell wall–bound phenolics in strawberry leaves provided increased resistance (Hukkanen *et al*., 2007).

INA is also effective in promoting increased resistance to a number of pathogens such as *Alternaria macrospora*, the cause of alternaria leaf spot in cotton where treatments of between 5 and 25 μg/mL showed a reduction in disease development (Colson-Hanks & Deverall, 2000). Similarly, a study by Ward *et al*., (1991) revealed that the use of INA in tobacco leaves reduced the lesion size associated with infection by TMV. Despite these promising outcomes widespread use of BTH and INA as systemic inducers of defence has been limited due to variation in host-pathogen responses, phytotoxicity, cost and also bio-safety regulations of the potential user countries.

While SA can stimulate resistance to biotrophic pathogens, there is little evidence to indicate a role in defence against necrotrophic pathogens. Necrotrophic pathogens live on dead or dying tissue and an IR or SAR response induced by SA would ultimately aid necrotrophic pathogens by providing dead cells and tissues via the hypersensitive response (Glazebrook, 2005). A number of studies have shown that for necrotrophic pathogens it is jasmonic acid (JA) that plays the crucial role in defence activation. For example, Fugate *et al*., (2012) found treating sugarbeet roots with JA decreased rotted tissue associated with the necrotrophic pathogens, *Botrytis cinerea*, *Penicillium claviforme* and *Phoma betae*. The authors concluded that JA application effectively reduced the

progression of disease and did not have a direct effect on the pathogen, as incidence of disease did not change.

It is widely held that the accumulation of SA and JA cannot occur together. Both these phytohormones are thought to act antagonistically, that is, the accumulation of SA will prevent the accumulation of JA and vice versa (Kunkel and Brooks, 2002). Recent work by El Oirdi *et al*., (2011) has shown that *Botrytis cinerea* manipulates both pathways to enable infection and host colonisation. Thus, in the interaction with tomato, *B. cinerea* first induced the SA pathway which then antagonised the JA signalling pathway to effectively suppress JA-induced defences, leading to susceptibility. Birkenbihl *et al*., (2012) have since found that the antagonism between these two pathways in *Arabidopsis* infected by *B. cinerea* is regulated by the WRKY33 transcription factor. Clearly the role of SA in plant-pathogen interactions cannot be treated as separate from that of JA.

There are currently no chemicals in use in Australia which actively initiate a defence response in host plants to provide resistance against *P. brassicae.* I investigated SA and its potential to stimulate defence in *Brassica oleracea* var. *italica* (Broccoli) against *P. brassicae* and found that SA reduced galling associated with infection by this pathogen. In combination with JA, SA also significantly reduced disease in glasshouse grown plants. I propose that with further development of treatment regimes, SA could be used as an activator of defence in *B. oleracea* prior to farmers planting seedlings in the field and that,

additionally, manipulation of SA-regulated pathways may provide new avenues for disease control.

4.3 Materials and Methods

4.3.1 Selection of suitable soil for disease analysis in glasshouse trials

Preliminary pot trials were performed under controlled conditions (see 3.3.2) to determine the appropriate soil type to use in the analysis of disease symptoms prior to commencement of glasshouse trials. Plants were either grown in a commercial soil mix (Debco) or in a mixture of sand and peat moss with the addition of Osmocote®, as these soils have been regularly used in growth of plants. Initially, *B. oleracea* seeds were sown and plants grown in 48 cell trays in debco potting mix for a period of two weeks before being transferred to pots containing either commercial potting mix or a sand and peat moss (1:3) mixture.

A first trial was performed to determine the effect of soil type on plant growth, plants were grown in pots containing soil for a period of 4 weeks after which time plant weight was assessed. For analysis of disease caused by *P. brassicae* plants were transferred to pots with desired soils and left for 2 days and then inoculated with 250 µl of *P. brassicae* spores (1×10^7), by making a small hole in soil surface at the base of the stem and pipetting spore suspension into hole, and left to grow for a period of six weeks with watering every second day, before analyzing galling.

4.3.2 Plant growth and maintenance

For both glasshouse and field trials, broccoli (cv. Marathon) plants were grown under the same conditions until transferred from trays in which plants were subjected to SA treatment to either pots (glass house trials) or field plots (field trials). Glasshouse (Figure 4. 1) and field trials were performed at the Department of Primary Industries, Knoxfield, Victoria, in 2010, 2011 and 2012 during the months of January – March, and utilised two- and six-week-old seedlings grown from seed obtained from the Department of Primary Industries.

Seeds were sown as described previously for the controlled environment experiments but in pasteurised vegetable seed raising mix (Biogro Pty Ltd, Bayswater North, Victoria), which has the same texture, density and nutrients as the Debco mix. Plants were grown within a glasshouse (22°C ± 3°C) under ambient light and watered once daily for one minute using overhead irrigation. Sowing of seeds was staggered to allow for simultaneous treatment of plants with SA at two and six weeks of age.

For glass house experiments, plants were removed from the cells at either 24 or 72 h following SA treatment and transferred to pots (10cm x 8cm) with two plants of the same age per pot, plants continued to grow under the same conditions, 36 plants were used for each treatment group. For field trials, plants were removed from cell trays at either 24 or 72 h and transferred to a sand bed containing the pathogen, which was separated out into 6 replicate grids containing 8 individual plots (Figure 4. 2 & Figure 4. 3).

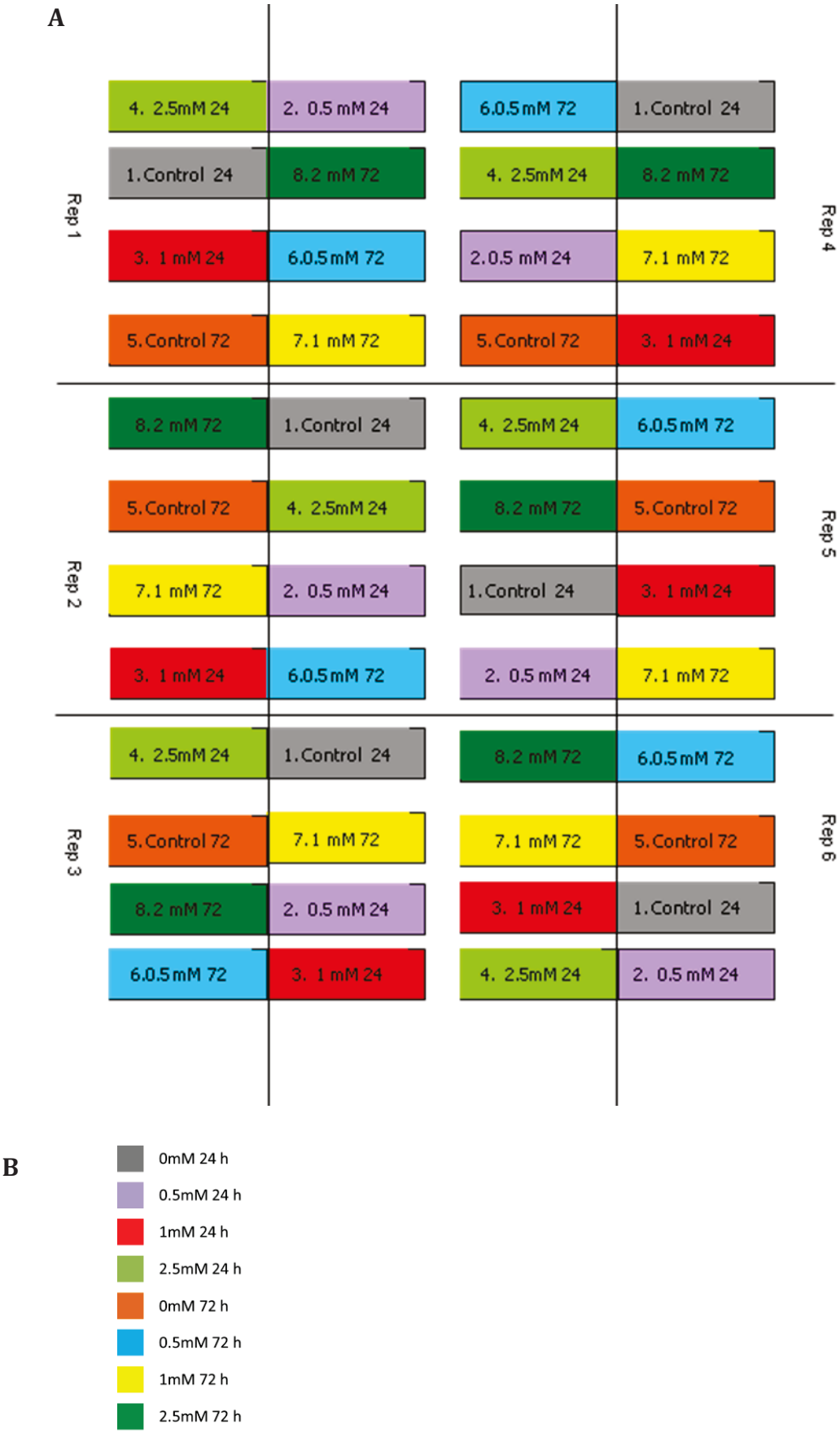
Five plants both 2 and 6 week old from a single treatment group and time-point were planted into a randomly selected plot in all replicate grids. The trial was a completely randomized block design, and a total of 30 plants per age, per treatment group were used. Following planting, snail-bait and fertiliser (Rustica plus® 12-5-14; applied at 400kg/ha) were cast over the plots and netting was placed over the top to prevent animal disturbance. To reduce chemical interaction in the field trials, pests were removed manually and herbicide was not applied.

Figure 4. 1: Glasshouse trial set-up

Brassica oleracea plants were placed on benches in a random design to ensure any bench potentially lost to unforeseen circumstances would only account for a single repeat of each treatment group.

