Enhancing the plant immune response for improved disease control

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Victorian Department of Primary Industries (VICDPI)

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Enhancing the plant immune response for improved disease control

Final report for project VG07010
Caroline Donald *et al.*
(July 2010)

Project Details

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Final report for Horticulture Australia Ltd project VG07010
July 2010

This report details research directed towards the induction of systemic acquired resistance in vegetable *Brassica* crops using salicylic acid. The work has progressed through three stages. Firstly 'proof of concept' studies were conducted to determine whether SAR could be induced using salicylic acid in the model plant *Arabidopsis*. Secondly SAR induction was demonstrated and optimised in a vegetable *Brassica* crop, broccoli. Finally, trials were conducted to determine whether any observed effects were specific to the broccoli-*P.brassicae* host-pathogen pair or could be applied more widely across a range of *Brassica* vegetable crops and *Brassica* pathogens.

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Improved disease control

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Media summary

This project is the key project within the innovative science component (subprogram 6) of the 'National Vegetable IPM Diseases Program'. The key aim of this sub program is to identify new technologies and innovative science that may be of future benefit to control plant pathogens within the vegetable industry.

This project has shown for the first time that under controlled conditions in the glasshouse salicylic acid can boost the natural plant immune response against clubroot of broccoli. Further, it has shown that this response, known as systemic acquired resistance, may be a feasible method to provide suppression of clubroot and other diseases in *Brassica* vegetable crops in the field. To assist future development of the method, the project also evaluated the optimum rate and method of application, efficacy, potential for phytotoxicity and commercial suitability of the method.

The project showed that a single 15 minute dip of commercial seedlings in 0.5 mM salicylic acid solution 72 hours before transplanting proved to be the optimal rate, although this rate was phytotoxic to younger seedlings (10-14 day old) and lower rates (0.1-0.25 mM) needed to be applied. In pot trials artificially inoculated with a low to moderate levels of the pathogen, salicylic acid reduced the severity of visual symptoms of clubroot disease on broccoli by up to two thirds. The systemic nature of the response was confirmed by upregulation of three genes (chitinase, PR-1 and PR-2), known to be associated with plant defence, following treatment with salicylic acid. Biochemical studies showed their levels were highest 72 hours after treatment.

In addition to clubroot (*Plasmodiophora brassicae*) control, trials have also indicated that the systemic acquired resistance was effective against white blister (*Albugo candida*) and *Rhizoctonia solani* AG 2.1, but attempts to replicate the disease suppressive effects observed in broccoli in other *Brassica* vegetables (cabbage and cauliflower) were unsuccessful.

This work demonstrates the potential for a whole new and, until now, relatively unexplored area of disease management to be opened up to the vegetable industry. Similar to the concept of vaccination in humans, this work shows that a simple treatment applied in the nursery has the potential to prime plants to defend themselves against pathogens. An industry partner (for example a chemical company) is required to further develop and commercialise the concept.

Technical summary

This project is the key project within subprogram 6, the innovative science subprogram, of the 'National Vegetable IPM Diseases Program'. It is directed towards identifying new technologies and innovative science that may be of future benefit to the vegetable industry. Research has focussed on developing and establishing the potential of induction of systemic acquired resistance as a means to 'boost the plant immune response' and provide long-lasting suppression of disease in *Brassica* vegetables. It has demonstrated for the first time the successful use of induction of systemic acquired resistance to control clubroot in broccoli.

Systemic acquired resistance (SAR) is a form of heightened defensive capacity in plant species. It is associated with the accumulation of salicylic acid which leads to the formation of pathogenesis related (PR) proteins. Historically this ability of the plant to defend itself has been largely overlooked and underutilised as a disease management strategy.

Preliminary studies using microarray analysis and the model *Brassica* weed *Arabidopsis thaliana* measured changes in gene expression and identified that the salicylic acid pathway was down regulated in plants with clubroot disease. It was proposed that manipulating the pathway by supplying salicylic acid (SA) to the plant externally might reverse this effect and enable enhanced resistance to the pathogen *P. brassicae*. Application of 0.5 mM SA applied to *A. thaliana* roots as a 1 minute dip halved the number of plants infected by *P. brassicae* and caused a 76% reduction in the severity of symptoms of root galling.

The results of the proof-of-concept study were extended to a vegetable brassica crop, broccoli. Since broccoli roots are much thicker than *A. thaliana* the duration of the dip treatment was increased from 1 minute to 15 minutes. Application of 0.1 mM of SA (neutralised, pH 7) as a single 15 minute seedling dip significantly reduced the severity of symptoms of clubroot in broccoli under low-moderate disease pressure. At higher disease pressure a triple dip (three 15 minute dips in 0.1 mM SA applied 72, 48 and 24 hours before transplanting) improved the efficacy of treatment.

The systemic nature of the response was confirmed by the upregulation of three genes (chitinase, PR-1 and PR-2) known to be associated with plant defence responses. Reverse phase high performance liquid chromatography (RP-HPLC) analysis of salicylic acid in plant roots indicated that levels were highest 72 hours after treatment. Young seedlings (10-14 day old) were more susceptible to phytotoxicity from SA treatment with one third of plants dead following treatment with the highest rate of SA used (1 mM). A single 15 minute dip of commercial (6 week old) seedlings in 0.5 mM salicylic acid solution 72 hours before transplanting proved to be the optimal rate, although this rate was phytotoxic to younger seedlings (10-14 day old) and lower rates (0.1-0.25 mM) needed to be applied.

Preliminary trials indicated that the systemic acquired resistance was also effective against white blister (*Albugo candida*) and *Rhizoctonia solani* AG 2.1 in addition to clubroot (*Plasmodiophora brassicae*) but attempts to replicate the disease suppressive effects observed in broccoli in other *Brassica* vegetables (cabbage and cauliflower) were unsuccessful. Many of the anticipated changes in the expression of key plant defence genes observed in broccoli were not observed in these crops.

Variation in individual plant responses to SA, observed as a range of disease symptoms or fold changes in gene expression within a treatment group, was consistently observed. A minimum of 10 replicate plants in a treatment group is recommended in any future experiments to account for this variation.

This work demonstrates the potential for SAR to be used to manage diseases in broccoli. Further work is required to reduce the variation in treatment effects, prove the effects in the field and to achieve a similar outcome across a range of vegetable *Brassica* crops.

Recommendations

Based on the findings presented in this report the following recommendations are made:

- Where significant commercial interest can be demonstrated (ie. involvement and financial support from a commercial partner such as a chemical company or other interested party with the capacity to deliver products to market) the vegetable industry continue to support research into systemic acquired resistance (SAR) to capitalise on the potential that has been demonstrated for induction of SAR to be used as a disease control tool.

- That rates of salicylic acid between 0.1 and 0.5 mM are optimum applied as a 15 minute seedling dip. The actual rate used will depend upon the age of the seedlings with lower rates 0.1-0.25 mM being optimum for treatment of younger seedlings (0-3 weeks) that are more susceptible to phytotoxicity and higher rates 0.5 mM being suitable for older (6 week) seedlings.

- That transplanting or inoculation (exposure to pathogen) does not occur until 72 hours after SA treatment to allow SAR to establish fully in the plant.

- That salicylic acid solutions are neutralised (pH 7) before use to eliminate any direct effects of an acidic solution on the host or pathogen.

- That studies of changes in gene expression are conducted concurrently with disease control trials to establish that any effects are occurring systemically and are not a direct effect of the inducer on either the host or pathogen.

Any future research should focus on understanding why so much plant-to-plant variation is observed within a treatment group and whether this can be minimised, further proofing of the outcomes in large field trials and seeking to modify the methods developed for broccoli to induce similar responses across a range of vegetable *Brassica* crops.

This project was a foundation project of the vegetable IPM disease program. The program approach has brought together researchers from all over Australia. It has created opportunity for interaction, critical discussions and planning. It has also provided a focus for and coordinated approach to technology transfer/communication activities. The program approach should be strongly encouraged as we enter Phase II.

Introduction

1. Introduction

Systemic acquired resistance is a form of heightened defensive capacity in plant species. It is associated with the accumulation of salicylic acid which leads to the formation of pathogenesis related (PR) proteins. Previous studies using *Arabidopsis* as a model plant identified a number of changes in gene expression that occur in this plant in response to infection by *Plasmodiophora brassicae*, the pathogen causing clubroot disease of vegetable *Brassicas*. These studies revealed that within four days of infection, genes involved in the biosynthesis of salicylic acid (SA) were suppressed. Since SA is suppressed in the compatible host-pathogen interaction, upregulation of SA biosynthesis or application of exogenous SA may have the reverse effect causing an incompatible host-pathogen interaction mediated by systemic acquired resistance (SAR). This report details studies directed towards proving this hypothesis and demonstrating the potential for SAR to be developed as a crop protection tool in the vegetable industry.

1.1 Self defence – the ability of the plant to defend itself against pathogens

A complex and coordinated resistance response is activated in plants upon recognition of a pathogen. This leads to the induction of a range of host responses including phytoalexin synthesis, physical barriers such as hypersensitive cell death, callose and lignin deposition, and the production of chemicals such as proteinase inhibitors and lytic enzymes (eg. glucanases and chitinases). When the plant fails to recognise the pathogen or the pathogen is able to avoid or overcome the plant resistance response, disease develops.

Historically this ability of the plant to defend itself has often been overlooked and under exploited as a disease management strategy. Increasingly however, crop protection experts are looking within the plant as they seek to accelerate and exploit the plant resistance response and search for novel chemistries. Understanding the mechanisms of plant defence against pathogen invasion is critical as these dictate which chemistries are likely to accelerate or amplify an effective host response against which pathogens. Broadly pathogens can be classified as biotrophs, those that feed on living host tissue, and necrotrophs, those that kill host tissue and feed on its remains. Hemibiotrophy defined by Perfect and Green (2001) as an initial period of biotrophy followed by necrotrophic hyphae is a subsidiary definition. Considering pathogens as either biotrophic or nectrotophic, it is easy to understand how plants might respond to their invasion differently. A hypersensitive response with programmed cell death for example might be an effective strategy against a biotrophic pathogen but may well support the growth of a necrotroph by supplying an instant food source.

Plasmodiophora brassicae, the cause of clubroot disease of vegetable brassicas, is a classic biotrophic soilborne pathogen but many other soilborne plant pathogens are predominantly necrotrophic. *Sclerotinia* spp. are classic necrotrophs. These ascomycete fungi kill tissue as they spread. Pathogenicity results from the production of oxalic acid which is toxic to most plants and polygalacturonase which breaks down plant cell walls (Wang and Fristensky 2001). Likewise the basidiomycete fungus *Rhizoctonia solani* is also considered to be a necrotropic pathogen. Wilt fungi including the ascomycete fungi of the genus *Fusarium* are generally considered to be necrotrophic. However, these fungi initially live biotrophically before reaching and colonizing the xylem vessels. Invasion of other tissues occurs only after the xylem vessels have become blocked by fungal and degraded cell wall material causing wilting and cell death. They may therefore be considered closer to biotrophs than necrotrophs (Thaler *et al.* 2004). *Pythium* spp. are oomycetes and as such are taxonomically distinct from fungi, although they use infection strategies that have

much in common (Latijnhouwers *et al.* 2003). Members of this genus exhibit necrotrophic or hemibiotrophic lifestyles.

The molecular mechanisms underlying the defence responses of biotrophic and necrotrophic pathogens have been extensively reviewed by a number of research groups, for example, Hammond-Kosack and Jones (1997), Oliver and Ipcho (2004) and Glazebrook (2005). According to Glazebrook (2005), with respect to several subtleties, in general, gene-for-gene resistance and salicylic acid dependent signalling are effective against biotrophs whereas jasmonate and/or ethelyene signalling is effective against necrotrophic pathogens of *Arabidopsis thaliana.* Oliver and Ipcho (2004) also report the ability of this 'mode-of-defence division' to distinguish necrotroph from biotroph but caution that this limits the biotroph class to those pathogens that possess haustoria.

Most of the work in this field has utilised *Arabidopsis thaliana* and the suite of pathogens to which it is susceptible as a model plant-pathogen system (Glazebrook 2005; Oliver and Ipcho 2004; Thomma *et al.* 2001; Thomma *et al.* 1998; Vijayan *et al.* 1998). However, recent work conducted using *Lycopersicon esculentum* (tomato) reports that the jasmonate response is involved in limiting susceptibility to pathogens from a wide range of taxonomic groupings and lifestyles thus challenging the generalised biotrophic and necrotrophic *Arabidopsis* models of plant defence (Thaler *et al.* 2004). Perhaps this may be because true biotrophs and true necrotrophs are relatively rare with far more pathogens, described by Parbery (1996) as 'predominantly biotrophic hemibiotrophs' or 'predominantly necrotrophic hemibiotrophs', falling somewhere along the continuum between biotroph and necrotroph. Further, not all species within a particular genus will group together on this continuum.

Whilst it is not possible to generalize plant defence responses based on the mode of nutrition of the pathogen or any other predictive indicator, plants are equipped with a host of defence mechanisms. These are activated by salicylic acid, jasmonate and/or ethylene dependant signalling pathways (Fig. 1.1). Understanding which pathway is most important for which pathogen and how the pathogen regulates these pathways will lead to improved crop protection as techniques to accelerate or upregulate plant defence responses are further developed.

1.2 Induced resistance

Induced resistance is a state of heightened defensive capacity in plant species. It can be mediated by biotic and abiotic elicitors (Lyon *et al.* 1995) and results in long-lasting protection against a broad spectrum of microorganisms including viruses, bacteria, fungi and oomycetes (Ryals *et al.* 1996; Sticher *et al.* 1997). The two most clearly defined forms of induced resistance are systemic acquired resistance (SAR) and induced systemic resistance (ISR), which can be differentiated on the basis of the nature of the elicitor and the regulatory pathways involved (Vallad and Goodman 2004).