Figure 4. 2: Randomized field plot

(**A**) Field plot set-up, which includes 6 replicate sites with randomized positions for the 8 treatment groups. (B) Colour codes for each treatment group.


A
Rep 1
4. 2.5mM 24
2. 0.5 mM 24
6.0.5 mM 72
1.Control 24
1.Control 24
8.2 mM 72
4. 2.5mM 24
8.2 mM 72
3. 1 mM 24
6.0.5 mM 72
2.0.5 mM 24
7.1 mM 72
5.Control 72
7.1 mM 72
5.Control 72
3. 1 mM 24
Rep 4
Rep 2
8.2 mM 72
1.Control 24
4. 2.5mM 24
6.0.5 mM 72
5.Control 72
4. 2.5mM 24
8.2 mM 72
5.Control 72
7.1 mM 72
2. 0.5 mM 24
1.Control 24
3. 1 mM 24
3. 1 mM 24
6.0.5 mM 72
2. 0.5 mM 24
7.1 mM 72
Rep 5
Rep 3
4. 2.5mM 24
1.Control 24
8.2 mM 72
6.0.5 mM 72
5.Control 72
7.1 mM 72
7.1 mM 72
5.Control 72
8.2 mM 72
2. 0.5 mM 24
3. 1 mM 24
1.Control 24
6.0.5 mM 72
3. 1 mM 24
4. 2.5mM 24
2. 0.5 mM 24
Rep 6
B
0mM 24 h
0.5mM 24 h
1mM 24 h
2.5mM 24 h
0mM 72 h
0.5mM 72 h
1mM 72 h
2.5mM 72 h

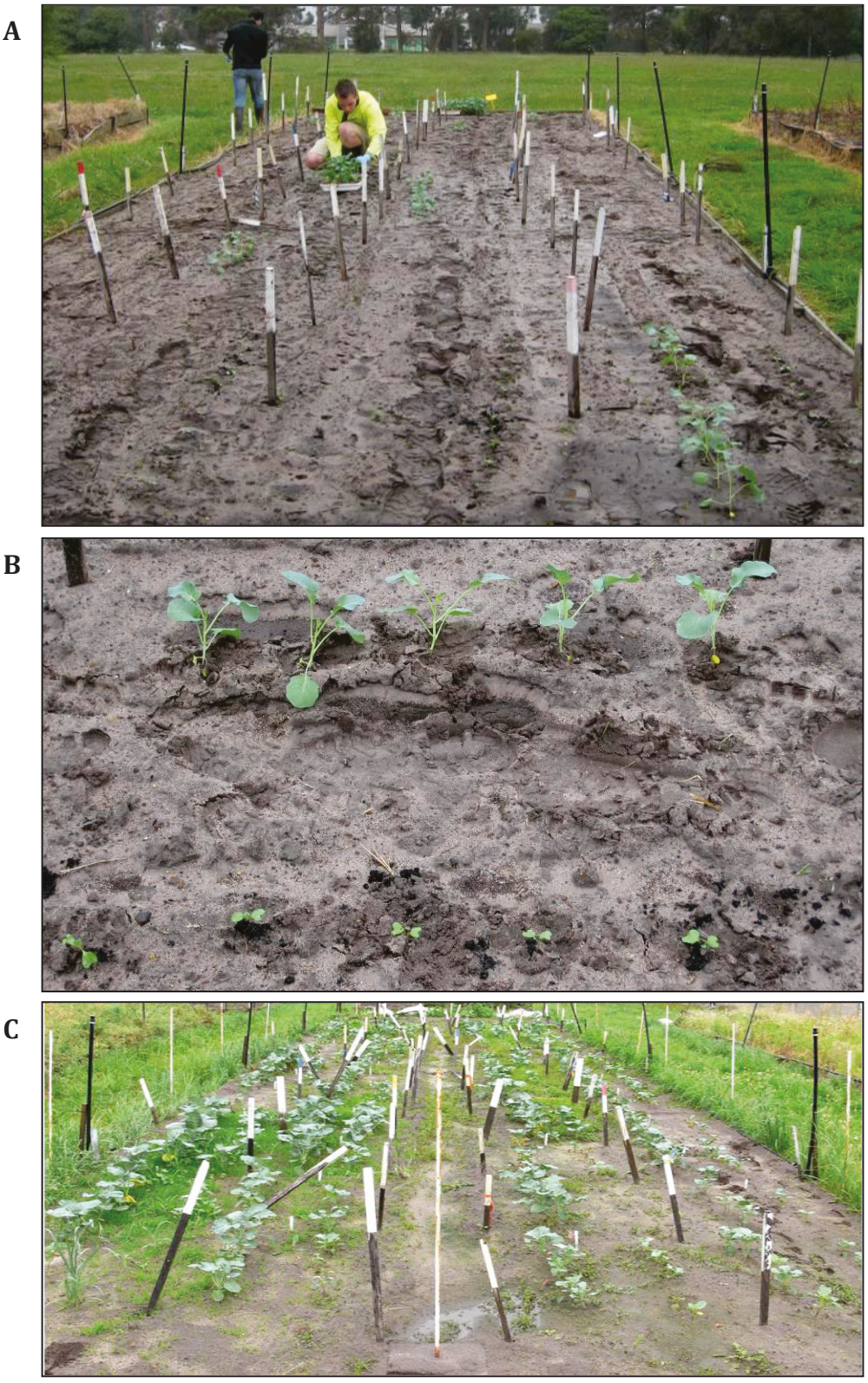


Figure 4. 3: Field plot set-up.

(**A**) Field plots during planting stage, (**B**) separation of 2 and 6 week old plants and (**B**) field plots 6 weeks post planting.

4.3.3 Treatment of plants with salicylic acid and jasmonic acid

A stock solution of salicylic acid (SA) (99% plant extract, Sigma Aldrich) was prepared in MeOH/sdH_2O (v/v1:1) and dilutions made with H_2O, for jasmonic acid (JA) (95%, Sigma Aldrich) treated plants, JA was added directly to H_2O at desired concentrations and used either alone or in combination with SA. Two and 6-week-old plants were treated with SA at concentrations that ranged from 0.5 to 25 mM, while plants were treated with JA at either 1 or 2.5 mM. Where a combination of SA and JA was used the concentration of SA and JA added was adjusted to give an equivalent concentration to that when either chemical was used alone. Plant roots were exposed to SA and JA by application directly to the soil.

Treatment of plants growing in the 48 cell trays with SA and JA was as previously described (see 3.3.3) for SA but rather than the plants being removed to small pots they remained in the trays during treatment. The tray cells had a smaller volume than the pots used in the controlled cabinet experiments so 20 mL of SA or JA or the combination of SA and JA (10mL of each) was gently poured into each of the 48 cells and then 1.04 L was added to the holding tray to give a total volume of SA or JA added of 2 L. The SA solution in the holding tray was left in place for 30 min before being removed and cell trays allowed to drain freely.

The control group of plants were treated with an equivalent volume of H_20 without added SA. Immediately after treatment the pH of the soil within pots ranged from 5 (25 mM SA treatment) to 6 - 6.5 (20 mM – 0.50 mM SA treatment;

the pH of the soil in the water control was 7.0. Commencing 24 h after treatment and for each day thereafter, at the same time, plants were watered with H_20.

Plants were then transferred at 24 or 72 h post treatment with SA and/or JA into larger pots (10cm x 8cm) with two plants of the same age in a single pot, 36 plants were used for each treatment group at each age. For field trials, plants were removed at the same time-points and placed in the field plots as described in 4.3.2. Inoculation of plants was carried out as described below (4.3.4) for glasshouse grown plants.

4.3.4 Inoculation of plants with *P. brassicae* and root gall scoring

For inoculation of glasshouse grown plants a *Plasmodiophora brassicae* spore suspension was obtained from infected cabbage from Lindenow, Vic (Department of Primary Industries; ECD code 16/2/31). Following treatment with SA broccoli plants were inoculated 24 or 72 hours post-treatment with a suspension of 10^8 spores mL^{-1} by pipetting 200µl of the spore suspension (approximately 2×10^7 spores) into close proximity to the roots via a 1cm deep hole made in the soil surface at the base of the stem. Once roots were inoculated the hole was re-covered with soil and the plants watered.

Root galling was assessed 6 weeks post-treatment. Disease severity was assessed using a scoring system of 0-9 modified from Donald *et al*., (2001). For field trial inoculums, soil beds had been artificially inoculated with a mixed population of *P. brassicae* several years prior and this had been maintained by repeated cultivation of brassicas.

4.3.5 Statistical analysis

Statistical analysis was performed using SPSS (PASW 18.0) (IBM, Armonk, New York, USA) and a statistics package (Microsoft Excel 2010). In all experiments, One-Way ANOVAs, specifically Tukey's test and Fisher's least significant difference (LSD) test with a P value = 0.05, were used to analyse significant differences between each treatment group.

4.4 Results

4.4.1 Soil suppression of broccoli growth and *P. brassicae* infection

The effect of soil type on broccoli growth was analysed six weeks post – treatment with *P. brassicae*. Growth of broccoli was impeded in a soil mixture of sand and peat moss (pH 4), with a reduction in shoot development observed compared to broccoli grown in a commercial soil mixture (pH 6.5) (Figure 4. 4). Plant weight was also assessed, with plants grown in the commercial soil mixture significantly weighing more than those grown in the sand and peat moss (Table 4. 1). Soil type was tested to determine the effect on pathogen viability by treating broccoli with *P. brassicae* and assessing disease symptoms 6 weeks post – inoculation (Table 4. 2). Plants inoculated with the pathogen in a commercial soil mixture were found to have a high root score with an average of 6.8. Plants grown in a mixture of sand and peat moss were found to have no disease symptoms with a score of 0 for all plants analysed (Figure 4. 5).