SAR can be triggered by microbial infection (virulent, avirulent or non-pathogenic microbes) or with chemicals. It results in the accumulation of salicylic acid (SA) and pathogenesis-related proteins and is effective across a wide variety of plant species. By contrast ISR is triggered by plant growth-promoting rhizobacteria (PGPR) (eg. *Pseudomonas* spp, (Zhou and Paulitz 1994)), is mediated by jasmonate and ethylene and the ability of certain PGPR to elicit ISR is specific for certain plant species and genotypes. Common to both of these mechanisms is the lack of any direct antimicrobial effect therefore they offer a means of controlling plant disease without asserting direct selective pressure on pathogen populations (Vallad and Goodman 2004). In

addition, the biological and synthetic elicitors compare very favourably with current pesticides in terms of their environmental impact. For these reasons induced resistance, including SAR and ISR is very compatible with sustainable integrated pest management based approaches to plant disease control.
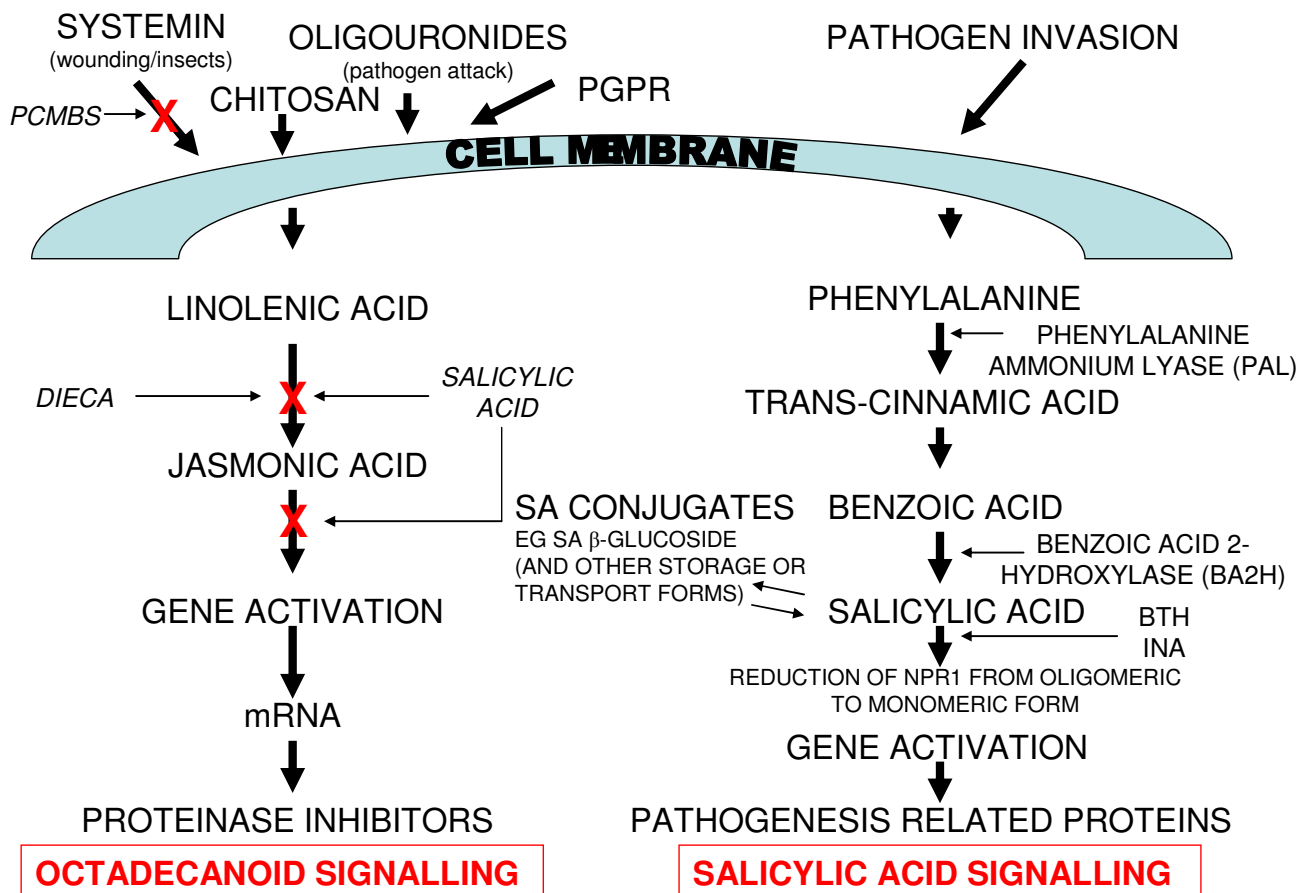
Figure 1.1 Plant defence responses mediated by jasmonic acid via the octadecanoid signalling pathway or by Salicylic acid. Inhibitors (sodium *p*-chloromurcuribenzenesulfonate, PCMBS; sodium diethyldithiocarbamate, DIECA; salicylic acid) shown in italics. (Based on Doares *et al.* 1995; Mauch-Mani and Métraux 1998; Ryals *et al.* 1996).

1.3 Clubroot disease of vegetable brassicas

Clubroot caused by *Plasmodiophora brassicae* a biotrophic obligate plant pathogen, affects the Brassicaceae family of plants including many important vegetable crops. In Australia, clubroot is managed in vegetable brassicas using a combination of integrated control methods and recently introduced resistant varieties (Donald and Porter 2009). However, previous experience has shown

that the genetic variation in field populations of *P. brassicae* may enable the pathogen to overcome the resistance developed and this poses a major and ongoing challenge for the development of clubroot-resistant cultivars (Hirai 2006).

The life cycle of *P. brassicae* occurs in two distinct phases; the primary phase occurs predominantly in root hairs while the secondary phase occurs predominantly in the root cortex (Ingram and Tommerup 1972). Susceptibility to *P. brassicae* is characterised by the development of enlarged, severely malformed roots (clubroots), and physiological and morphological abnormalities that lead to stunting and wilting of the plant. Symptoms are associated with alterations in the synthesis of cytokinins and auxins (Ludwig-Müller and Schuller 2008). However, resistance is characterised by the restriction of the pathogen within a defined area of the infected root, or failure of the pathogen to complete its life cycle within the host.

1.4 Arabidopsis a model plant

The flowering plant *Arabidopsis thaliana* is a weed belonging to the family Brassicaceae (Fig. 1.2). The genome of this species has been fully sequenced and the function of many genes determined therefore it is an important model plant for studying host-pathogen interactions at the genetic, cellular and molecular levels. *A. thaliana* is a host of *P. brassicae* (Koch *et al.* 1991) and presents a unique model system to examine the pathogen's life cycle and the development of the disease in this complex host-pathogen interaction.

1.5 *Arabidopsis-P. brassicae* host-pathogen interaction

A detailed host–pathogen interaction study has been conducted in a known clubroot susceptible *Arabidopsis* ecotype Col-0 with a highly virulent Victorian field population of *P. brassicae* and disease development was monitored at both cellular and molecular levels (Agarwal *et al.* 2009). Pathogen penetration occurred from day 4 onwards and the disease progressed within 28 days from root infection to gall development in *Arabidopsis* hosts (Fig. 1.3). Using real-time quantitative Polymerase Chain Reaction (qPCR) assay and *P. brassicae* specific primers developed by Faggian *et al.* (1999) disease progression was monitored in *Arabidopsis* roots inoculated with *P. brassicae* (Agarwal, 2009). Pathogen was detected at 4 days after inoculation confirming pathogen penetration. Plasmodia were detected within the root hair by day 7 and by day 10 these plasmodia had developed into zoosporangia containing zoospores. These three time points corresponding to important developmental stages within the primary phase of the lifecycle of the pathogen were used in a subsequent microarray study of changes in gene expression during the compatible *Arabidopsis-P. brassicae* interaction.

Figure 1.2 Mature *Arabidopsis thaliana*, a small fast growing weed belonging to the family Brassicaceae. The genome of this species has been sequenced and the function of many genes determined making it a good model plant system to study host-pathogen interactions at the genetic, cellular and molecular levels.

0	4	7	10	15	23	28
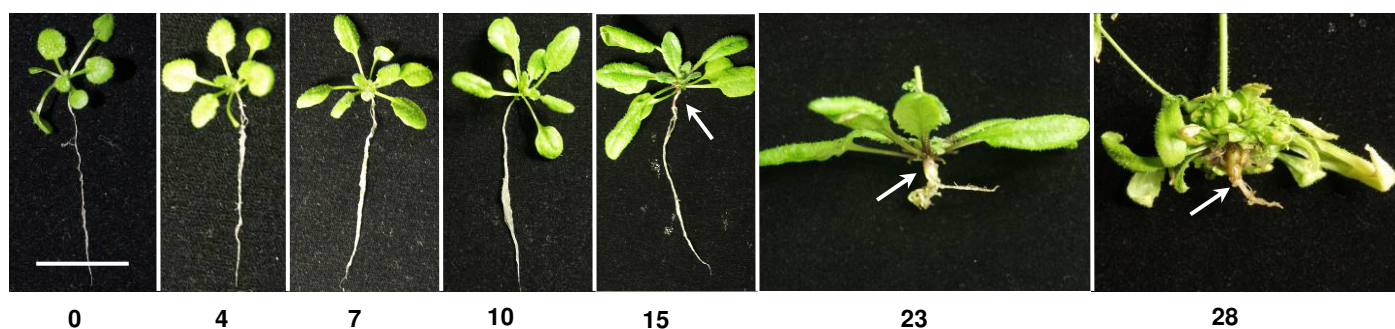
Figure 1.3 Clubroot disease development in a susceptible Arabidopsis ecotype Col-0 between 0 day (14 day old inoculated seedling) and 28 days grown in soil under growth cabinet conditions. Plants were assessed for disease symptoms at 4, 7, 10, 15, 23 and 28 days after inoculation (dai). Root and shoot growth was normal at 4, 7, and 10 dai. Swelling in the hypocotyl was observed 15 dai (arrow) which developed into whitish root galls by 28 days (arrow). Lateral roots were completely destroyed and the whole plant was stressed with a proliferation of multiple rosette leaves at 28 dai. Bar represents 2 cms for all the figures.

1.6 Microarray analysis to study changes in gene expression

Results of the microarray analysis revealed that at day 4 the expression of several genes known to be critical for pathogen recognition and signal transduction in other resistant host pathogen interactions were induced in this compatible interaction. However, genes involved in the biosynthesis of phenylpropanoids, ethylene, cytokinin, reactive oxygen species and a PR protein were repressed. The induction of phenylalanine ammonia lyase (PAL), the first enzyme of phenylpropanoid biosynthesis, is important in SA biosynthesis, as well as in the synthesis of flavonoids and lignins (Zhao *et al.* 2005). Interestingly, at the 4 day time point PAL4 gene involved in the SA biosynthesis pathway was suppressed and the lipoxygenase gene (LOX4) involved in the jasmonic acid biosynthesis pathway was induced. Since the SA pathway was down-regulated in this compatible interaction, manipulation of the SA signaling pathway may enable enhanced resistance to *P. brassicae* in the field.

1.7 Salicylic acid (SA) and systemic acquired resistance (SAR)

The phytohormone salicylic acid (SA) is required for a number of physiological processes within plants but primarily it is an important signalling molecule in plant defence, at both cellular and tissue levels but also systemically (Vallad and Goodman 2004). Salicylic acid is implicated as a signal in defence against pathogens via systemic acquired resistance (SAR), a mechanism of induced defence that confers long-lasting protection against a broad spectrum of microorganisms (Durant and Dong 2004). Salicylic acid, produced endogenously or exogenously applied, is a potent inducer of SAR and can protect plants against pathogens (Thatcher *et al.* 2005).

The phenolic molecule salicylic acid (SA) is synthesised by plants in response to challenge by a diverse range of pathogens and is an essential signalling molecule regulating changes in gene expression. The molecular basis of SA synthesis and regulation of the SA-dependant signalling pathway leading to defense gene expression is reviewed in detail by Durrant and Dong (2004) and Katagiri (2004). Salicylic acid is produced in response to pathogen invasion by conversion of phenylalanine to *trans*-cinnamic acid, catalysed by phenylalanine lyase. *Trans*-cinnamic acid is converted to benzoic acid. Hydroxylation of this molecule, catalysed by benzoic acid 2-hydroxylase, results in the production of salicylic acid (Ryals *et al.* 1996) (Fig. 1.1). Most of the pathogen induced SA is glucosylated to form SA β-glucoside that is sequestered in vacuoles and serves as a readily hydrolysable source of SA (Loake and Grant 2007). Other modified forms such as methylated SA and amino acid conjugated SA have been implicated as transport forms of SA (Loake and Grant 2007).

SAR requires changes in gene expression in plants (Fig. 1.1). Accumulation of SA changes cellular redox potential (Fobert and Després 2005). This triggers reduction of NON-EXPRESSOR of PATHOGENESIS RELATED1 (NPR1) protein from disulphide-bound oligomers to active monomers (Mou *et al.* 2003). These translocate to the nucleus where they interact with TGA transcription factors. These TGA factors bind to SA-responsive elements in the promoters of PATHOGENESIS RELATED (PR) genes resulting in SAR (Grant and Lamb 2006). Activation of the PR genes leads to an increase in the production of antimicrobial pathogenesis-related (PR) proteins throughout the plant (van Loon et al. 2006). The PR-1 proteins are often used as markers for SA signalling and systemic acquired resistance but their biological role is not known. The PR-2 proteins (β-1.3-glucanase) are also used as markers for SA signalling and SAR and play an important role in limiting pathogen activity, growth and spread. The PR-3, -4, -8, and -11 proteins (chitinase) are used as markers for JA signalling and systemic acquired resistance.

1.8 SAR as a crop protection tool

Research directed towards the development of SAR as a means of preventing crop diseases has centred on the use of SA, a derivative, acetylsalicylic acid (aspirin) and several synthetic functional analogues of SA (2,6-dichloroisonicotinic acid (INA) and benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) also known as acibenzolar-S-methyl or benzothiadiazole). Salicylic acid and acetylsalicylic acid as a seed dressing or soil drench have been shown to significantly reduce the percentage root rot incidence at both pre- and post- emergence stages of lupin plant growth in soils artificially infested with *Fusarium solani, Rhizoctonia solani* and *Sclerotium rolfsii* (El-Mougy 2004). In addition to any SAR effects, a direct inhibitory effect of SA and acetyl SA on the growth of these pathogens has been demonstrated (El-Mougy 2002). A significant reduction in *Fusarium* head blight in wheat has been reported following treatment with a sodium salt of SA (Zhang *et al.* 2007). In kiwifruit vines SA caused a 35 % reduction in disease caused by *Sclerotinia sclerotiorum* (Reglinski *et al.* 2001). Elsewhere exogenous application of SA failed to induce systemic resistance to cucumber root rot caused by *Pythium aphanidermatum* (Chen *et al.* 1999). SA is available in Australia as ReZist® (Stoller) and has been used effectively to induce SAR in melon trials (McConchie 2007).

Use of the synthetic functional analogues INA and BTH has been reviewed by Vallad and Goodman (2004). This work reviews 37 field studies using a range of host-pathogen pairs. With the exception of only two studies (both increased the severity of late leaf spot caused by *Cercosporidium personatum* in peanut), application of either INA or BTH reduced disease relative to the untreated control by between 4 and 99 %.

SAR responses elicited by INA or BTH have been demonstrated to be effective against a number of soilborne pathogens for a range of host crops. White mold (caused by *Sclerotinia sclerotiorum*) of soybean was reduced by 46 and 59 % using INA and BTH respectively to elicit SAR (Dann *et al.* 1998). A significant reduction in *Fusarium* head blight in wheat has also been reported following treatment with INA or β-amino-*n*-butyric acid (BABA), another functional analogue of SA (Zhang *et al.* 2007). In tomato plants BTH protects plants against disease caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* by restricting the pathogen to the epidermis and outer cortex through the formation of callose-enriched wall appositions at sites of fungal invasion (Benhamou and Bélanger 1998).

Phytotoxicity issues have hindered the commercial development of INA. However, BTH has been commercialized as Bion® (Syngenta). This product is available in Australia and it is currently marketed as a seed treatment to the cotton industry.

1.9 Aims of the current study

The current study was undertaken to determine whether there is potential for SAR to be developed as a disease prevention strategy in vegetable brassicas. The work progressed through several stages:
1. Proof-of-concept using the *Arabidopsis* model plant
2. Demonstration of SAR in a vegetable brassica crop (broccoli-*P. brassicae* host-pathogen system)
3. Optimisation of rates, timing and methods of application in broccoli
4. Evaluation of efficacy in other vegetable brassicas
5. Evaluation of efficacy against other pathogens of brassica crops (*Albugo candida*, a foliar pathogen causing white blister and *Rhizoctonia solani* a necrotrophic soilborne pathogen).

Proof-of-concept study

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2. Proof-of-concept study

A proof-of-concept study was conducted using the model plant *Arabidopsis thaliana* ecotype Col-0 to investigate the effect of SA as an inducer of systemic acquired resistance and determine the effectiveness of this response against clubroot. Application of 0.5 mM salicylic acid as a 1 minute root dip reduced the percentage of plants displaying visual symptoms of clubroot disease by half and the severity of symptoms (expressed as a disease index) from 81.5 out of a possible 100 to 20. This is the first evidence of successful SAR induction reported against clubroot.

2.1 Materials and methods

2.1.1 Inoculum preparation - *P. brassicae* spore suspension

Plasmodiophora brassicae inoculum was prepared from mature galls on *Brassica oleracea* (broccoli) roots collected from a vegetable farm in Werribee South, Victoria. Root material was stored at -20 ^{o}C (Department of Primary Industries, Knoxfield, Victoria) for over 5 years. The ECD code (pathotype) 16/02/31 has been previously reported for this population (Donald *et al*. 2006). Galls picked from 2 to 3 different broccoli plants were used for the extraction of resting spores by homogenisation in deionised water (1:3) (w/v) followed by filtration of the crude extract through a double gauze filter (25 µm pore width). Spore density was determined using a haemocytometer and suspensions of 10^{8} spores/mL in water were prepared to be used for inoculation.

2.1.2 Plant growth, inoculation and SA treatment of Arabidopsis plants

Arabidopsis seeds of ecotype Col-0 were germinated and grown within pots in seed raising mix (Debco, Australia). The seeds were stratified at 4°C in the dark for 4 days then pots were transferred to a growth room with controlled environment conditions at 20°C, with 75% relative humidity and a 16-h photoperiod at 100 µE/m^{2}.s. Fourteen-day-old seedlings were thinned to 1 seedling per pot (60-mm-diameter pots) and 20 pots arranged randomly within trays. For treatment with salicylic acid, 14 day old seedlings were carefully removed from their pots, gently washed and dipped in 0.5 mM unbuffered salicylic acid (BDH Chemicals, Australia) for 60 seconds before being transferred back to soil. After 4 h equilibration in the growth chamber one third of the plants were either i) inoculated with 200 µL suspension of *P. brassicae* resting spores (10^{8} spores/mL) or ii) mock-inoculated with 200 µL of sterile distilled water. Another subset of control plants, which were not treated with salicylic acid, was also inoculated with *P. brassicae* as described above. All the three trays set up with plants were left in the growth cabinet (conditions described above) to grow for 50 days.

2.1.3 Phytopathological analysis of Arabidopsis plants infected with *P. brassicae*

Disease was assessed 50 days after inoculation by visually examining all 20 plants within a treatment group for symptoms of disease. Fresh shoot weight was also recorded. The plants were gently removed from soil, roots washed with water and visually assessed. Disease Index for *Arabidopsis* (DI_A) was calculated as a function of both disease incidence and disease severity.

Disease severity was based on a scale consisting of five classes (0–4) according to published protocols (Kobelt *et al.,* 2000) where: 0 = no symptoms; 1 = very small clubs mainly on lateral roots, that do not impair the main root; 2 = small clubs covering the main root and few lateral roots; 3 = medium-sized to bigger clubs, also including the main root (up to 2/3), plant growth might be impaired; and 4 = severe clubs in lateral roots and main root, fine roots completely destroyed, plant growth also affected. The disease index in *Arabidopsis* (DI_A) was calculated using the five-grade scale according to the formula: $DI_A = (0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4) \times 100/4N_t$, where n_0 to n_4 is the number of plants in the indicated class and N_t the total number of plants tested. Depending on the DI calculated, the plant was classified as resistant ($DI_A = 0$) or susceptible ($DI_A > 30$). A DI_A of 100 = full susceptibility and $0 < DI < 30$ = partial resistance (restriction of pathogen growth in host) or tolerance (no restriction of pathogen but no yield loss). The percentage of infected plants (disease incidence) was calculated by dividing the total number of plants infected by the total number of plants tested.

2.1.4 Statistical analyses

The statistical software GenStat (12th edition) was used for statistical analysis. Analysis of Variance was used to determine the effect of SA treatment on clubroot disease. An LSD (least significant difference) was calculated when differences were significant (p = 0.05) to compare treatment means.

2.2 Results and Discussion

Clubroot disease was strongly suppressed in SA treated *Arabidopsis* plants (Table 2.1). SA treated plants infected with *P. brassicae* were much healthier compared with infected untreated plants, which had high levels of clubroot infection (Fig. 2.1). Fifty days after inoculation only 50% of the SA treated plants displayed visual symptoms of infection (Table 2.1). Symptoms of clubroot were mild in 20% of SA treated plants and only 30% of the plants exhibited severe clubroot symptoms. The SA treated plants had a disease index (DI_A) of 20 out of a possible 100, and a higher shoot weight of 9.5 gm. By comparison all the untreated plants were severely infected, stunted in growth with multiple rosette leaves. Untreated plants had a much higher disease index of 81.5 and lower shoot weight of 8 gm compared with treated plants. However, SA treated plants were delayed in flowering showing a reduction in the number of inflorescence stalks to 2 or 3 per plant compared with untreated control plants which produced 4 or 5 stalks per plant.

This study of the effects of SA treatment of *Arabidopsis* plants on subsequent clubroot disease development confirms the outcome of a previous study of gene expression in the compatible *Arabidopsis*-*P. brassicae* host-pathogen system (Agarwal, 2009). In that study genes for salicylic acid biosynthesis were down-regulated four days after inoculation. It was proposed that through exogenous application of SA the opposite effect (ie. an incompatible host-pathogen interaction) might result from SA mediated systemic acquired resistance (SAR). This is the first evidence of successful SAR induction reported against clubroot.

Table 2.1. Disease severity, disease index and percentage of plants with visual symptoms of clubroot after a 1 minute root dip in 0.5 mM salicylic acid

Treatment	Disease severity (0-4)	Disease Index	Disease incidence (%)
Control (uninoculated)	0	0	0
- SA (inoculated)	3.5	81.5	100
+ SA (inoculated)	0.8	20	50
lsd (p=0.05)	0.2	na	na

na – statistical analysis not applicable.

Figure 2.1 Disease assessment of salicylic acid treated *Arabidopsis* plants 50 days after inoculation with *P. brassicae*. Comparison of clubroot symptoms in roots and shoots (A) and plant vigour (B) - uninoculated control plants, inoculated untreated plants and inoculated SA treated plants (left to right). Bar represents (A) 1 cm and (B) 5 cm.

2.3 Conclusions

This is the first evidence of successful SAR induction reported against clubroot. Application of 0.5 mM salicylic acid as a 1 minute root dip reduced the percentage of plants displaying visual symptoms of clubroot disease by half and the severity of symptoms (expressed as a disease index) from 81.5 out of a possible 100 to 20.

Development and optimisation of a SAR induction system for broccoli

3. Development and optimisation of a SAR induction system for broccoli

The second stage of the work required that the proof-of-concept study be extended to a vegetable crop, broccoli. In initial experiments seedlings were washed and dipped for one minute in salicylic acid and inoculated 4 hours later. Whilst this method was effective for *Arabidopsis* with its very fine root system, one minute exposure was not sufficient for the thicker broccoli roots. In order to achieve SAR response in broccoli initially a range of trials investigating rate (0.1 – 1.0 mM), methods of application (seedling tray dips, drenches and foliar sprays) and timing (single dip and triple dip) of the inducer were conducted. A single 0.1 mM SA root dip applied for 15 minutes 24 hrs before transplanting significantly reduced the severity of symptoms of clubroot in broccoli plants when inoculated with a low rate of inoculum (10^4 spores/ml *P. brassicae*). Application of the triple dip (three applications 72, 48 and 24 hrs before transplanting) improved the efficacy of treatment at the highest rate of inoculum. Symptoms of disease were significantly reduced using the triple dip treatment at the highest rate of inoculum (10^6 spores/mL). The systemic nature of the effect was confirmed by the upregulation of three genes (chitinase, PR-1 and PR-2) known to be associated with plant defence responses in root and/or leaf tissue after SA treatment. There was considerable variation in the level of upregulation between replicates. This variation was lowest in samples collected 72 hours after SA treatment. Likewise, variability of effect within a treatment group was a consistent feature of disease assessments and the number of replicates of each treatment had to be increased to 10 to accommodate this variability. There was evidence that the 0.1 mM rate is marginal and a higher rate, closer to 0.5 mM is more likely to be consistently effective.

3.1 Materials and methods

3.1.1 Seedling growth, inoculation and general experimental conditions

Resting spore suspensions of *P. brassicae* (the inoculum) were prepared as described previously (2.1.1) and used in preliminary experiments. For subsequent optimising experiments *P. brassicae* inoculum was prepared from mature galls on *Brassica oleracea* (cabbage) roots collected from a vegetable farm in Lindenow, Victoria. Spore density was determined using a haemocytometer and suspensions of 10^8 spores/mL in water were prepared and stored at -20^oC for use in later experiments.

Broccoli seeds were germinated in cell trays. Each tray contained 48 cells (4 cm x 3.5 cm in size) filled with steam sterilized vegetable seed raising mixture (Biogro, Bayswater). Broccoli cv. Greenbelt was used in the preliminary experiments and cv. Marathon was used in all subsequent optimising trials reported in this chapter. Treatments were applied to ten to fourteen day old broccoli seedlings. SA solutions were prepared by dissolving SA (BDH chemicals) in hot deionised water with stirring. SA solutions used in the preliminary experiments were not neutralised. The pH of the SA solution was neutralised (pH 7) in subsequent optimising experiments to eliminate any direct effects on the pathogen or host of an acidic solution. Treated and untreated control seedlings were planted into pots containing vegetable seed raising mixture (Biogro, Bayswater VIC) and were inoculated after treatment by pipetting 200μL of *P. brassicae* spore suspension (containing 10^5-10^8 resting spores/mL) into a small depression at the base of the seedling. Each experiment consisted of between 4 and 20 replicates per treatment.

The experiments were set up either in a growth room with controlled environment conditions (25°C with a 12-hr photoperiod) or in a glasshouse (25°C, natural day and night cycle).

Plants raised in the growth room were placed in trays which retained any water running through pots and ensured that the pots remained moist, increasing the likelihood of infection by *P. brassicae*. Plants raised in the glasshouse were watered for 1 minute twice daily and the pots drained naturally.

3.1.2 Disease assessment

Disease was assessed six weeks after inoculation by examining all the plants within a treatment group. The plants were removed from soil, roots washed with water and visually assessed using a 0-9 severity scale: (0) no visible root galling; (1) single small gall on lateral roots; (2) 2-3 small galls on lateral roots; (3) multiple small galls on lateral roots (plant appears healthy); (4) mild galling of the taproot, multiple small galls on lateral roots; (5) moderate galling of the taproot, many small or several large galls on lateral roots; (6) severe galling of the taproot, many large galls on laterals; (7) severely galled, several healthy roots remaining; (8) severely galled, few healthy roots remaining; (9) severely galled, no healthy roots present.

3.1.3 Extraction of RNA, cDNA synthesis and gene expression analysis

For gene expression studies root and/or leaf samples were collected from 3 plants in the preliminary experiments and 5 plants in later optimising experiments between 24 and 72 hours after SA treatment for RNA extraction. Initially a liquid nitrogen method was used for RNA extraction. This method was subsequently replaced by a Mackenzie buffer method (see below) to avoid problems caused by the inconsistent supply of liquid nitrogen to the laboratory.

Liquid nitrogen RNA extraction
Root samples were washed and flash-frozen in liquid nitrogen. Total RNA was isolated from roots that had been stored at -80°C. RNAqueous RNA isolation kit (Ambion) was used for total RNA isolation. RNA was isolated from each sample of 3 treated replicates and one control. Firstly RNA was treated with DNase1 (DNA-*free*^{TM} Kit, Ambion) to get rid of the DNA contamination and then reverse transcribed into cDNA (High Capacity cDNA Reverse Transcription kit, Applied Biosystems) for quantitative real-time reverse transcriptase PCR (RT-qPCR). RT-qPCR was performed according to the protocol described below using only two sets of primers - the reference gene (Actin 8) and the chitinase gene (Table 3.1).

Mackenzie buffer RNA extraction
Soil particles were removed from the roots by gently washing the root system in water. Roots (midsection of the root system) and leaves (one true leaf) were harvested pairwise from the same plant and kept in a prelabelled extraction bag (Bioreba). Two mL of McKenzie buffer and 20 µL of Mercapto-ethanol were added to each bag in the fume hood. The homex grinder (Bioreba) was used for grinding the plant tissue sample in the bag and the bags then stored at -80°C for future RNA extraction. RNA was isolated from each sample for 5 replicates per treatment from roots and leaves that had been stored at -80°C. To precede with RNA extraction bioreba bags containing ground samples were thawed at room temperature. Once thawed in the fume hood 900 µL of the sample was transferred to a 1.5 mL centrifuge tube to which was added 100 µL of 20% Sarkosyl (lauryl sarcosine sodium salt) and the tube shaken to mix the contents. The tubes were then

incubated at 70°C for 15 minutes with intermittent shaking followed by centrifugation for 2 minutes at maximum speed. Then 450 µL of the supernatant from each tube was mixed with 225 µL of 100% ethanol and transferred to the Filter Cartridge (which came with the Ambion RNAqueous kit) placed in a 2 mL collection tube (maximum volume 700 uL) and centrifuged for 1 min at maximum speed. From this step onwards RNA was extracted following the manufacturers instructions (RNAqueous, Ambion). The Filter Cartridge was washed once with 700 µL of Wash Solution #1 and twice with 500 µL of Wash Solution #2 each followed by 1 minute centrifugation. RNA was eluted in a pre-heated (70°C) elution solution (50-60 µL). The quantity and the integrity of all the RNA samples were determined using a spectrophotometer (NanoDrop Technologies, Wilmington, USA). Samples were stored at -20°C.

RNA samples stored previously at -20°C were treated with DNase 1 (DNA-*free*™ Kit, Ambion) to get rid of the DNA contamination and then synthesized into cDNA (High Capacity cDNA Reverse Transcription kit, Applied Biosystems). Samples of cDNA were quantified by spectrophotometry (NanoDrop Technologies) and stored as above. Gene specific primer sequences for all the 4 target genes (PR-1, PR-2, Chitinase and PAL) and one reference gene (Actin 8) are listed in Table 3.1.