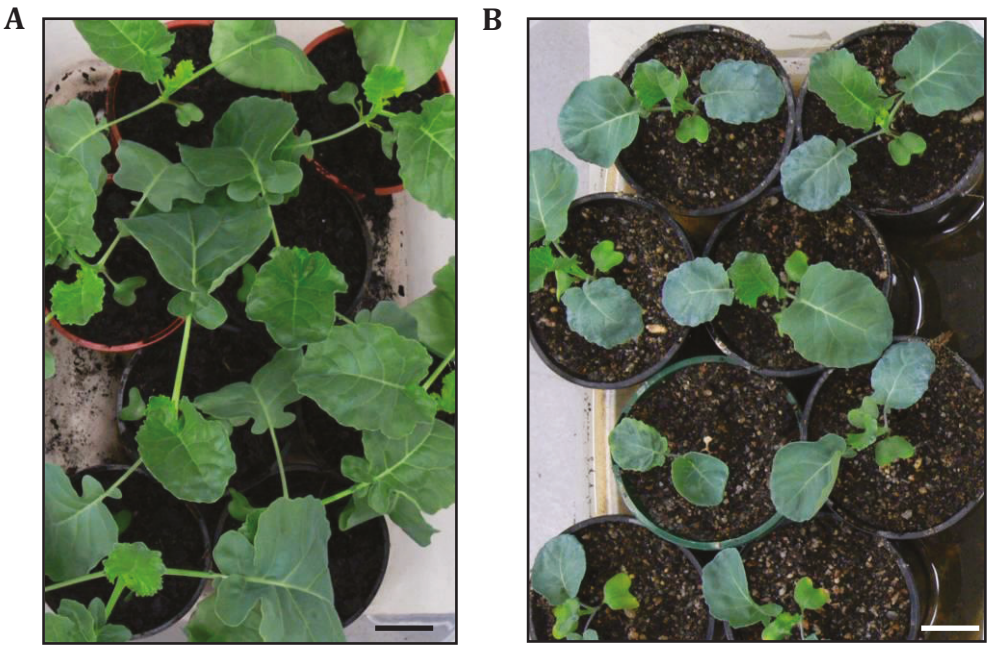


Figure 4. 4: Effect of soil type on *B. oleracea* growth

Effect of soil type on shoot biomass 4 weeks post-planting reveals plants grown in a (**A**) commercial soil mix are much more developed compared to those grown in a (**B**) soil mixture of sand and peat-moss (right) (scale = 2.5cm).

Table 4. 1: Shoot weight of *B. oleracea* grown in either a commercial soil mix or sand/peat moss mixture.

	Shoot Weight	
Repeat	**Commercial Soil Mix**	**Sand & Peat Moss**
1	22.5	12.2
2	25.5	15.5
3	19.1	16.3
Average	**22.3**	**14.6**

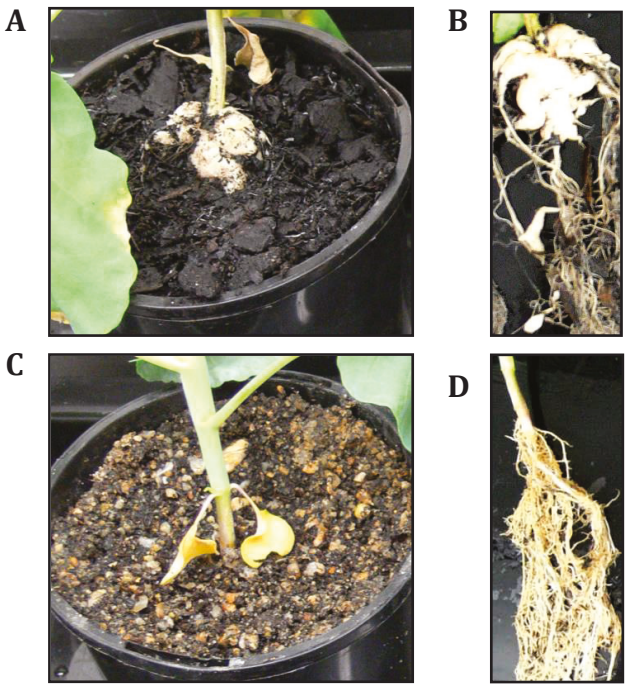


Figure 4. 5: Effect of soil type on *P. brassicae* viability

Effect of soil type on the ability of *P. brassicae* to infect *B. oleracea* roots reveals a commercial soil mix is ideal for *P. brassicae* infection (**A** & **B**), while a sand/peat moss mixture does not accommodate *P. brassicae* (**C** & **D**).

Table 4. 2: Gall scores of *B. oleracea* grown in a commercial soil mix or sand/peat moss mixture.

	Gall Score	
Repeat	**Commercial Soil Mix**	**Sand & Peat Moss**
1	6.4	0
2	6.3	0
3	7.7	0
Average	**6.8**	**0**

4.4.2 Survival of seedlings and young plants folowing SA treatment

The survival of 2 and 6 week old *B. oleracea* seedlings following SA treatment was noted (Table 4. 3) (Figure 4. 6 & Figure 4. 7). Following treatment with 5 mM SA, survival of 2 week old seedlings at 24 and 72 h post-treatment was observed to be 89% and 94% respectively; 6 week old plants were not affected. Following treatment with 10 mM SA a significant reduction in survival rate at both 24 and 72 h was observed, 2 week old seedlings had a survival rate of 53 and 55% respectively, while 6 week old seedlings had a survival rate of 75 and 92% respectively. Treatment of seedlings with 25 mM had the biggest impact on survival rate at 24 and 72 h, 2 week old seedlings had a survival rate of 39 and 53% respectively, while 6 week old seedlings had a survival rate of 50 and 94% respectively.

Table 4. 3: The percentage of 2 and 6 week old plants which survived respective treatments.

Two week old Brassica oleracea plants are able to withstand an SA treatment of 5 mM, with a loss of 89 & 94% at 24 & 72 h respectively, 6 week old plants show no loss in plant numbers. At 10 mM almost half the 2 week old plants are lost at both 24 & 72 h, with a loss of 53 & 55% respectively, while 6 week old plant numbers are reduced to 75 & 92% at 24 & 72 h respectively. When treated with 25 mM there is a greater reduction for both 2 week old plants with 39 & 53% surviving at 24 & 72 h respectively, while 6 week old plants are reduced by 50% at 24 h and only 6% at 72 h.

Treatment	**2 week old**		**6 week old**	
mM	**24hr**	**72hr**	**24hr**	**72hr**
0	100%	100%	100%	100%
5	89%	94%	100%	100%
10	53%	55%	75%	92%
25	39%	53%	50%	94%

Figure 4. 6: Effect of SA on broccoli seedlings at low concentrations

The effect of salicylic acid on 2 and 6 week old broccoli seedlings 24 h post-treatment reveals little change in morphology and health of plants at 0, 0.5 and 1 mM and 2.5 mM.