Quantitative real-time reverse transcriptase PCR (RT-qPCR)

A serial dilution was prepared from a control sample to obtain final concentrations of 500 ng/µL, 250 ng/µL, 125 ng/µL and 62.5 ng/uL of cDNA for use as standards. The PCR reaction was set up in a 25 µL volume containing 12.5 µL of SybrGreen (Qiagen), 1 µL each of forward and reverse primers (30 ng each), 5.5 µL of RNA free water (Ambion) and 5 µL of the template cDNA at a concentration of 100 ng/µL. A no template control was used as negative control to check for contamination. All the samples were set up in duplicates or triplicates using 1 reference gene and 3 or 4 target genes in the same run. Real-time Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR) was performed in a Rotor–Gene Q (Qiagen, Australia). Cycling conditions were 95°C for 15 minutes of initial denaturation followed by 40 cycles of amplification in a three step procedure; 40 s at 95°C, 30 s at 55°C and 30 s at 72°C, and 1 three step cycle of product melting (60-92°C with 30 s hold on first and next steps). The Delta Delta C_t method was used for calculating the relative fold change of a target gene in comparison with a reference gene. The fold change of reference gene in control is calculated as 1.

Table 3.1 Primers designed for SAR gene expression study

Gene	Gene ID	Sequence size	Product size	Primer sequence (5' to 3')
Actin 8	*A. thaliana* At1g49240	476	133	Forward TGC AGA CCG TAT GAG CAA AG Reverse CTG GAA AGT GCT GAG GGA AG
Chitinase	*B.oleracea* 1 AF261098	392	133	Forward CAC CAG GCA AAG GCT ACT TC Reverse GCG ACA GTT GGG TTG CTA CT
PAL	*B.oleracea* AB281591	486	129	Forward AAC GGT CTT CCT TCG AAC CT Reverse ATG GCT AGT CAC TGG GTT GG
PR-1	*B.oleracea* EF423806.1	556	105	Forward GCG ACT GCA GAC TCG TAC AC Reverse TCT CGT TGA CCC AAA GGT TC
PR-2	*B.oleracea* EF484879	1277	111	Forward ACA TTC ATG GGA GCC TTC AC Reverse AGA TCG CTC GCT TAC CAA GA

3.1.4 Statistical analyses

The statistical software GenStat (12th edition) was used for statistical analysis in all the experiments reported. Analysis of Variance was used to determine the effect of SA treatment, timing and rate of inoculum on clubroot disease. An LSD was calculated when differences were significant ($p = 0.05$) to compare treatment means.

3.1.5 Preliminary experiments

Seven preliminary experiments were conducted primarily to develop a method of application for SA that would induce SAR and suppress disease in broccoli but also to evaluate a range of potential SAR inducers (Table 3.2). All of the experiments were conducted in a glasshouse and plants in all trials were inoculated by pipetting 200 μL of resting spore suspension of *P. brassicae* into a small depression in the soil at the base of the seedling.

Treatment efficacy was measured as a reduction in the severity of symptoms of disease (3.1.2). SAR induction was confirmed by measuring changes in the expression of the chitinase gene, a gene known to be associated with plant defence responses (3.1.3).

Proof-of-concept studies (chapter 2) were conducted using *Arabidopsis* which is a small weedy *Brassica* plant with a very fine root system. Since broccoli roots are much thicker than those of *Arabidopsis* the rate of SA was doubled to 1 mM in the first experiment compared to the effective rate for *Arabidopsis* (0.5 mM). All other variables including the method and duration of

dip application (1 minute dip applied to washed roots) remained as per the proof-of-concept study (2.1.2). In subsequent experiments a range of treatment methods were evaluated including root drenches, leaf sprays and direct incorporation into the soil (Table 3.2). The duration of the SA root dip was increased to 15 mins to facilitate greater penetration of the SA into the broccoli roots and the rate of SA was reduced to minimise phytotoxicity.

Several other potential SAR inducers (mostly commercially available products) were also evaluated. ReZist™ (Stoller Enterprises Inc) is promoted in the USA as a product that 'increases the natural ability all plants have to withstand stress'. It contains 1.75% copper, manganese and zinc together with unspecified polyamines and plant extracts. Bion® (Syngenta) is promoted as a 'plant activator'. It contains 500 g/L acibenzolar-s-methyl (a functional analogue of SA). This product is available in Australia and it is currently marketed as a seed treatment to the cotton industry. Liquid chitin (Ellis and Associates) and chitosan (Sigma) are complex carbohydrate elicitors derived from shrimp and crab processing wastes.

Table 3.2. Details of preliminary experiments conducted to develop a suitable method of SAR induction in broccoli and evaluate a range of potential SAR inducers.

Expt. no.	SAR elicitor	Rate	Method of application	Duration of dip applications	Rate and timing of inoculation	Number of replicates	Sample collection for gene expression
1	Salicylic acid[A]	1 mM	Root dip, washed roots (as per 2.1.2)	1 min	10^8 sp/mL, 4hrs post trt	5	3
2	ReZist™ (Stoller)[B] Salicylic acid	0.1% 0.1 mM	Root drench 5 mL/plug	na	10^6 sp/mL	20	3
3	ReZist™ (Stoller)	0.1%	1, 2 or 3 leaf sprays applied weekly	na	10^5 sp/mL, 7 days after 1st spray	18	not measured
4	Bion® (Syngenta)[C]	0.01% 0.001%	Root drench 5 mL/plug	na	10^5 sp/mL, 3 days post trt	5	3
5	Salicylic acid	0.1 mM 0.25 mM 0.5 mM 1.0 mM	Root drench 5 mL/plug	na	10^5 sp/mL, 3 days post trt	5	3
6	Salicylic acid	0.1 mM 0.25 mM 0.5 mM 1.0 mM	Root dip in seedling trays	15 mins	10^5 sp/mL, 24, 48 or72 hrs post trt.	4	1
7	Chitosan (Sigma)[D] Liquid chitin (Ellis and associates)[D]	1 g/kg 2.5 g/kg 5 g/kg 0.5% 1.5% 3%	Incorporated into soil	na	10^6 sp/mL, 1 week post planting into treated soil	4	not measured

[A] Preliminary experiments were conducted using unamended salicylic acid. In all subsequent optimising experiments (3.2.2) the SA solution was neutralised to pH 7 before application to eliminate the possibility of any direct effect on the host or pathogen resulting from the acidity of the solution.
[B] Contains 1.75% copper, manganese and zinc together with unspecified polyamines and plant extracts.
[C] Contains 500 g/L acibenzolar-s-methyl and is a functional analogue of SA.
[D] Complex carbohydrate derived from shrip/crab shells.

3.1.6　Optimising salicylic acid dip rates for induction of SAR in broccoli

Trays containing ten to fourteen day old broccoli seedlings cv. Marathon in cell plugs were dipped for 15 minutes in 0.1, 0.2, 0.3, 0.4 or 0.5 mM SA solution (pH 7). Control trays were dipped in water for 15 minutes. Twenty-four hours after SA treatment 3 roots and 3 leaf samples were collected from plants within each treatment group. The expression of the chitinase gene, a gene known to be associated with SAR, was studied (for method see above 3.1.3). The experiment was conducted twice. In the first instance root and leaf samples were collected but these were not from the same plant and only one replicate was tested. In the second trial three replicates of paired root and leaf samples (ie. samples from the same plant) were used to study gene expression. Four replicate plants per treatment were maintained and visually assessed for symptoms of disease six weeks after inoculation (see 3.1.2).

3.1.7　Optimising the timing and method of applying SA dips

Trays containing ten to fourteen day old broccoli seedlings cv. Marathon in cell plugs were dipped for 15 minutes in 0.1 mM SA solution (pH 7) once (single dip, 24 hrs before transplanting and inoculation) or three times (triple dip, 72, 48 and 24 hrs before transplanting and inoculation). Plants were inoculated by pipetting 200 µL of *P. brassicae* spore suspension containing 10^4, 10^5 or 10^6 spores/mL into a small depression in the soil at the base of the seedling immediately after transplanting into pots. Root and leaf sample pairs collected from the same plant (5 replicate plants) were harvested 24, 48 and 72 hours post treatment and processed as described previously (3.1.3) for gene expression studies. In addition to the chitinase gene (reported previously 3.1.6) two new primer pairs for PR-1 and PR-2 defence related genes were designed from the *B. oleracea* genome sequence. The PR-1 proteins are often used as markers for SA signalling and the induction of this gene would help in confirming the up-regulation of SA pathway.

Remaining seedlings were planted into pots filled with steam sterilized vegetable seed raising mixture (Biogro, Bayswater) and inoculated with 200 µL of *P. brassicae* spore suspension using the same spore densities (10^4-10^6 spores/ml). Pots were placed in a completely randomised block design in a controlled environment growth room.

A further two trials were conducted in the controlled environment growth room using 10-14 day old broccoli cv. Marathon seedlings. The first trial was identical to the one described above. It was conducted to confirm the initial results. The second trial was conducted to determine the effect of the time between treatment and inoculation. Plants were treated with a single 15 minute dip in 0.1 mM SA and inoculated using spore densities 10^4-10^6 spores/ml 24, 48 and 72 hr after treatment.

There were 10 replicates of each treatment in each trial. Disease development was assessed 6 weeks after inoculation (for method see 3.1.2) and compared with untreated inoculated control plants. Statistical analysis was conducted as described in section 3.1.4.

3.1.8 Establishing direct and indirect effects of organic amendments

The potential for organic amendments to be used to enhance SAR was determined in a series of glasshouse trials. Organic amendments could induce SAR directly (ie these products may induce a SAR response) or indirectly (by enhancing the activity of chemical inducers). These two possibilities were examined in separate experiments.

(i) Direct SAR induction

Broccoli seedlings cv. Marathon were dipped for 24 hours in 4 different organic amendments (10 % liquid chitosan (Ellis and associates), 10 % fish emulsion (Organic Excel-crop), 0.5 % liquid silicon product (Stand SKH, Agrichem), 10 % liquid seaweed (Seasol)). Treated and untreated seedlings were transferred into pots containing pasteurised vegetable seed raising mix (Biogro, Bayswater, VIC) and inoculated with 200 µL *P. brassicae* spores (10^7 spores/mL). Disease severity was assessed 6 weeks after inoculation (for method see 3.1.2). This trial consisted of 10 replicates of each treatment.

(ii) Indirect SAR induction

Broccoli seedlings cv. Marathon were dipped for 24 hours in 0.1 mM salicylic acid and transferred into pots containing pasteurised vegetable seed raising mix (Biogro, Bayswater, VIC) containing one of the four organic amendments (200 ml of 5 % liquid chitosan (Ellis and associates), 1 % fish emulsion (Organic Excel-crop), 1 % liquid silicon product (Stand SKH, Agrichem), or 5 % liquid seaweed (Seasol) per kg soil) and *P. brassicae* spores (10^7 spores/mL) mixed in the soil. Untreated seedlings were also transferred into pots containing the same soil mix with inoculum and served as control. Disease severity was assessed 6 weeks after inoculation (for method see 3.1.2). This trial consisted of 10 replicates of each treatment.

3.1.9 Confirming SAR induction in older seedlings

For speed and convenience, all of the glasshouse and controlled environment trials used during development and optimisation of the induction method were conducted using young (10-14 day old) seedlings. Commercially grown *Brassica* seedlings are significantly older than this when sent to farms for planting (approx 4-6 weeks). Separate glasshouse trials were conducted using commercial age (6 week old) and older (11 week old) broccoli cv. Marathon seedlings to determine whether SAR could be induced in older plants.

Broccoli seedlings cv. Marathon were dipped in 0.1 mM SA (pH 7) once (single dip, 24 hrs before transplanting) or three times (triple dip, 72, 48 and 24 hrs before transplanting). Six week old seedlings were dipped for 15 minutes but the duration of the dip was increased from 15 mins to 1 hour to allow penetration of SA into thicker roots of older (11 week old) seedlings. Treated seedlings were transferred into pots containing pasteurised vegetable seed raising mix (Biogro, Bayswater VIC) and inoculated with 200 µL *P. brassicae* spores (10^4 - 10^6 spores/mL). The trials consisted of 10 replicates per SA/inoculum density treatment combination. Disease development was assessed 6 weeks after inoculation (as described in section 3.1.2) and compared with untreated inoculated control plants. Statistical analysis was conducted as described in section (3.1.4).

3.2 Results and Discussion

A large number of trials (16) have been conducted. These trials can be classed broadly as those directed towards demonstrating a SAR effect in broccoli (the preliminary trials) and those directed towards optimising rates, method and timing of application. Results from these trials are summarised in Tables 3.3 and 3.4.

3.2.1 Preliminary experiments

In initial experiments seedlings were washed and dipped for one minute in salicylic acid following inoculation after 4 hours. Whilst this method was effective for *Arabidopsis* with its very fine root system, one minute exposure was not sufficient for the thicker broccoli roots (expt 1, Table 3.3). The first evidence of SAR induction in broccoli plants was confirmed in broccoli seedlings dipped in 1 mM SA for 15 minutes. In these plants expression of the chitinase gene increased in roots by up to 42 fold three days after treatment and disease was suppressed even at 0.1 mM SA, the lowest rate used (expt 6, Table 3.3 & Fig 3.1). At 1 mM SA symptoms of phytotoxicity were observed and the level of disease control was reduced compared to the lower rates (expt 6, Table 3.3 and Fig 3.1). Rates of SA were therefore reduced to 0.1 – 0.5 mM in a subsequent trial (3.2.2) and neutralised SA (pH, 7) was used to eliminate any possible direct effects of the acid solution on the host plant or pathogen.

There was no significant effect of Bion® or ReZist™ at the rates and methods of application used. The experiment with chitin and chitosan was inconclusive as there was no disease expression even in the inoculated control plants (Table 3.3). It became evident that there were potentially an infinite number of inducers, rates, methods and duration of treatment that could be investigated. A decision was taken to seek to optimise the effect using one product, salicylic acid. Root dips were used in subsequent optimising experiments as liquid treatments delivered as leaf sprays tended to run off the waxy broccoli leaf surface and it was therefore impossible to estimate the actual rate of SA applied. Likewise it was difficult to be certain how much SA was penetrating through the growing medium to the root surface when seedling drenches were used. All subsequent trials were conducted by dipping seedling roots contained in cell trays in the SA solution.

Table 3.3. Summary of results of preliminary experiments conducted to demonstrate SAR induction in broccoli cv. Greenbelt.

Expt. no.	SAR elicitor	Rate	Method of application	Disease severity (0-9)			Fold change in chitinase gene expression
1	Salicylic acid[A]	1 mM Inoculated control	Root dip, 1 min	All plants severely diseased			Inconclusive[*]
2	ReZist™ (Stoller)[B] Salicylic acid (pH7) 	0.1% 0.1 mM Inoculated control lsd (p≤0.05)	Root drench 5 mL/plug	4.1 3.8 6.3 0.7			No change in roots
3	ReZist™ (Stoller)	0.1% 1 spray 0.1% 2 sprays 0.1% 3 sprays Inoculated control lsd (p≤0.05)	1, 2 or 3 leaf sprays applied weekly	2.5 1.9 1.8 2.5 0.6			not measured
4	Bion® (Syngenta)[C]	0.01% 0.001% Inoculated control	Root drench 5 mL/plug	9 9 9			No change in roots
5	Salicylic acid[A]	0.1 mM 0.25 mM 0.5 mM 1.0 mM Inoculated control	Root drench 5 mL/plug	0 0 0 0 0			No change in roots or leaves

Expt. no.	SAR elicitor	Rate	Method of application	24hr	48hr	72hr	Fold change in roots 3 days after SA trt.
6	Salicylic acid[A]	0.1 mM 0.25 mM 0.5 mM 1.0 mM Inoculated control lsd (p≤0.05)	Root dip in seedling trays	1.0 1.0 0.8 1.8 4.5 2.5	0.3 2.0 2.3 2.0 4.5 2.5	0.3 0.5 1.3 1.8 3.5 2.5	1.6 3.4 13 42 1

Expt. no.	SAR elicitor	Rate	Method of application	Disease severity (0-9)			Fold change in chitinase gene expression
7	Chitosan (Sigma)[D] Liquid chitin (Ellis and associates)[D] 	1 g/kg soil 2.5 g/kg soil 5 g/kg soil 0.5% 1.5% 3% Inoculated control	Incorporated into soil	0 0 0 0 0 0 0			not measured

[A] Preliminary experiments were conducted using unamended salicylic acid. In all subsequent optimising experiments (3.2.2) the SA solution was neutralised to pH 7 before application to eliminate the possibility of any direct effect on the host or pathogen resulting from the acidity of the solution.
[B] Contains 1.75% copper, manganese and zinc together with unspecified polyamines and plant extracts.
[C] Contains 500 g/L acibenzolar-s-methyl and is a functional analogue of SA.
[D] Complex carbohydrate derived from shrip/crab shells.
[*] Primers designed for *Arabidopsis* did not work for broccoli. New primers were designed and used in all subsequent trials.
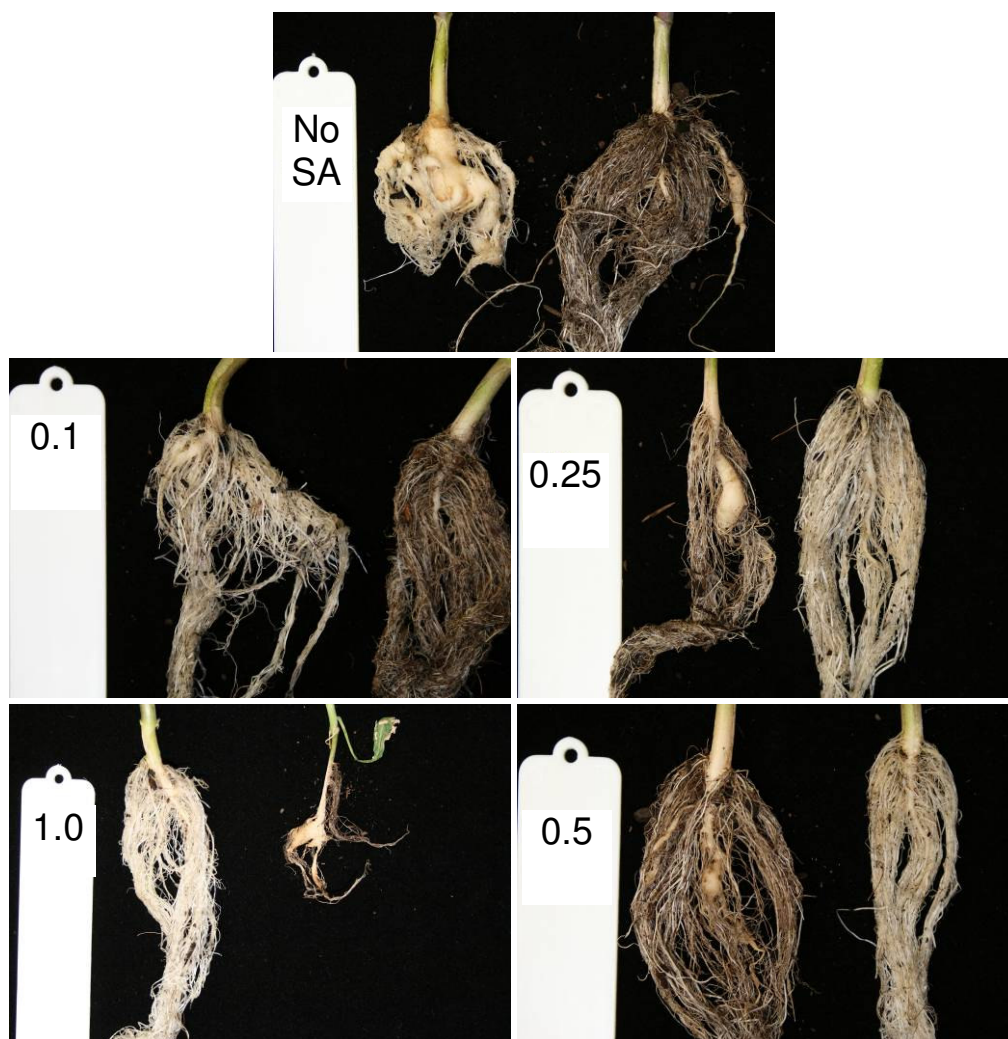
Figure 3.1. Disease assessment of broccoli cv. Greenbelt plants 6 weeks after inoculation with *P. brassicae* spore suspension. Plants were treated with (clockwise from top left 0.1, 0.25, 0.5 and 1 mM unbuffered SA - 15 minute root dip) 24 hours before inoculation (Preliminary expt 6). Each image shows the range of symptoms within a treatment group. The untreated plants showed severe root galling.

Table 3.4 Summary of results of optimising experiments using SA (pH 7) to induce SAR in broccoli cv. Marathon.

Purpose	Crop age when treated	Conditions	Treatment/s	Outcome
Optimise dip rates	10-14 day	Controlled environment with RT-qPCR analysis of chitinase gene	0.1, 0.2, 0.3, 0.4 & 0.5 mM SA, 15 min root dip	Chitinase gene expression consistently increased in roots & leaves at 0.1 mM SA.
Optimise method of application	10-14 day	Controlled environment with RT-qPCR analysis of PR-1, PR-2 and chitinase genes	Single dip – 0.1 mM SA, 15 minute dip applied 24 hours before transplanting Triple dip – 0.1 mM SA, 15 minute dip applied 72, 48 and 24 hours before transplanting	Disease suppression at low to moderate (10^4 spores/ml) and moderate to high (10^5 & 10^6) spore densities for single and triple dip respectively. PR-1, PR-2 and chitinase defence genes expressed in roots and leaves of plants from both treatment groups.
Optimise timing of application	10-14 day	Controlled environment with RT-qPCR analysis of PR-1, PR-2 and chitinase genes	Single & triple dips (0.1 mM SA applied for 15 mins (as above) for gene expression study	Gene expression was maximum 72 hours after treatment indicating that a single dip may be as effective as triple dip if the treatment is applied earlier (ie. 72 hours before inoculation).
Confirm disease suppression in older seedlings	6 week	Glasshouse	Single & triple dips (0.1 mM SA) applied for 15 mins as described previously.	Disease significantly (p=0.05) reduced in plants inoculated at the lowest rate of inoculum using the single and triple dip treatment.
	11 week	Glasshouse	Single & triple dips (0.1 mM SA) applied for 1 hour as described previously. Note duration of dip increased from 15 mins to 1 hour to allow penetration of SA into thicker roots of older seedlings (previous expts. conducted with 10-14 day old seedlings)	Disease significantly (p=0.05) reduced in plants inoculated at the higher rate (10^5 spores/mL and 10^6 spores/mL) using the triple dip treatment.

3.2.2 Optimising salicylic acid dip rates for induction of SAR in broccoli

Using the reduced dip rates (0.1-0.5 mM) the systemic nature of the response was demonstrated as an increase in the expression of the chitinase gene in leaves by up to 13.9 fold 24 hours after treatment of the roots with 0.5 mM salicylic acid (Table 3.5). This was not a replicated experiment. A replicated trial was subsequently conducted using paired root and leaf samples to minimise the effect of plant to plant variation (Table 3.6).

Table 3.5. Measurement of chitinase gene expression activity 24 hours after treatment of the roots of broccoli seedlings with salicylic acid (15 minute dip).

Concentration of salicylic acid (mM)	Expression of chitinase gene (fold change)[A]	
	Root	Leaf
0.1	1	0.4
0.25	2.6	0.9
0.5	1.4	13.9

[A] One replicate only and roots and leaves were not pairwise

The results from the replicated trial indicate that at lower concentrations of SA treatment (0.1 mM) the chitinase gene was expressed in both roots and leaves whereas at higher SA treatments (0.2, 0.3, 0.4 and 0.5 mM) chitinase gene expression was less consistent (Table 3.6). At 0.1 mM SA chitinase gene expression consistently increased (2.3 to 5.5 folds) in roots and leaves confirming a SAR response. At higher concentrations SA might not be translocated or the rates used may be so high that they altered the physiology of the plant.

Table 3.6 Measurement of chitinase gene expression activity 24 hours after treatment of the roots of broccoli seedlings with salicylic acid (15 min root dip).

Concentration of salicylic acid (mM)	Expression of chitinase gene (fold change)	
	Root	Leaf
0	1.0	1.0
0.1	2.5	2.5
	2.6	5.5
	2.3	2.6
0.2	1.4	0.1
	1.0	0.1
	0.6	0.1
0.3	1.0	2.9
	2.4	2.8
	1.0	4.0
0.4	0.4	4.6
	0.9	0.4
0.5	0.4	0.5
	0.6	0.2

3.2.3 Optimising the timing and method of applying SA dips

Based on the results of the previous study (3.2.2), only one rate of SA (0.1 mM) was used but single and triple dip applications were evaluated in an effort to optimise the SAR effect. A single 0.1 mM SA root dip for 15 minutes 24 hrs before transplanting significantly reduced the severity of symptoms of clubroot in broccoli plants when inoculated with 10^4 spores/ml *P. brassicae* (p = 0.05) (Table 3.7 & Fig. 3.2). This was the lowest concentration of inoculum used and is approximately ten times higher than what is generally considered to be the threshold for disease in the field (1000 spores per gram of soil, the point at which visual symptoms of disease are evident). The effect was not significant at the higher rates of inoculum (10^5 & 10^6 spores/mL).

Application of the triple dip (three applications 72, 48 and 24 hrs before transplanting) improved the efficacy of treatment at the highest rate of inoculum. Symptoms of disease were significantly (p = 0.05) reduced using the triple dip treatment at the highest rate of inoculum used (10^6 spores/mL, Table 3.7 & Fig. 3.2). Plants treated with SA (triple dip) showed no symptoms of phytotoxicity when compared with untreated plants. Within a treatment group a range of symptoms were consistently observed (eg. Fig. 3.3). A large number of replicate pots (10) were used to ensure significant effects could be detected.

Fold changes in gene expression analysed 24 and 48 hours after treatment were not consistent in all the 5 replicates tested. However, fold changes in gene expression analysed 72 hour after treatment in roots and leaves were consistently higher in all five replicates tested. This result supports the conclusion of HPLC studies of plant tissue (chapter 4) that SAR may take 2-3 days post SA treatment to fully establish in the plant and that plants left for 72 h after SA treatment might have increased resistance to *P. brassicae*. The RT-qPCR results indicate that following SA treatment all 3 SAR marker genes were induced in the leaves but only PR-1 was induced in the roots (Fig. 3.4 and Table 3.8).

Table 3.7 The effect of 0.1 mM SA applied as a 15 minute single (24 hrs before transplanting) or triple (three applications 72, 48 and 24 hrs before transplanting) root dip on clubroot disease severity on broccoli cv. Marathon (visually assessed on 0-9 scale six weeks after inoculation).

	Inoculum concentration		
Treatment	10^4	10^5	10^6
- SA (single dip)	7.1	7.9	9.0
+ SA (single dip)	2.4	6.6	8.5
lsd (p=0.05)	1.1	ns	ns
- SA (triple dip)	2.4	4.2	7.1
+ SA (triple dip)	1.8	3.9	4.4
lsd (p=0.05)	ns	ns	1.7

ns means that the differences between treatments were not statistically different.