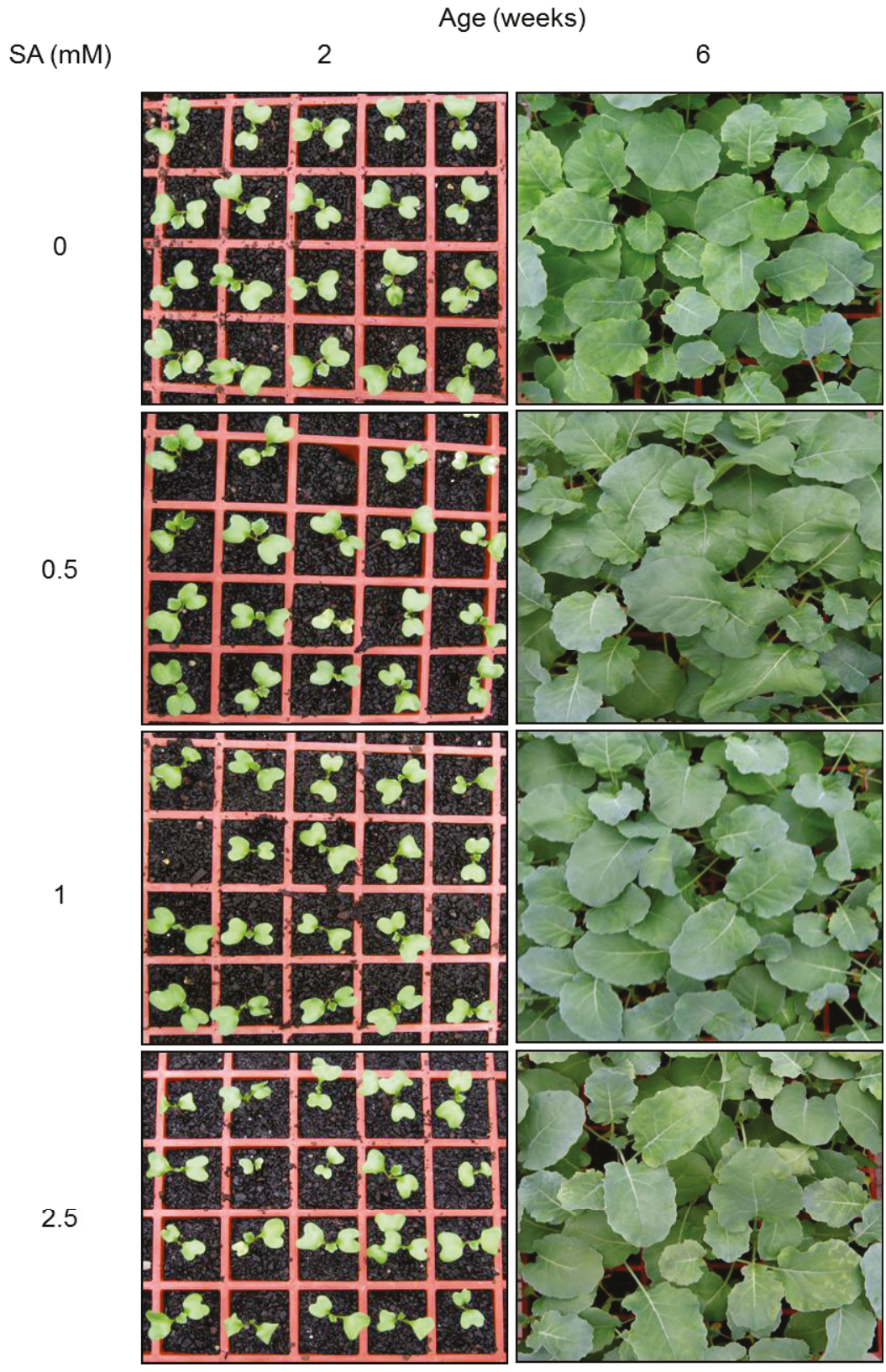
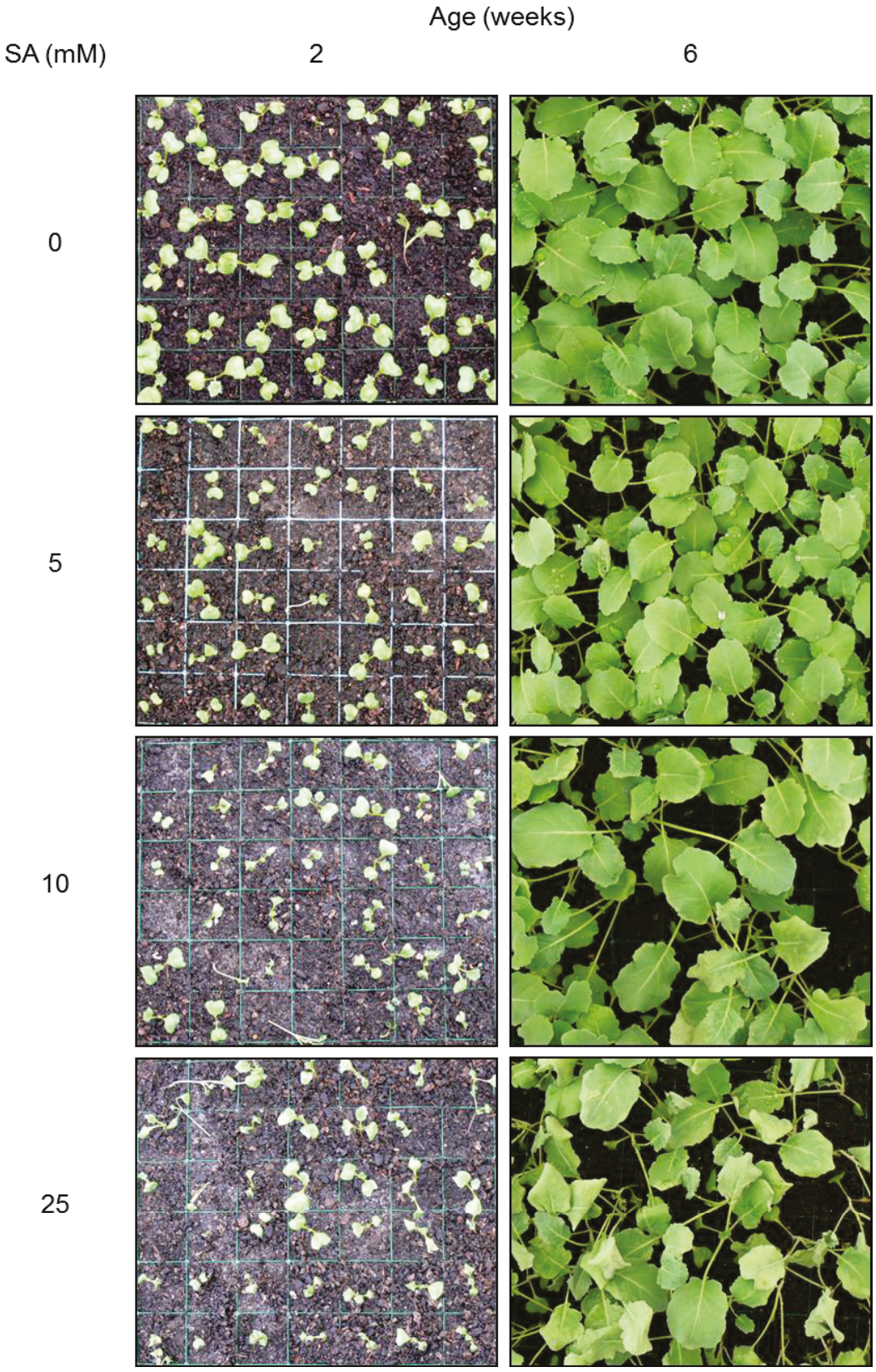

Age (weeks)
SA (mM)
2
6
0
0.5
1
2.5

Figure 4. 7: Effect of SA on *B. oleracea* seedlings at high concentrations

The effect of salicylic acid on 2 and 6 week old broccoli seedlings 24 h post-treatment reveals little change in morphology and health of plants at 0, 5 mM, while at 10 and 25 mM plant morphology begins to change with a clear reduction in plant density observed.


Age (weeks)
SA (mM)
2
6
0
5
10
25

4.4.3 Response of plants to infection by *P. brassicae* in the glasshouse and field to treatment with SA

The formation of root galls was assessed six weeks after treatment of 2 and 6 week old seedlings with SA 24 and 72 h prior to inoculation with *P. brassicae* in glass house (Figure 4. 8) and field trials (Figure 4. 10). Glass house trials of SA treated *B. oleracea* plants at low concentrations (Figure 4. 8 A & D) (0.5, 1 and 2.5 mM) revealed that 2 week old plants inoculated 24 h post treatment showed no reduction in gall formation compared to the control.

At 72 h post-treatment, the only treatment which significantly reduced gall formation was 2.5 mM. However, a clear response to SA was found in 6 week old plants inoculated at both 24 and 72 h post-treatment as a significant reduction in gall formation associated with *P. brassicae* is noted. A decrease in disease symptoms associated with *P. brassicae* was observed in 6 week old plants, with galling greatly reduced when treated with 1 and 2.5 mM SA 24 hr post-inoculation (Figure 4. 9). Increasing the concentration of SA to 5, 10 and 25 mM leads to an increase in disease suppression in both 2 and 6 week old seedlings, (Figure 4. 8 C & D).

Field trials of 2 week old *B. oleracea* plants treated with SA at 0.5, 1 and 2.5 mM, revealed that at 24 and 72 h post treatment only 2.5 mM showed a reduction in gall formation compared to the control. A similar response to SA is seen in 6 week old plants inoculated at both 24 and 72 h post-treatment as a significant reduction in gall formation associated with *P. brassicae* is only seen at a concentration of 2.5 mM.

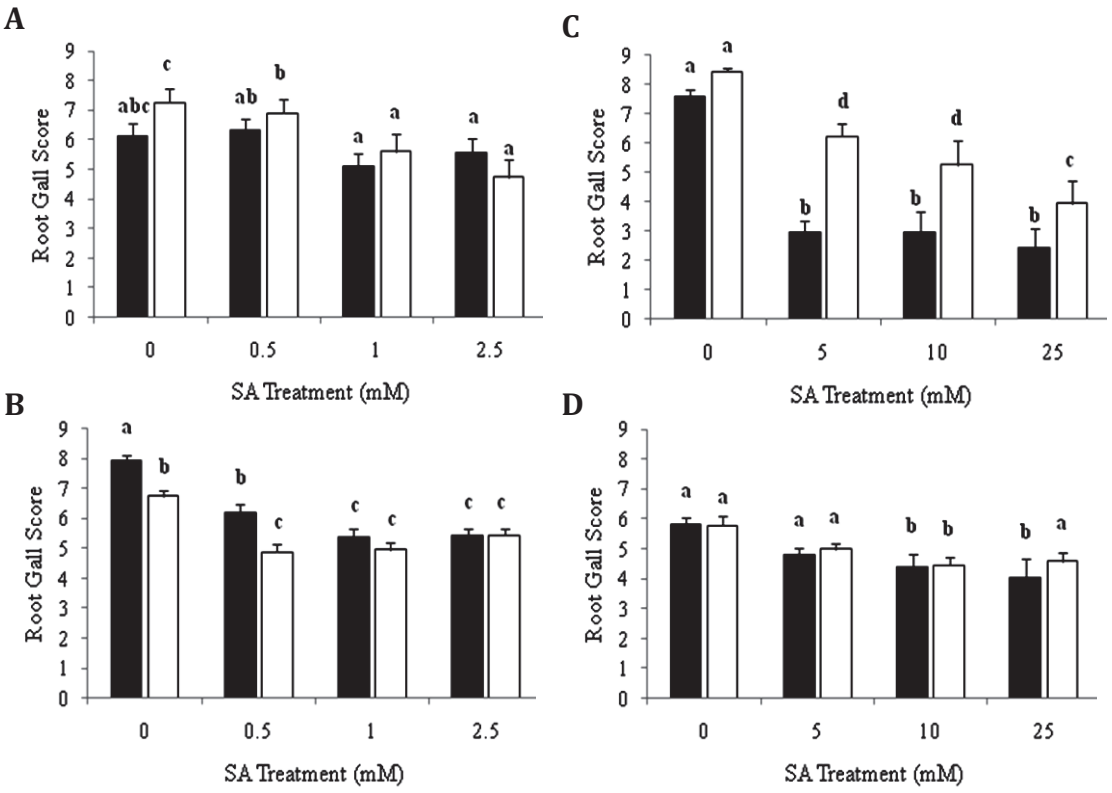


Figure 4. 8: Glass house trial of the effect of SA on root galling

Glass house trials reveal a reduction in root gall scores when 2 and 6 week old *B. oleracea* are treated with SA either 24 or 72 h prior to inoculation with *P. brassicae*. (**A**) Two week and (**B**) 6 week old broccoli plants inoculated with *P. brassicae* at 24 h and 72 h post-treatment with salicylic acid at 0 mM, 0.5 mM, 1 mM and 2.5 mM. (**C**) Two week and (**D**) six week old broccoli plants inoculated with *P. brassicae* at 24 h and 72 h post-treatment with salicylic acid at 0 mM, 5 mM, 10 mM and 25 mM. Error bars represent standard error of the mean, letters represent significant difference (n = 36) (p = 0.05). (Black bars: 24 h; White bars: 72 h).