There was no significant effect of SA applied at 0.1 mM on clubroot disease severity in two subsequent trials. The first of these trials was a duplicate of the one described above. In the second trial only the single dip (0.1 mM) was used but plants were inoculated using the three spore densities (10^4, 10^5 and 10^6 spores/ml) 24, 48 and 72 hours after treatment. It was concluded that the 0.1 mM rate is marginal and that higher rates 0.25 – 0.5 mM might provide more consistent results. Since there was no effect on clubroot disease severity, gene expression studies were not conducted for these two trials to minimise costs.
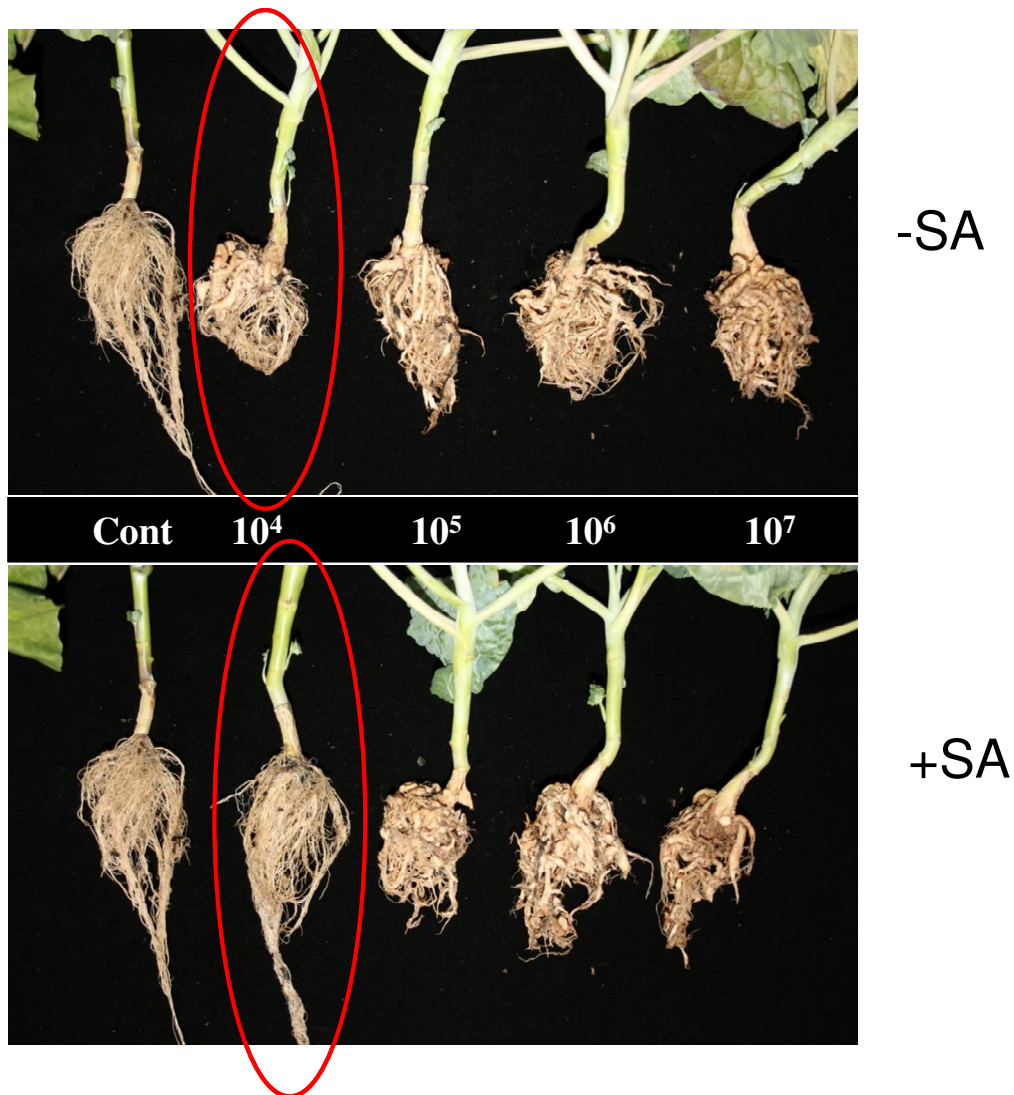
Figure 3.2 Disease assessment of broccoli plants 6 weeks after inoculation with *P. brassicae* spore suspension (10^4, 10^5, 10^6 and 10^7 spores/mL). Untreated roots shown above developed root galls at all of the four spore densities used. Plants shown below were treated with 0.1 mM SA (15 minute root dip) 24 hours before inoculation. SA treatment caused a significant ($p = 0.05$) reduction in symptoms of clubroot only at 10^4 spores/ml *P. brassicae* (highlighted red).
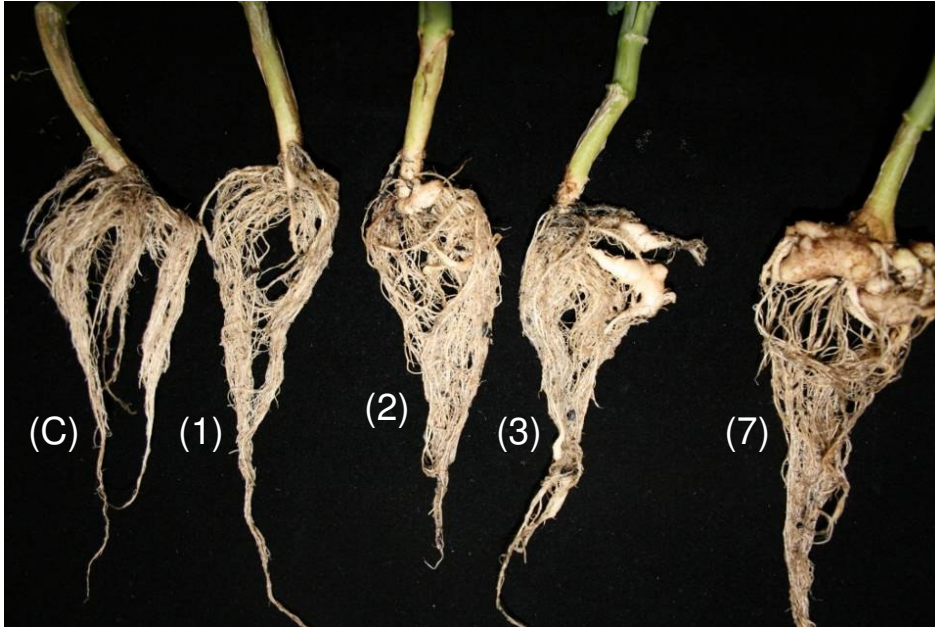
Figure 3.3 Range of root galling symptoms observed on SA treated (0.1 mM SA, triple dip) broccoli plants 6 weeks after inoculation with 10^6 spores/mL, the highest rate of inoculum used. Numbers in brackets refer to the clubroot root gall severity rating assessed visually on a 0-9 scale where 1 = no root galling, 9 = severe root galling, no healthy roots. (C) is the control, uninoculated plant.
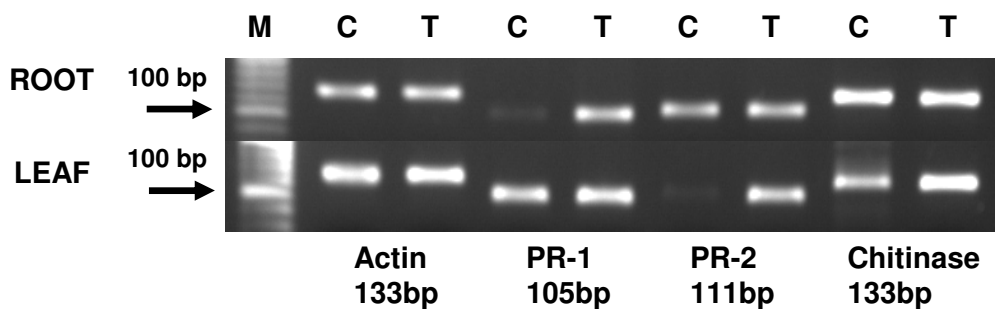
Figure 3.4 Agarose gel electrophoresis of RT-qPCR products of control (C) and 0.1 mM SA triple dip treated (T) broccoli plants (roots and leaves) 72 h after treatment. M = DNA Ladder.

Table 3.8. Fold changes in gene expression in roots and leaves 72 h after 0.1 mM SA triple dip treatment of broccoli seedlings compared with control (data from figure 3.4)

Genes	Root	Leaf
Actin 8	No change	No change
PR-1	38 fold change	4 fold change
PR-2	Not induced	60 fold change
Chitinase	Not induced	23 fold change

3.2.4 Establishing direct and indirect effects of organic amendments

None of the four organic amendments tested (liquid chitosan, fish emulsion, liquid silicon and liquid seaweed) had any direct or indirect effect on SAR induction nor did they reduce clubroot disease in broccoli.

At the rates used fish emulsion caused purple discoloration, wilting and eventual death of the seedlings. None of the other organic amendment root dip treatments were phytotoxic.

3.2.5 Confirming SAR induction in older seedlings

Salicylic acid treatment of 6 week old broccoli cv. Marathon seedlings reduced disease severity only at the lowest inoculum concentrations indicating that a higher rate of SA might be required for SAR induction in older seedlings and at higher rates of inoculum (Table 3.9).

Table 3.9 The effect of SA (0.1 mM, dip applied for 15 min once (single dip) 24 hours before inoculation or three times (triple dip) 72, 48 and 24 hours before inoculation) on clubroot disease severity on broccoli cv. Marathon on older seedlings (6weeks)

Treatment	10^4		10^5		10^6	
	Single dip	Triple dip	Single dip	Triple dip	Single dip	Triple dip
- SA	3.6	5.5	7.6	8.5	7.9	8.4
+ SA	2.2	3.2	6.2	8.8	8.7	8.6
lsd (p=0.05)	1.4	1.4	1.4	ns	ns	ns

In the second trial using 11 week old seedlings the duration of the SA dip was increased from 15 minutes to 1 hour. In this trial symptoms of disease were significantly (p = 0.05) reduced in plants inoculated at the higher rate (10^5 spores/mL and 10^6 spores/mL) using the triple dip treatment

(Table 3.10). Since a significant reduction in clubroot severity was obtained only using the triple dip treatment this result provides further evidence that the 0.1 mM rate is marginal.

Table 3.10 The effect of SA (0.1 mM, dip applied for 1 hour once (single dip) 24 hours before inoculation or three times (triple dip) 72, 48 and 24 hours before inoculation) on clubroot disease severity on broccoli cv. Marathon on older seedlings (11 weeks)

Treatment	10^4		10^5		10^6	
	Single dip	Triple dip	Single dip	Triple dip	Single dip	Triple dip
- SA	3.0	4.7	5.4	8.0	7.9	8.9
+ SA	4.0	4.2	6.1	5.9	7.3	6.9
lsd (p=0.05)	ns	ns	ns	1.3	ns	1.3

3.3 Conclusions

A systemic acquired resistance response was demonstrated in broccoli. A single 0.1 mM SA root dip applied for 15 minutes 24 hrs before transplanting significantly reduced the severity of symptoms of clubroot in broccoli plants when inoculated with a low rate of inoculum (10^4 spores/ml *P. brassicae*). Application of the triple dip (three applications 72, 48 and 24 hrs before transplanting) improved the efficacy of treatment at the highest rate of inoculum. Symptoms of disease were significantly reduced using the triple dip treatment at the highest rate of inoculum (10^6 spores/mL). The systemic nature of the effect was confirmed by the upregulation of three genes (chitinase, PR-1 and PR-2) known to be associated with plant defence responses in root and/or leaf tissue after SA treatment. There was considerable variation in the level of upregulation between replicates. This variation was lowest in samples collected 72 hours after SA treatment indicating that SAR may take up to 72 hours to establish and stabilise in plants. Likewise, variability of effect within a treatment group was a consistent feature of disease assessments and the number of replicates of each treatment had to be increased to 10 to accommodate this variability. There was evidence that the 0.1 mM rate is marginal and a higher rate, closer to 0.5 mM is more likely to be consistently effective.

Biochemical analysis of changes in plant tissue salicylic acid levels

Enhancing the plant immune response for
improved disease control

4. Biochemical analysis of changes in plant tissue salicylic acid levels

Changes in root salicylic acid levels were measured after SA treatment using reverse phase-high-performance liquid chromatography. Exogenous application of a high rate of SA (1 mM) to plants as a single or triple 15 minute dip showed that root SA reached a maximum 72 hours after treatment. This finding supports gene expression studies (chapter 3) which indicate that fold changes in gene expression are inconsistent until 72 hours after SA treatment. It is proposed that the efficacy of SA treatments may therefore be optimised if they are applied 72 hours before being exposed to inoculum (ie. 72 hours before planting into the field). Exogenous applications of lower rates of SA (0.05 mM & 0.1 mM) appear not to increase endogenous SA. A higher rate of 0.25 mM was the minimum rate required to increase endogenous SA above basal levels. In spite of this it is possible that a number of genes involved in SAR are being switched on as a result of low rate applications. Concurrent analysis of gene expression and endogenous SA in the same plant samples is required to further investigate this possibility.

The work detailed in this chapter was conducted by David Lovelock as part of a PhD project undertaken as part of the wider program.

4.1 Materials and Methods

4.1.1 Plant growth conditions and salicylic acid treatment

Seeds of *Brassica oleracea* broccoli cv. Marathon were sown into an autoclaved potting mix in 3.5cm x 3.5cm x 5.5cm plastic pots and grown in a temperature controlled cabinet (Thermoline Scientific Equipment, Australia) under 12 hr light/12 hour (450μmol m^{-2} s^{-1}) dark cycle with a constant temperature of 23.5°C. The potting mix comprised of propagation sand/Peat Moss/Vermiculite (3:3:4) with the addition of 2 parts water; following autoclaving, 5g/L Osmocote was added. Plants were watered every 3 days in the first two weeks of growth, then daily from 2-4 weeks growth.

SA treatments were performed on 4 week old seedlings. Pots containing broccoli seedlings cv. Marathon were treated by carefully pouring 1 litre of the desired SA concentration over 40-50 seedlings, an additional 1 litre of SA was poured into the receptacle containing the pots. Seedlings were left to soak in the SA for 15 minutes before being removed and placed into a new receptacle. Seedlings were watered approximately 5 hours after treatment and were not inoculated.

In the first experiment seedlings were treated with 1 mM SA solution (pH 7) once (single dip, 24 hrs before transplanting) or three times (triple dip, 72, 48 and 24 hrs before transplanting). Control trays were dipped in water for 15 minutes. Samples were taken at 0, 24, 48, 72 and 216 hours after treatment for reverse phase-high-performance liquid chromatography (RP-HPLC). There were five replicates of each treatment.

In the second experiment a single application of reduced rates of SA was applied at concentrations of 0 mM, 0.05 mM, 0.1 mM and 0.25 mM. For RP-HPLC 4 replicate samples were collected at day 0, 1, 2, 3 and 7 post-treatment. A further 10 replicate plants were sampled at days 0, 2, 4 and 8 post-treatment for weight and plant length measurements. Plant length (from root tip

to shoot tip) was measured using the software 'Image J' version 1.43 (National Institutes of Health, USA).

4.1.2 Salicylic acid extraction

SA was extracted from broccoli roots using a method modified from Li *et al.* (1999) and Pan *et al.* (2010). Root material was ground to a fine powder in liquid nitrogen. After addition of 3 ml of methanol (90%) the mixture was vortexed then shaken on an orbital shaker at 130 r.p.m for 30 minutes at 4°C. The mixture was then centrifuged at 7500g for 10 min at 4°C and the resulting pellet was re-suspended in 100% methanol. The supernatant from the original mixture was kept. The methanol/pellet mix was centrifuged at 7500g for 10 min at 4°C and the resulting supernatant was extracted with 1:1 ethylacetate (v/v) and combined with the original supernatant. The supernatants were air dried using N_2.

Once samples were dried, 2.5 ml trichloroacetic acid (5%) was added and the samples were vortexed and placed on an orbital shaker at 130 r.p.m for 30 minutes at 4°C. The samples were then centrifuged at 7500g for 10 min at 4°C and supernatants were collected and extracted in a 1:1 (v/v) ethylacetate by vortexing for 10 min. The organic phase (top layer) was transferred to a new tube. The aqueous phase was acidified with 1.5 ml concentrated HCl and heated at 75°C for 1hr. The released SA from this step was then extracted by partitioning against 1:1 (v/v) ethylacetate and combined with the previous organic phase; N_2 was used to dry samples. The dried extract was re-suspended in 250µl of mobile phase (0.2 M KAc, 0.5 mM EDTA [pH 5]) by vortexing and placed on an orbital shaker at 130 r.p.m for 10 min at 4°C. Samples were then filtered by centrifugation using Nanosep MF GHP (0.45µM) centrifugal devices (PALL Scientific, Australia).

4.1.3 High performance liquid chromatography conditions

High Performance Liquid Chromatography (HPLC) separations were performed on an Agilent Technologies 1200 series consisting of a solvent degasser, autosampler, column heater diode array and fluorescence detector. All separations were performed on an Altima C_{18} 250x4.6 mm 5µm particle size column supplied by Alltech. A solvent gradient was applied starting at 0 min: 95 % H_2O, 5 % Acetonitrile, and finishing at 20 min: 5 % H_2O, 95 % Acetonitrile, a flow rate of 1 ml/min was applied throughout. Samples were detected using fluorescence excitation at 295nm and emission at 405nm.

4.1.4 Statistical analyses

One-way ANOVAs and T-tests were performed to determine differences between treatments using the program SPSS 17.0.

4.2 Results and Discussion

4.2.1 Broccoli root salicylic acid

Experiment 1
Following 15 minute single or triple dip treatment with 1 mM SA the SA concentration in roots increased with time. Maximum concentrations of SA were found in roots 72 hrs after treatment in

both single dip and triple dip treated plants (Fig. 4.1). This result compliments the gene expression studies reported in chapter 3 (3.2.3) and indicates that it may require 3 days post treatment for full expression of systemic acquired resistance in plants. Therefore if a single dip treatment is preferred for ease of commercial application, the effect might be optimised if it is applied 3 days (72 hours) before planting.

The amount of SA in roots treated with the triple dip was consistently higher after 24, 48 and 72 hours than that of roots treated with the single dip (Fig. 4.1). However after 216 hrs roots had the same amount of SA regardless of the single or triple dip treatment. With the exception of gene expression studies, the fate of endogenous SA was not studied in detail however; this result indicates that there may be no long term benefit of additional SA treatments as a triple dip. Since the triple dip is difficult to apply commercially, and in field trials (5.2.1) application of a triple dip reduced plant growth, only the single dip was used in subsequent studies.
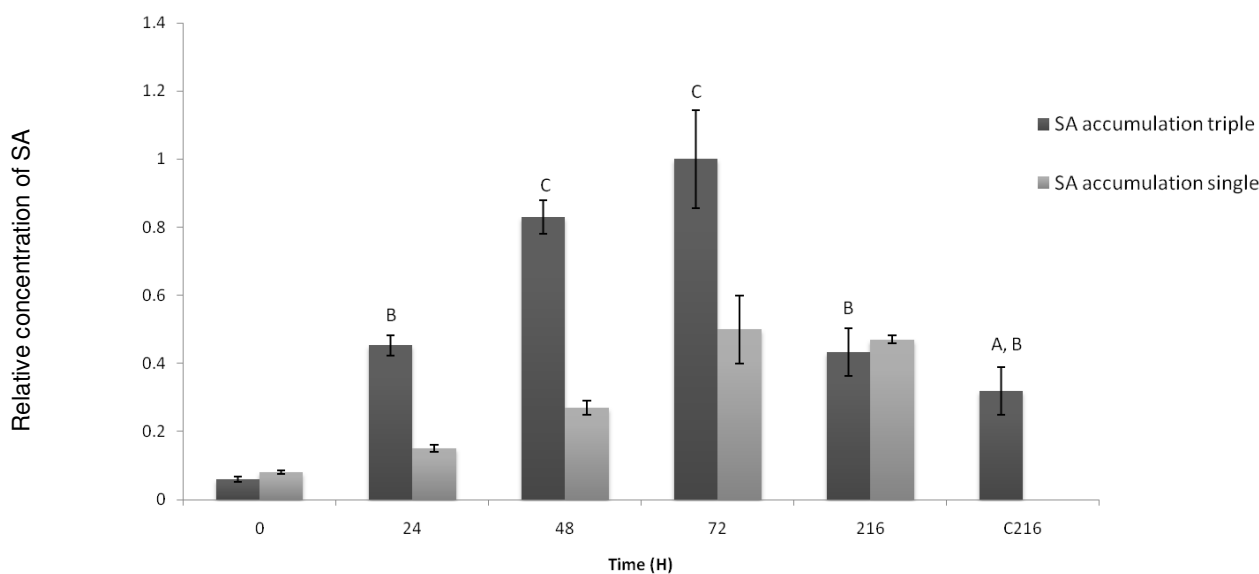
Figure 4.1. Relative concentration of SA within roots in both single and triple dip treated plants obtained from HPLC analysis at 0, 24, 48, 72 & 216 h after treatment. 0 h is the untreated control. Values relative to the highest (1). Letters indicate a significant difference (p = 0.05). Error bars represent the standard error, n=5. C216 is the untreated control 216 hours after the beginning of the experiment.

Experiment 2
Reverse phase-high-performance liquid chromatography (RP-HPLC) analysis of SA pre-treated broccoli roots at concentrations of 0 mM, 0.05 mM and 0.1 mM revealed no significant difference between these concentrations over the first 72 hours post-treatment (Fig. 4.2). Plants in these three

treatment groups (including the untreated control) showed a similar trend for increasing SA concentration over time with peak tissue concentrations of SA occurring at day 7 (Fig. 4.3). Plants treated with the highest rate (0.25 mM SA) had significantly higher root tissue SA than all other treatments over the first 3 days post-treatment (Fig. 4.2) and tissue SA peaked at day 3, earlier than any of the other treatments (Fig. 4.3). After three days post-treatment there was no significant difference in SA concentration between treatment groups.

Since the 0.25 mM SA treatment, the highest rate used, was the only one to differ significantly from the other treatments over the first 3 days post-treatment, it is likely that the other rates used were too low to observe any changes in tissue SA. It is unlikely that an applied concentration of 0.05 mM or 0.1 mM SA will induce systemic acquired resistance (SAR) as the reported levels of endogenous SA, analysed over a period of a week, closely resemble those of the untreated group (Fig. 4.3). At an applied concentration of 0.25 mM, SA appears to provide an increased level of endogenous SA in *B. oleracea* over the first 3 days post-treatment (Fig. 4.2). This work suggests that 0.25 mM is the minimum rate required to observe changes in plant tissue SA. This further supports the conclusions of chapter 3 which indicated that an applied rate of 0.1 mM is marginal. The elevated plant tissue SA observed following treatment with 0.25 mM SA returned to basal levels after 7 days. Further work is required using higher rates of SA 0.25 – 0.5 mM to confirm this finding and to study the fate of SA that is taken up by the plants to determine whether the genetic and biochemical changes that occur in the early days after SA treatment are sufficient to adequately protect the plants from disease. A high concentration of applied SA such as 1 mM can cause symptoms of phytotoxicity (see chapters 3 & 5) which may increase susceptibility to disease. However, if the concentration is too low, the plant may break down the SA via numerous metabolic pathways which utilise the plant hormone; therefore the endogenous SA would remain at basal levels.

Since differences in plant tissue SA over time were observed only at the highest rate of SA this study also highlights the possibility that like potato *Solanum tuberosum* L. cv. Désirée (Yu *et al.* 1997), *B. oleracea,* or at least some crops or cultivars of this species, may have a naturally high basal level of SA. Increased SA is associated with induction of SAR in *Arabidopsis* and tobacco plants as both these species contain low basal levels of SA (Malamy *et al.* 1990; Uknes *et al.* 1992). To date there has been insufficient work done with vegetable brassica species. For example, higher basal levels of SA in the cabbage and cauliflower cultivars used in chapter 6 could potentially explain the observed lack of effect reported for these crops.
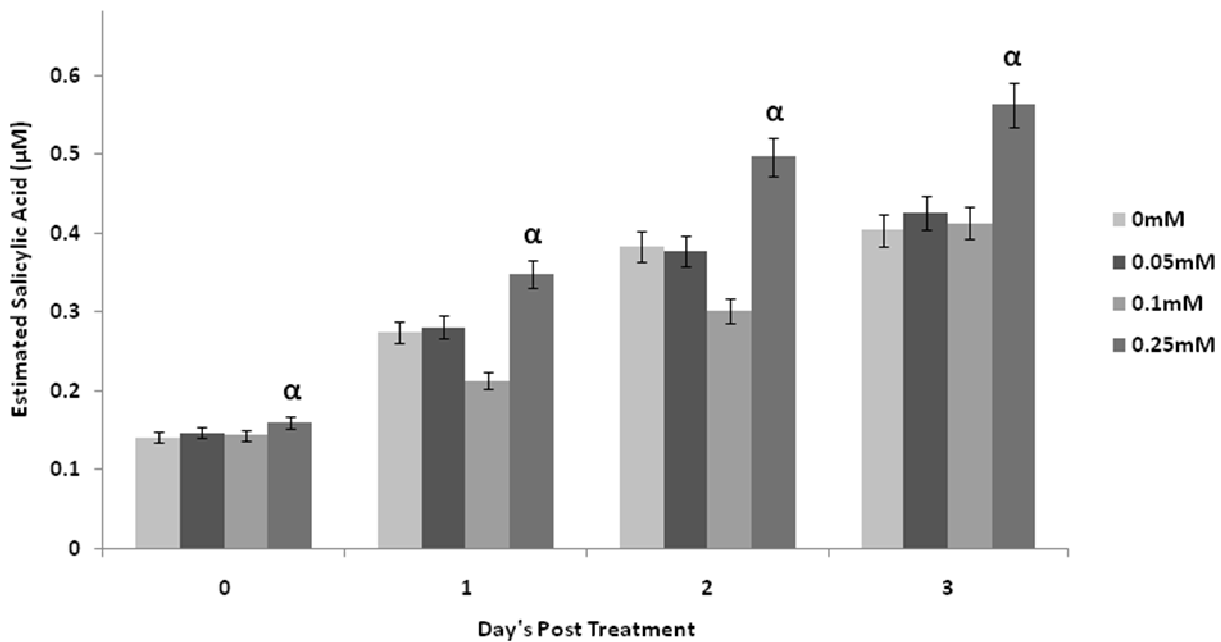
Figure 4.2. RP-HPLC analysis showing total SA accumulation in broccoli roots revealed a difference (p=0.05) between 0.25 mM and all other treatment groups (0 mM, 0.05 mM and 0.1 mM) at days 0, 1, 2 and 3. 'α' represents significant difference to the control, n=4; error bars represent standard error of the mean.
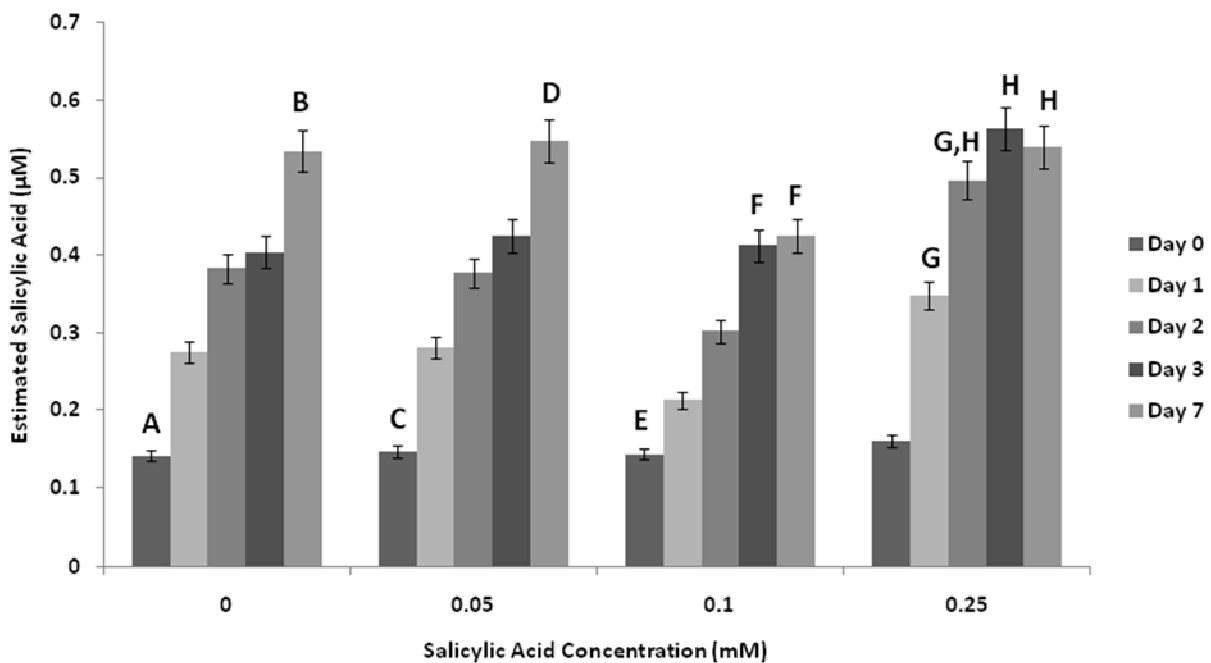
Figure 4.3. Further analysis of total SA accumulation in broccoli roots at individual concentrations revealed differences (p=0.05) within each group after RP-HPLC. Letters indicate significant difference between treatment groups, n=4; error bars represent standard error of the mean.

4.2.2 Effects of salicylic acid on plant growth

At the rates used salicylic acid pre-treatment appeared to have no direct effect on the growth rate of broccoli measured as plant length (shoot to root tip, Fig. 4.4) and weight (Fig. 4.5). Untreated plants grew at a similar rate to those of the SA-pre-treated groups (Figs. 4.4 &4.5). The growth rate appeared to remain relatively constant over the 4 sampling days.
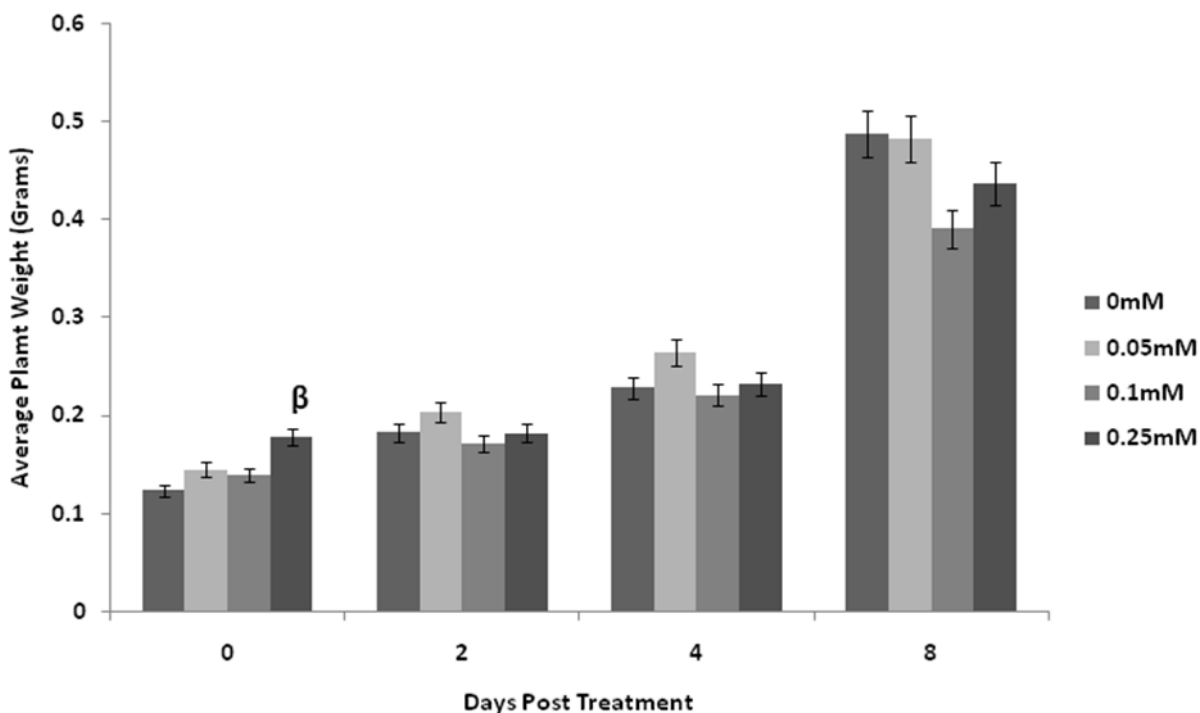
Some significant differences (p=0.05) in mean plant length were observed between SA treatment rates at days 0 and 2 however no consistent trends were observed (ie. plant length did not consistently decrease with increasing rates of SA, Fig. 4.4). At 4 and 8 days after SA treatment there was no significant difference in mean plant length between SA concentrations (Fig 4.4).

With the exception of the highest treatment rate (0.25 mM on day 0) there were no significant differences in plant weight due to SA treatment at any of the assessment time points (Fig. 4.5).