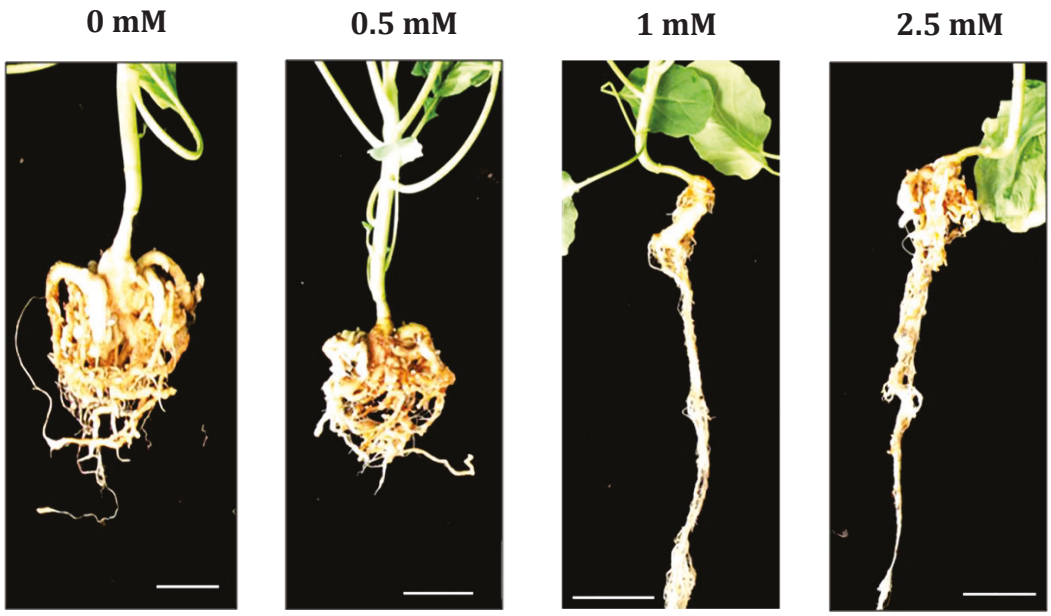


Figure 4. 9: Effect of SA on the interaction between *P. brassicae* and *B. oleracea*

A reduction in root galling in 6 week old *B. oleracea* plants treated with SA 24 hours prior to incoculation with *P. brassicae*, was not observed at 0 mM and 0.5 mM, however at 1 mM and 2.5 mM, a reduction was observed.

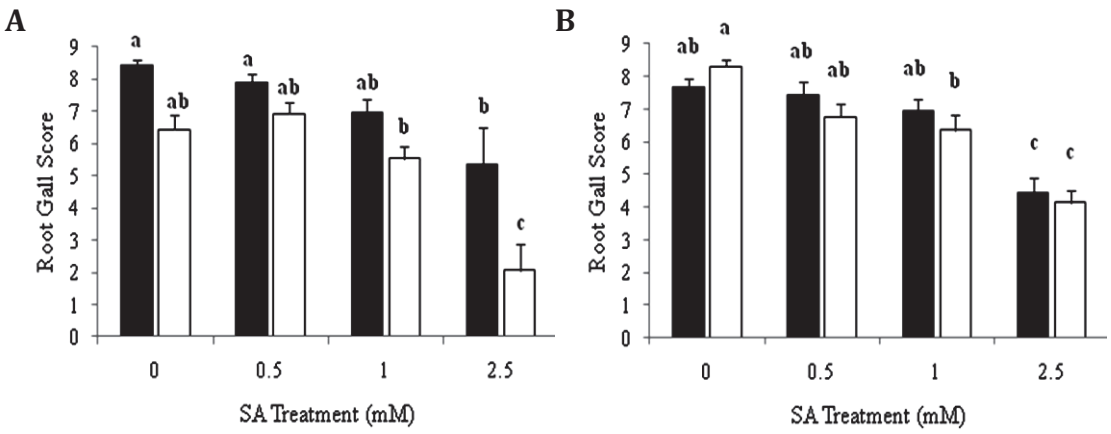


Figure 4. 10: Field trial results

Field trial results with 2 and 6 week old B. oleracea plants revealed root galling was reduced following treatment with SA. (**A**) Two and (**B**) six week old *B. oleracea* plant's inoculated with *P. brassicae* at 24 h and 72 h post-treatment with salicylic acid at 0 mM, 0.5 mM, 1 mM and 2.5 mM. Error bars represent standard error of the mean, symbols represent significant difference (n = 30) (p = 0.05) (Black bars: 24 h; White bars: 72 h).

4.4.4 Reduction in plant weight following treatment with SA and *P. brassicae*

Plant weight of glass house trials was assessed 6 weeks post-treatment with SA and *P. brassicae* to determine the effectiveness of the treatment (Figure 4. 11). Two week old *B. oleracea* plants treated at low concentrations (0.5, 1 and 2.5 mM) differ greatly in their weights, 24 and 72 h post-treatment. At 24 h, there is a significant reduction in weight in all treatment groups, whilst at 72 h, there is no significant reduction in weight compared the control, however the plant weights are significantly lower than those of the 24 h group. When treated with high concentrations (5, 10 and 25 mM) of SA, 2 week old plants exhibit a similar trend in weight reduction at 24 and 72 h post-treatment. Six week old plants do not show as a dramatic change in weight compared to 2 week old *B. oleracea*. At low concentrations, there is a significant reduction at 72 h, but a significant increase is seen 24 h post-treatment. While at high concentrations, there is only a significant reduction in plants treated with 25 mM SA, 24 h post-treatment.

4.4.5 Response of the interaction following treatment with SA or JA or a combination of both

B. oleracea plants were treated with SA and JA, both separately and as a mixture, 24 h prior to inoculation with *P. brassicae*. Plant growth and symptom expression was assessed at both 21 and 42 day's post treatment with the pathogen present or absent from the soil. Treatment of *B. oleracea* with SA (2.5 mM) and JA (1 or 2.5 mM) either separately or in a mixture, caused a reduction in plant weight compared to the control 21 days post treatment in inoculated and uninoculated treatment groups (Figure 4. 12).

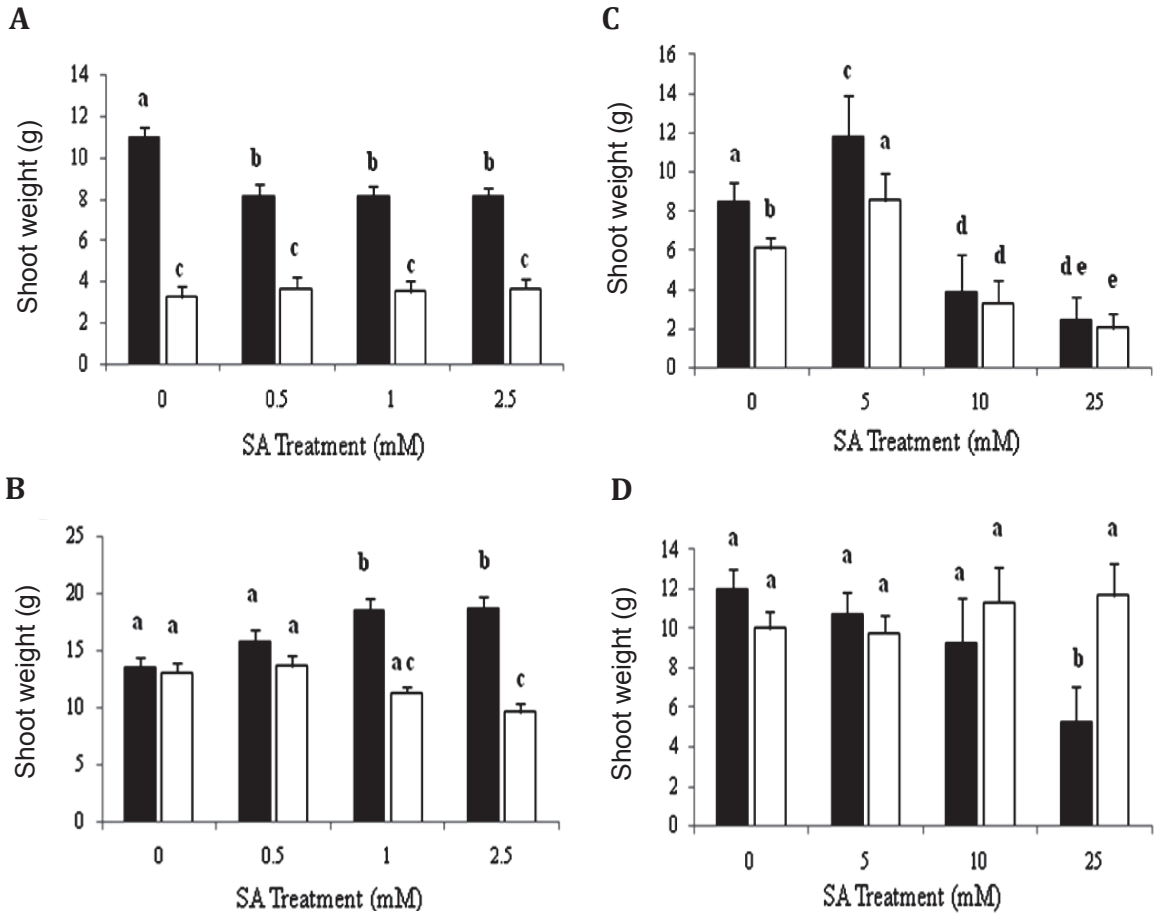


Figure 4. 11: Effect of SA and *P. brassicae* on the weight of glass house plants

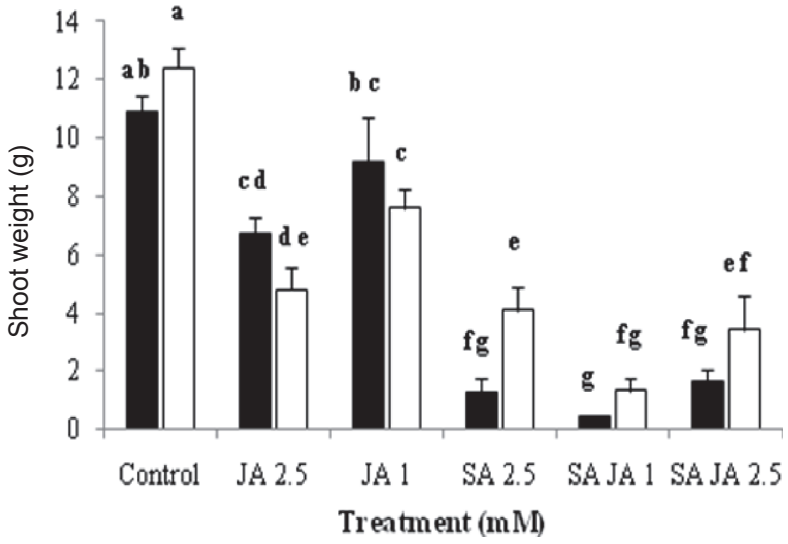
Glass house trial results revealing a reduction in plant weight when 2 and 6 week old *B. oleracea* are treated with SA either 24 or 72 h prior to inoculation with *P. brassicae*. (**A**) Two week and (**B**) 6 week old broccoli plants inoculated with *P. brassicae* at 24 h and 72 h post-treatment with salicylic acid at 0 mM, 0.5 mM, 1 mM and 2.5 mM. (**C**) Two week and (**D**) six week old broccoli plants inoculated with *P. brassicae* at 24 h and 72 h post-treatment with salicylic acid at 0 mM, 5 mM, 10 mM and 25 mM. Error bars represent standard error of the mean, symbols represent significant difference (n = 36) (p = 0.05). (Black bars: 24 h; White bars: 72 h).

Figure 4. 12: Effect of SA/JA on *B. oleracea* weight

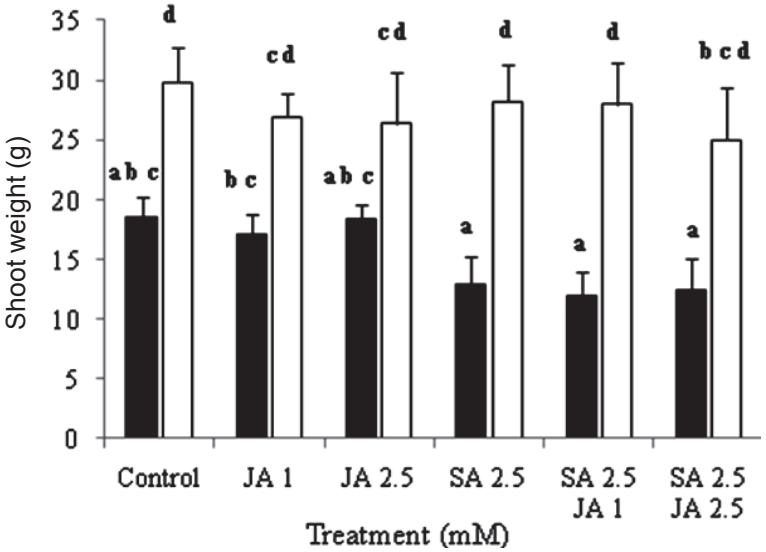
The effect of phytohormone treatments on plant growth with and without the addition of *P. brassicae*, 21 days (3 weeks) and 42 days (6 weeks) post-treatment.

At (**A**) 21 days, there is a significant hormone dependent reduction in weight for those treated with and without *P. brassicae*. However at (**B**) 42 days, there is no significant difference in weight in hormone treated plants, but there is a significant reduction in weight when *P. brassicae* is added. Symbols represent a significant difference (N = 24) (p = 0.05) (Black bars: + *P. brassicae*; White bars: - *P .brassicae*).

A


Shoot weight (g)
14
12
10
8
6
4
2
0
a
ab
bc
c
cd
de
e
fg
g
ef
Control
JA 2.5
JA 1
SA 2.5
SA JA 1
SA JA 2.5
Treatment (mM)

B


Shoot weight (g)
35
30
25
20
15
10
5
0
d
cd
abc
bc
a
bcd
Control
JA 1
JA 2.5
SA 2.5
SA 2.5 JA 1
SA 2.5 JA 2.5
Treatment (mM)

In the absence of the pathogen, plant weight was similar regardless of the treatment 42 day post-treatment (Figure 4. 12). When the pathogen was present, a reduction in the fresh weight of above ground plant parts was observed in all treatment groups including the control. Gall severity was reduced compared to the control 42 day post-treatment when SA and JA were added as a mixture (Figure 4. 13).

4.4.6 Response of *B. oleracea* to different *P. brassicae* isolates

The single spore *P. brassicae* isolate 'e3' was tested for its virulence to *B. oleracea* and compared to an Australian isolate. Four week old plants were inoculated with a 10^6 spore suspension of either 'e3' (see 2.3.1.3 for details of inoculum preparation) or an Australian isolate of *P. brassicae* and root galling was assessed 6 weeks post-inoculation (Figure 4. 14). *B. oleracea* inoculated with 'e3' did not produce root galls 6 weeks post-inoculation, while plants treated with the Australian isolate, produced typical root galls.

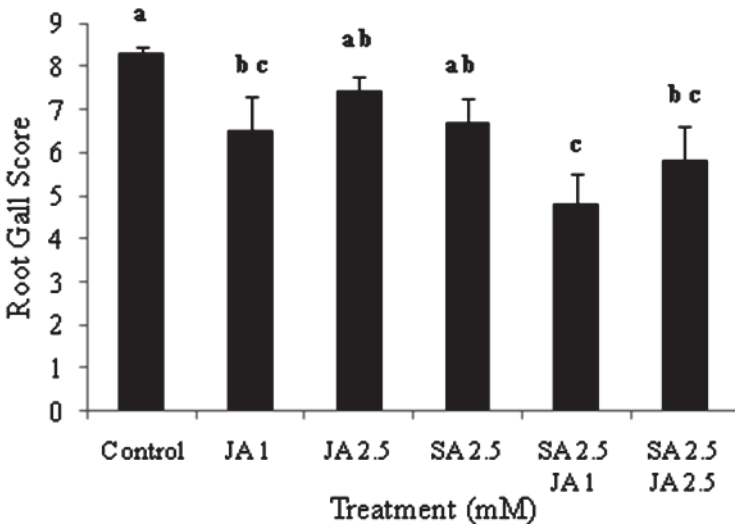


Figure 4. 13: Root galling following SA and JA treatment

Root gall scores of 12 week old *B. oleracea* plant's treated with the phytohormones SA and JA, 6 weeks post-treatment with *P. brassicae*. In combination, SA and JA provide the plants with a significant reduction in galling ($N = 24$, $p = 0.05$).

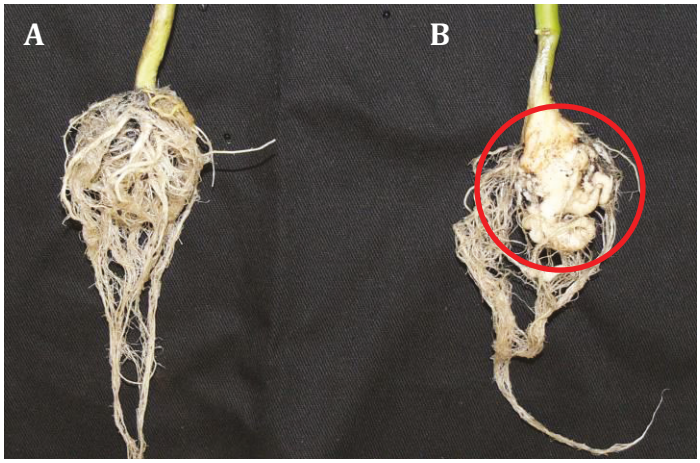


Figure 4. 14: Galling on *B. oleracea* from 2 different *P. brassicae* isolates

Root galling (circle) of 10 week old *B. oleracea* inoculated with either the (**A**) single spore isolate ('e3') or an (**B**) Australian isolate of *P. brassicae*, 6 weeks post-inoculation.

4.5 Discussion

I set out to investigate the potential of SA to control clubroot disease in *Brassica oleracea* caused by the biotroph *P. brassicae.* Despite the effects of SA on various plant growth parameters when used at relatively high concentration, as described in Chapter 3, SA application significantly reduced disease, as measured by the extent of root galling, caused by *P. brassicae* in young, 2-week-old, seedlings grown in the glasshouse across a range of concentrations and most dramatically in those seedlings that had been inoculated with *P. brassicae* 24 h after treatment with SA. The same reduction in disease was not observed in older plants. A similar reduction with increasing concentration of SA was found for plants grown in the field, under high disease pressure, and for both the shorter (24 h) and longer period (72 h) following treatment with SA. In contrast to the glasshouse experiments there was some reduction in disease in the 6-week-old plants but only at the highest concentrations of SA tested. The reduction in disease across both glasshouse and field experiments and found most consistently for the younger plants is likely to be due to a combination of factors and a balance between the detrimental effects of SA on host physiology and its priming or protective effect and, possibly, the interaction of SA with JA.

In the experiments in which SA and JA were tested either alone or in combination a significant reduction in galling, from a gall score of above 8 to one less than 5, was found for the SA/JA combination. This reduction in gall score represents a dramatic reduction in disease and, as has been recently suggested for the interaction of *P. brassicae* with *B. oleracea* (Agarwal *et al*., 2011) may point to a role for not only SA but JA in disease development. Spoel *et al*., (2007)

examined the relationship between the two pathways by treating *Arabidopsis thaliana* leaves with SA, 24 h prior to inoculation with the necrotrophic pathogen, *Alternaria brassicicola*. When SA was present, the number of spores per lesion was high, indicating severe disease; however without additional SA the level of spores per lesion was reduced, indicating a reduction in the severity of disease. In combination, SA and JA increased susceptibility to *A. brassicicola* which is in direct contrast to our work with a biotroph. This relationship may prevent an overload of intracellular chemicals, which could become toxic to the plant; however, it is more likely that an antagonistic relationship provides the plant with the best opportunity to limit damage caused by the pathogen.

This interaction between SA and JA in plant disease has gained prominence as an example of feedback inhibition of one hormone over another but synergistic effects of two hormones on disease expression have also been recently reported for SA and cytokinins in *Arabidopsis* in interactions with biotrophic and necrotrophic pathogens (Argueso *et al*., 2012). Our results also suggest a synergistic interaction between SA and JA and that not only is SA effective in enhancing resistance, as might be expected for the interaction with the biotrophic *P. brassicae,* but that the combination of SA and JA enhances resistance.

Evidence for synergism of action of SA with JA has also recently been demonstrated in *Arabidopsis* in interactions with *Fusarium graminearum* (Makandar *et al*., 2010) and in tomato infected with *Botrytis cinerea* (El Oidi *et al*., 2011). Pinning down the roles of these two hormones in plant pathogen

interactions is proving difficult but the central idea that biotrophic interactions are simply controlled by SA and necrotrophic interactions by JA would appear to be under challenge, a view supported by recent work with bacterial and necrotrophic fungal pathogens of *Arabidopsis* (Tsuda *et al*., 2009; Wathugala *et al*., 2012).

Soil type was found to affect the interaction between *P. brassicae* and *B. oleracea*, which may have been a combination of pH and soil compostion. Myers & Campbell (1985) were able to demonstrate *P. brassicae* was successful at a pH range between 5.4 and 7.1; however when the pH rose above 7.3 infections did not occur. A recent review by Donald & Porter (2009) has highlighted the importance pH can have on disease reduction.

It is unclear as to whether a pH as low as 4 has a direct effect against *P. brassicae*, as very little research has been undertaken into pH effects below 5. Soil composition may have a direct effect against *P. brassicae*; with indications already present organisms can reduce disease severity (Murakami *et al*., 2000). Wallenhammer (1996) showed the presence of sand, both course and fine, were able to reduce disease severity to below 45%, while recent studies in Canada have shown that infection rates on canola grown in sand were at 100% (Kasinathan *et al*., 2011). Therefore it is unclear as to whether the presence of sand from my research has had any influence on the interaction between plant and pathogen.

The results presented in this chapter show that exogenous application off SA reduces disease severity associated with *P. brassicae* infection in the glasshouse and, importantly, in field trails. In particular field grown plants appear to respond better to SA treatment when comparing disease control, this is not a surprising result as field trial plants often develop a higher threshold for resistance compared to glasshouse grown plants due to constant exposure to changing environmental conditions (Kaur *et al.*, 2012). This opens up the possibility of SA being used as a clubroot disease control agent, something for which SA has not previously been examined. I have presented good evidence that SA is a regulatory molecule of this disease in a crop species. For practical application a balance needs to be struck between disease amelioration and direct effects on plant growth both of which will impact on crop productivity. Whether the level of reduction in disease severity I have found is useful in terms of maintenance of crop productivity in situations of high disease pressure is yet to be determined but will be the subject of further study.

Chapter 5: General Discussion

5.1 Summary

The research presented in this thesis will help in better understanding the interaction between the soil-borne biotroph, *Plasmodiophora brassicae* and two of its root hosts, *Arabidopsis thaliana* and *Brassica oleracea*. Of particular importance is the salicylic acid defence pathway and its involvement in the plant-pathogen interaction. Most research that surrounds the role that SA plays in plant-pathogen interactions has focused on leaf and stem pathogens, with little research conducted on defence against root pathogens. A major difficulty with assessment of root pathogen interactions is that unlike foliar pathogens, disease assessment in root pathogens can be more difficult to analyse and observe, as initial infection is very difficult to monitor. The Australian *P. brassicae* isolate used in the current research creates its own problems as it is highly virulent, making the control of the disease very tough.

The research presented here has provided evidence of the importance of the SA pathway in the interaction of *P. brassicae* with its hosts through the use of *A. thaliana* and its SA pathway mutants. Disease suppression was found in those mutants that over-expressed either SA or *PR-1*. The phytohormone SA can now be considered in avenues for control in the economically important crop, *B. oleracea*. Where switching on the defence pathways associated with the hormone may provide a useful level of disease resistance against *P. brassicae*.

5.2 Importance of *Arabidopsis* as a host for *P. brassicae*

The important role that *A. thaliana* ecotypes and pathway mutants play in understanding plant-pathogen interactions should not be underestimated, especially when examining the disease caused by *P. brassicae*. Although much research into plant-pathogen interactions has utilized *A. thaliana*, in the interaction with *P. brassicae* it becomes more vital as it belongs to the family Brassicaceae, the same family to which *B. oleracea* and other important crops belongs. Prior to this study there had been no observations of the interaction between the *A. thaliana* mutants *cpr1* and *dnd1* and *P. brassicae*.

The *Arabidopsis thaliana* pathway mutant *cpr1*, which confers constant pathogenesis related gene function and is a critical component of biotroph defence, was observed to provide a level of resistance against *P. brassicae*. The *A. thaliana* mutant *dnd1*, which undergoes all facets of enhanced disease resistance, but does not produce a hypersensitive response, was also observed to provide a level of resistance against the pathogen. The addition of SA to the system greatly increased this resistance response to *P. brassicae*, which again highlights the importance of SA in defence against *P. brassicae*.

The use of pathway mutants compromised in a component of the SA pathway has provided evidence of the need for SA when compared to over-expressed mutants. The mutant *NahG*, contains an enzyme which breaks down SA before it is allowed to accumulate to a significant level and was observed to be highly susceptible to the pathogen. This is not a surprising result as *PR-1* is unable to accumulate due to this reduction in SA content. *Npr1*, which is able to

accumulate SA, but unable to fully express pathogenesis related genes and was susceptible to the pathogen as was shown by Siemens *et al*., (2003). There is however evidence presented in this thesis that suggest *npr1* responds better to the pathogen with a reduced gall score compared to that found for *NahG*. As *npr1* is not restricted in the amount of intracellular SA it can accumulate, it is reasonable to suggest an important role for SA itself in the initiation of a resistance response against *P. brassicae*, through other defence routes. The use of a number of pathway mutants in the current research combined with what is already known about the salicylic acid pathway has enabled me to contribute to a scheme of the important genes in the regulation of a defence response against *P. brassicae* as described in Figure 5. 1.

5.3 Salicylic acid and its role in *B. oleracea* and *A. thaliana*

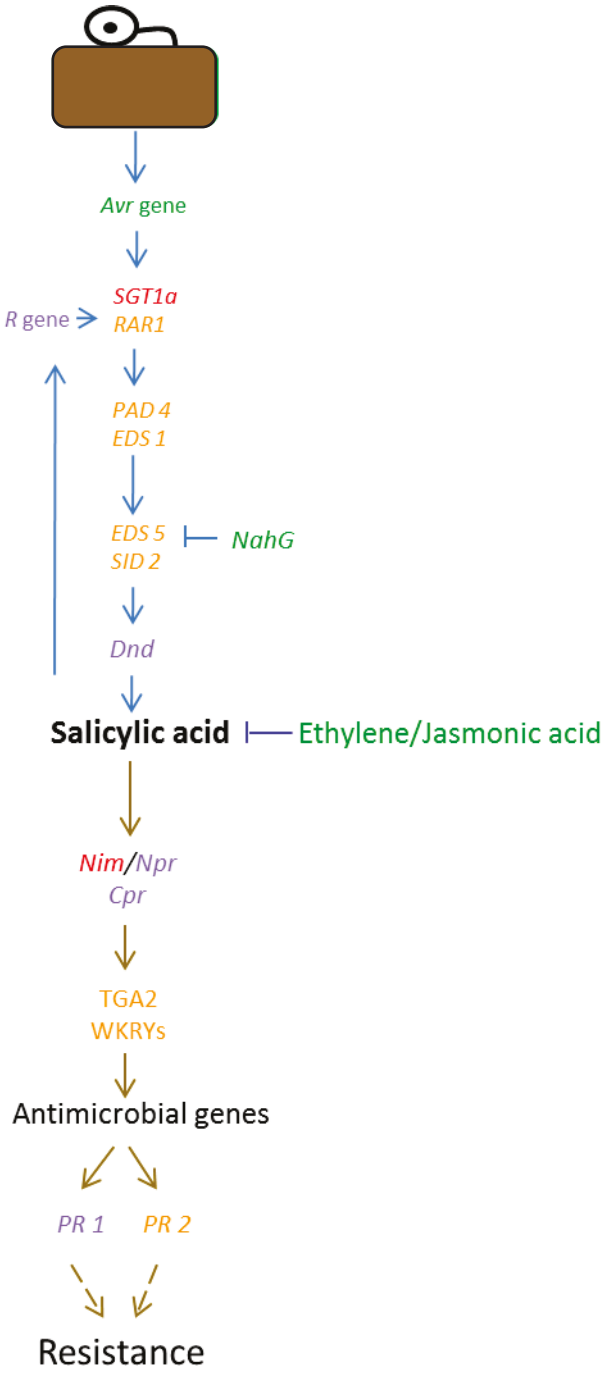
Salicylic acid has been recognized as a major phytohormone in plant defence and is most associated with biotrophic pathogens. The basal level of SA within a plant may play an important role in the early initiation and development of a defence response against an invading pathogen. There is little research, of the effect that SA plays in growth of plants and there has been no research into the growth effects on *B. oleracea*. *Arabidopsis thaliana* mutants may provide evidence of the role SA plays in plant development as observed in the different leaf biomass in this study with *NahG*, *cpr1* and *dnd1*. Rivas-San Vincente and Plasencia (2011) hypothesized that *NahG*, which produces large leaves, occurs due to a reduction in basal SA levels, while *cpr1* and *dnd1* were shown to produce quite small leaves in comparison, both of which are over-expressors of SA.

Figure 5. 1: Potential SA pathway in an interaction with *P. brassicae*

A generalised view of a potential SA signaling pathway in the interaction between *P. brassicae* and *A. thaliana*, following initial infection from a spore (top part of figure).

It is widely acknowledged that in a resistance interaction with a pathogen the *Avr* gene must be recognized by the plants *R* gene for a number of resistance genes to be switched on. It is believed the first genes switched on are *SGT1a*, *PAD4* and *EDS5* leading to an increase in intracellular SA levels. Following an SA increase *NPR* and *CPR* maybe up-regulated leading to the formation of the TGA2/WRKYs complex, which regulates antimicrobial genes such as *PR-1* and *PR-2*. Jasmonic acid and ethylene are known to act antagonistically against SA, reducing the intracellular levels available, while the *NahG* mutation prevents SA being synthesized.

Purple indicates genes and proteins found to be potentially involved in *P. brassicae* defence from this research, while red indicates genes found to be involved from previous research. Orange indicates genes and proteins in the SA pathway that are likely to be involved in *P. brassicae* defence. Figure modified from Thoma et al., (2001); Muskett & Parker, (2003); Shah (2003); Bari & Jones, (2009); Lu, (2009) & Vlot et al., (2009).


Avr gene
SGT1a
R gene
RAR1
PAD 4
EDS 1
EDS 5
NahG
SID 2
Dnd
Salicylic acid
Ethylene/Jasmonic acid
Nim/Npr
Cpr
TGA2
WKRYs
Antimicrobial genes
PR 1
PR 2
Resistance

The addition of SA to *B. oleracea* roots was shown to have a dose-dependent effect on the health of plants. A concentration below 1 mM SA however did not significantly impact on plant health and growth. When the concentration was increased to 5 mM and above there was a dose-dependent reduction in root and shoot weight and an increase in phytotoxicity to the phytohormone. Senaratna *et al*., (2000) tested SA and acetyl salicylic acid (ASA) at concentrations of 0.05, 0.1, 0.5, 1 and 5 mM on bean and tomato plants and observed adverse effects on plant health at concentrations above 1 mM. *A. thaliana* wild-types treated with SA at concentrations above 5 mM were unable to survive.

An important finding from this research was the ability of *B. oleracea* and *A. thaliana* to uptake and distribute exogenous SA from the root to the shoot to provide an SAR-type response as has been highlighted in a review by Hayat *et al*., (2010). Systemic distribution of the phytohormone was revealed in the present study by high performance liquid chromatography of plant extracts and evaluation of *PR-1* gene expression through PCR and real-time quantitative PCR. This evidence, the first in *B. oleracea*, provides information that suggests that SA is able to be transported systemically through the plant in relatively short time to induce a systemic defence response. Similarly for the *A. thaliana* wild-type Col-0 root drenching with SA resulted in elevated *PR-1* expression in leaves that is either due directly to elevated SA levels or a signal generated by SA.

5.4 Challenges associated with studying root pathogens

Plasmodiophora brassicae is an extremely difficult pathogen to not only work with, but also in studying the plant-pathogen interactions as has been

highlighted by Agarwal *et al*., (2009) and Siemens *et al*., (2003). Unlike many soil-borne pathogens, *P. brassicae* cannot be cultured outside of a living host, this makes for studying the multi-component life-cycle and host interactions rather difficult.

The 'e3' trials using both wild-type and mutant *Arabidopsis* highlights another potential problem as the observed results in this study do not match those observed in previous studies conducted by Siemens *et al*., (2002). Potential changes in lab conditions such as temperature, soil type and soil moisture can increase or decrease *P. brassicae* infection rates. However, it is believed the difference in responses is due to the 'e3' isolate obtained no longer being a single spore isolate, this may cause an increase in virulence.

There is an added complication when working with *P. brassicae*, the soil type used can influence the disease outcome. *B. oleracea* is able to grow in an acidic soil as low as pH 4.0 as was found in Chapter 4 when using a soil mixture, *P. brassicae* however is unable to infect at such an acidic pH. Although this could be used as a control method for *P. brassicae*, the effects on the plant and soil make-up are important factors that must be taken into consideration. Although *B. oleracea* plants do grow in low acidic pH, they are severely dwarfed when compared to those grown in neutral pH soils as shown in Chapter 4. This suggests the soil chemistry might be manipulated and nutrients and minerals once available to the plants become insoluble (Weaver & Hamill, 1985).

5.5 Salicylic acid and jasmonic acid and their roles in defence against *P. brassicae*

As has been discussed, SA is important in the development of a defence response in *B. oleracea* and *A. thaliana*. However, little evidence prior to this study had been conducted on a possible defence response in *B. oleracea* against *P. brassicae*. To clearly understand if this response is associated with a decrease in disease severity, the plant-pathogen interaction was studied following treatment with SA. An Australian isolate of *P. brassicae* is highly virulent to *B. oleracea* and initial experiments using *A. thaliana* ecotypes suggested the application of SA alone was not enough to reduce disease severity. Unlike *A. thaliana*, the root system of *B. oleracea* is much larger, thicker and tougher. SA applied to plants in the glasshouse at concentrations below 2.5 mM resulted in a slight decrease in disease severity in both 2 and 6 week old plants. The disease control associated with these concentrations may be as a result of the overall virulence of this particular isolate of *P. brassicae* to *B. oleracea*, rather than the application of SA itself. Importantly an increase in SA concentration from 5 mM to 25 mM did provide a significant reduction in disease severity in glasshouse grown plants, especially when applied to 6 week old plants. This suggests that to overcome a highly virulent isolate of *P. brassicae*, SA may need to be used in conjunction with other control methods.

There are indications that jasmonic acid plays a crucial role in disease development in biotrophic pathogens, including *P. brassicae* (Ludwig-Müller, 2008). There is a suggestion that an increase in JA will reduce the production of SA, through an antagonistic response (Koornneef & Pieterse, 2008), and allow

for pathogen infection. The use of the *jar Arabidopsis* mutant provided evidence that in an interaction with *P. brassicae*, the production of JA is not essential for disease development. The *Arabidopsis* ecotype Col-0, parental line of *jar*, exhibits a high level of susceptibility towards *P. brassicae* through galling and reduction in plant biomass.

5.6 Glass house and field grown plants differ in disease susceptibility

An important finding from this research was the differing responses of plants grown in a glasshouse to those grown in field plots, as has been commonly found in other systems. Plants grown in controlled environments often don't develop the 'hardness' associated with field grown plants. This arises from their lack of exposure to an ever changing environment, which causes the plant to continuously manipulate it's responses to these abiotic and some biotic stresses. The root systems themselves become much larger and thicker in field plots as they have more room to grow, this may aid the plants responses to the pathogens by making it more difficult for the pathogen to infect.

These observations were also detailed by Kaur *et al*., (2012) in which *Nicotiana attenuate* plants silenced in two *cinnamyl alcohol dehydrogenase* (CAD) genes often produced rubbery stems and low lignin content and small rooting making them unstable when grown in a glasshouse or in protected field trials. However when grown in unprotected field-trials, plants often lost these traits and grew with thick, stable stems and large thick root systems allowing them to withstand high winds as well as wild type plants. This led to the researchers hypothesising that a number of environmental conditions such as UV-B, wind,

temperature fluctuations, herbivory and drought can greatly influence the growth and structure of plants.

5.7 Conclusion

The findings of this research provide an important insight into the critical role salicylic acid plays in host defence against *P. brassicae*. The evidence found during this research suggests that SA is of major importance for a successful defence response in *B. oleracea*. However, there are still many gaps in the knowledge of the role of SA in the interaction between *P. brassicae* and *B. oleracea*. The use of *A. thaliana* mutants has provided vital information of this role as those that over-express SA and components of the SA pathway are more resistant to a highly virulent isolate of *P. brassicae*.

The SA defence pathway in this plant-pathogen interaction and many others is still unclear. It is an area of research that could open the door for better protected crops and other economically important plants with the knowledge and ability to target key aspects of the SA pathway which are critical for defence. Further investigation into SA must center on whether SA itself is transported systemically through the plant by using radio-labeled SA or if it is only a signal molecule. Further field trials must be undertaken, not only to confirm that SA provides a defence response, but also the impact of SA on soil chemistry, pH and other microbes

It is important that research into this pathogen continues as it threatens many of the economically important crops here in Australia but also throughout the

world. The application of SA to *B. oleracea* has been shown to reduce disease severity associated with the pathogen, however further work is required into its function in defence against *P. brassicae* and the longevity of action. A possible rout to further investigate SA's role could be through the use of SA enhanced *B. oleracea*, by using the *A. thaliana* SA pathway as a reference. This could be through the production of SA or antimicrobial enhanced Brassicaceae crops. Ultimately control against *P. brassicae* whether it's through a synthetic or natural chemical and in combination with other control practices must be found to reduce the economic impact of this pathogen.

Chapter 6: References

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