Similar studies have shown that SA applied exogenously does not have a direct effect on plant height, but may in fact increase leaf growth. When applied to corn, Kahn *et al.* (2003) observed that exogenous applications of SA sprayed onto plants had little effect on plant height and root growth, however leaf growth was observed to increase when sprayed with 10^{-5} mol/L SA.
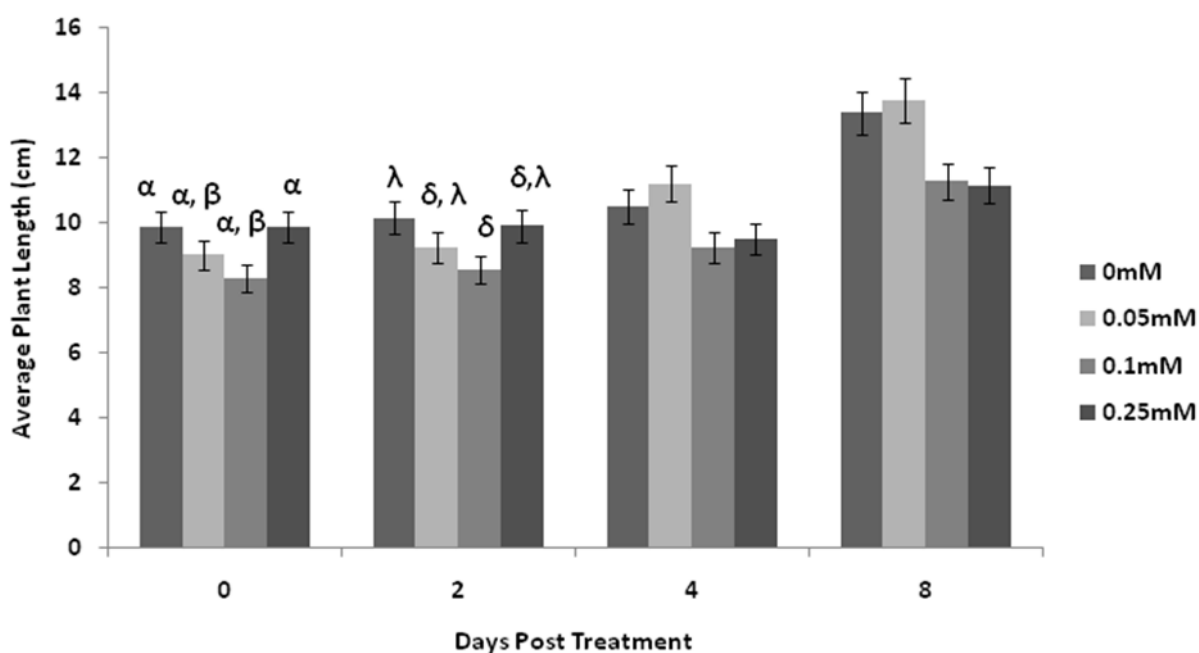
Figure 4.4. SA pre-treatment of broccoli roots revealed a difference (p=0.05) in mean plant length at days 0 and 2, days 4 and 8 are similar between treatment groups. Symbols represent significant difference between individual time points, n=10; error bars represent standard error of the mean.

Figure 4.5. Salicylic acid pre-treatment of broccoli roots revealed a difference (p=0.05) between mean plant weights at day 0 (0.25 mM). All other sample days revealed no difference between treatment groups. Symbol represents significant difference between time points, n=10; error bars represent standard error of the mean.

4.3 Conclusions

Exogenous application of SA (1 mM) to plants as a single or triple 15 minute dip showed that root SA reached a maximum 72 hours after treatment. The efficacy of treatments may therefore be optimised if they are applied 72 hours before being exposed to inoculum (ie. 72 hours before planting into the field). Exogenous applications of lower rates of SA (0.05 mM & 0.1 mM) appear not to increase endogenous SA. A higher rate of 0.25 mM was the minimum rate required to increase endogenous SA above basal levels. In spite of this it is possible that a number of genes involved in SAR are being switched on as a result of low rate applications. Concurrent analysis of gene expression and endogenous SA in the same plant samples is required to further investigate this possibility.

Broccoli field studies

5. Broccoli field studies

The dip techniques developed and optimized in glasshouse and controlled conditions were further evaluated in field trials. Small scale field trials were conducted with broccoli transplants at the Department of Primary Industries Knoxfield site. Field trials conducted using *P. brassicae* were inconclusive due to the extreme severity of disease and problems with residual herbicide damage. Field trials artificially inoculated with *Rhizoctonia solani* AG2.1 together with gene expression studies of the treated plants used in the second series of field trials indicate that the maximum rate of SA that can be applied as a 15 minute dip is 0.5 mM. At this rate symptom of phytotoxicity were only slight in the young (3 week old) seedlings and were not evident in the commercially produced (6 week old) seedlings. All four plant defence related genes were upregulated at 0.5 mM SA and a significant reduction in symptoms caused by *Rhizoctonia solani* AG2.1 on broccoli was evident. The higher rate of SA (1 mM) was extremely phytotoxic to young seedlings.

5.1 Materials and Methods

5.1.1 Establishment and design of first field trials

Two small scale field trials were conducted at the Department of Primary Industries Knoxfield site. The two trials were identical except that one was planted on a site preinoculated with *Plasmodiophora brassicae* (clubroot) whereas plants in the other were inoculated 24 hours after planting with a strain of *Rhizoctonia solani* (previously isolated from brassicas).

The trials were established in raised beds containing Cranbourne loam (Sherwin garden supplies). A *P. brassicae* resting spore suspension (25 L) was prepared by macerating root galls previously collected from a cabbage crop in a mechanical blender (1:3 with water). The resulting mixture was further diluted 1:1 with water and was distributed evenly over the site. The bed was rotary hoed to incorporate and distribute the inoculum.

Broccoli transplants cv. Atomic were sourced from a commercial nursery. Salicylic acid (SA) dip treatments were applied before planting as a single (15 minutes in 1 mM SA (pH 7) 24 hours before transplanting) or triple (three by 15 minutes in 1 mM SA (pH 7) 72, 48 and 24 hours before transplanting) dip. Half of the plants in each of the pre planting treatment groups were drenched with a further 100 ml of SA (1 mM, pH 7) applied around the base of each plant one week after transplanting. The trials were designed as completely randomised blocks consisting of eight replicates of each treatment (Table 5.1).

Table 5.1 First field trial design

Pre planting treatment	Post planting drench (100 ml of 1 mM SA pH7 applied 1 week after planting)
Control – no treatment	Yes
Control – no treatment	No
Single dip[1]	Yes
Single dip[1]	No
Triple dip[2]	Yes
Triple dip[2]	No

[1]Single dip – cell grown transplants dipped in 1 mM SA for 15 minutes 24 hours before transplanting.
[2]Triple dip – cell grown transplants dipped in 1 mM SA for 15 minutes three times, at 72, 48 and 24 hours before transplanting.

Inoculum of *Rhizoctonia* was prepared by homogenising cultures of *Rhizoctonia solani* AG 2.1 previously isolated from brassica with water in a blender (2 plates per 8 L water). Inoculum was applied to the plants by drenching 100 ml of the agar slurry over each plant 24 hours after transplanting.

Fertiliser (Rustica plus® 12-5-14) was applied one week after transplanting at 400 kg/ha. Plants were assessed for disease severity (as described previously, 3.1.2) and the weight of above ground material after six weeks. Statistical analysis was conducted as described in section 3.1.4.

5.1.2 Establishment and design of second field trials

Two further small scale field trials were conducted at Department of Primary Industries Knoxfield using the same beds as used for the first field trials. The two trials were again identical except that one was planted on a site preinoculated with *Plasmodiophora brassicae* (clubroot) whereas plants in the other were inoculated at planting with a strain of *Rhizoctonia solani* (previously isolated from brassicas).

Two cultivars of broccoli of different age cv. Marathon (3 weeks) and cv. Tyson (6 weeks) were tested. Seedlings of different ages were used because younger seedlings are more susceptible to phytotoxicity. Rates lower than and including 1 mM salicylic acid (0.1, 0.25 and 0.5 mM) were tested to determine their potential to induce SAR for disease control without causing phytotoxicity to the treated plants.

A bed with a known history of severe clubroot was rotary hoed and sprayed with Dual Gold® (pre-emergent herbicide a.i. 960 g/L S-metolachlor) 7 days before transplanting. The bed was rotary hoed a second time immediately before transplanting. Broccoli transplants cv Marathon (3 weeks old) were grown in the glasshouse using the same conditions described previously. Broccoli transplants cv. Tyson (6 weeks old) were sourced from a commercial nursery. Salicylic acid (SA) dip treatments (single 15 minute dip) were applied 24 hours before transplanting (0.1, 0.25, 0.5 and 1 mM SA, pH 7). The trials were designed as completely randomised blocks

consisting of 8 replicates of each treatment (Table 5.2). Each replicate consisted of 4 plants (one row) of each of the two cultivars of broccoli.

Table 5.2 Second field trial design

3 weeks old broccoli seedlings (cv Marathon grown in glasshouse)	6 weeks old broccoli seedlings (cv Tyson obtained from commercial nursery)
Control – no treatment	Control – no treatment
Control – no treatment	Control – no treatment
0.1 mM	0.1 mM
0.25 mM	0.25 mM
0.5 mM	0.5 mM
1.0 mM	1.0 mM

Fertiliser (Rustica plus® 12-5-14) was applied one week after transplanting at 400 kg/ha. Snail bait was also applied to the plot. Visual assessment of phytoxicity symptoms was conducted 24 hrs post treatment and 2 days after transplanting in the field. Plants were assessed for disease severity (as described in 3.1.2) and the weight of above ground material after six weeks. Gene expression studies were conducted to confirm SAR in the older commercially grown six week old seedlings (as described previously in section 3.1.3). Statistical analysis was conducted as described in section 3.1.4.

5.2 Results and Discussion

5.2.1 Disease expression and plant growth

In the first field trial the rate of SA used for all treatments (single dip, triple dip and post planting drench) was 1 mM (15 min dip). Disease severity was extreme and all plants were stunted in the clubroot trial with most plants having the maximum disease severity score. As a result there was no significant effect of SA treatment on either disease severity or plant weight in this trial. By contrast disease severity was low in the *Rhizoctonia* trial, insufficient for conclusions to be made regarding the efficacy of SA for management of *Rhizoctonia*. The effect of the preplant chemical dip treatment on plant growth was significant with the triple dip reducing plant weight by 29 and 26 g compared to the single dip (105 g) and control (102 g) respectively (l.s.d ($p=0.05$) = 26). The additional post transplanting SA drench consistently reduced plant weight (Fig. 5.1) but this effect was not statistically significant. There was no significant interaction between the pre and post transplanting treatments.
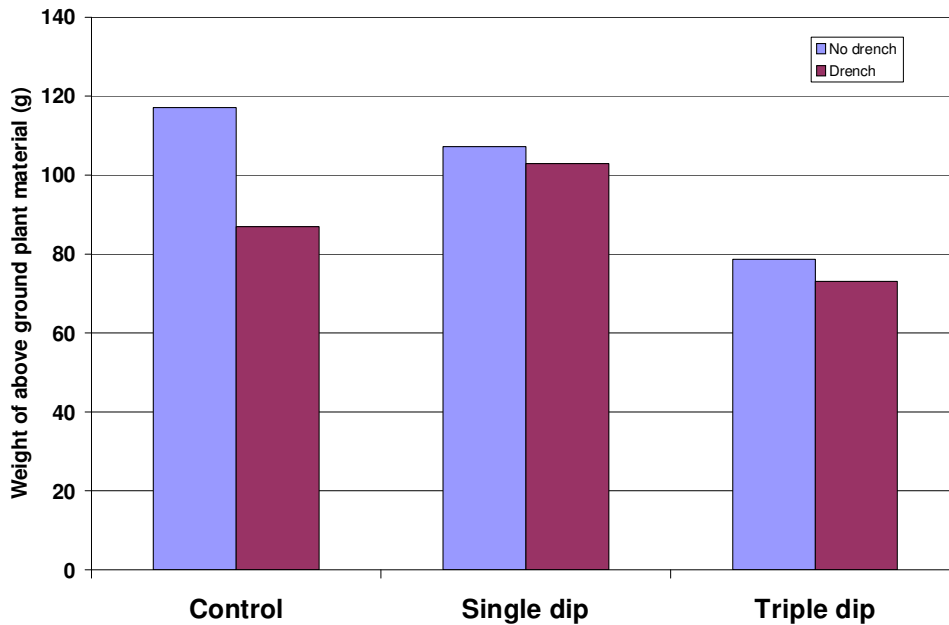
Figure 5.1. Effect of SA inducer treatments 1 mM dip for 15 minutes applied once (single dip) or three times (triple dip) 24 or 72, 48 and 24 hours respectively before transplanting. All plants were inoculated with *Rhizoctonia solani* 24 hours after transplanting and all treatments were applied with or without an additional post transplanting SA drench (1 mM SA, 100 mL applied one week after transplanting). The effect of the preplanting dip treatments was significant l.s.d (p=0.05) = 26.

In the second trials plants treated with 4 different rates of SA were assessed 24 hrs post treatment, before transplanting in the field. None of the rates used caused phytotoxic symptoms on the older seedlings (Fig. 5.2). However, in the younger seedlings, the 0.5 mM rate induced mild symptoms and the 1 mM rate induced severe symptoms of chlorosis and leaf burn (Figs 5.2 & 5.3). These symptoms were also evident 2 days after transplanting into the field (Fig. 5.4).

Figure 5.2 Broccoli seedlings 24 hrs post treatment (15 min dip in 0.1, 0.25, 0.5 and 1 mM SA) clockwise from top left. Each photo has two trays: 3 week old cv. Marathon seedlings (left tray), 6 week old cv. Tyson seedlings (right tray). Note symptoms of phytotoxicity evident in 3 week old seedlings at 0.5 and 1.0 mM rates.


Figure 5.3 Broccoli cv. Marathon seedlings (3 week old) 24 hrs post treatment (15 min dip in 0.1, 0.25, 0.5 and 1 mM SA) clockwise from top left. Note the phytotoxicity in 0.5 (mild) and 1 mM (severe) treated plants (arrows).

Figure 5.4 Broccoli transplants 72 hrs post treatment with 1 mM SA. Three week old seedlings showing symptoms of severe phytotoxicity and leaf burn (left) and 6 week old seedlings (right), no phytotoxicity.

All plants including the untreated controls grew poorly and unevenly in the clubroot bed and some residual herbicide damage was suspected. As a result there was no significant effect of SA treatment on disease severity or plant weight in this trial. The results were inconclusive and further field testing is required.

In the *Rhizoctonia* bed, significantly more of the younger seedlings died due to phytotoxicity following treatment with 1 mM salicylic acid than in any other treatment, including the control (Fig. 5.5). Application of SA at 0.5 mM caused a significant reduction in visual symptoms caused by *Rhizoctonia* in broccoli (Fig. 5.6) however, at this rate there was some evidence of mild phytotoxicity in the younger (3 week old) seedlings (Fig 5.2 & 5.3).
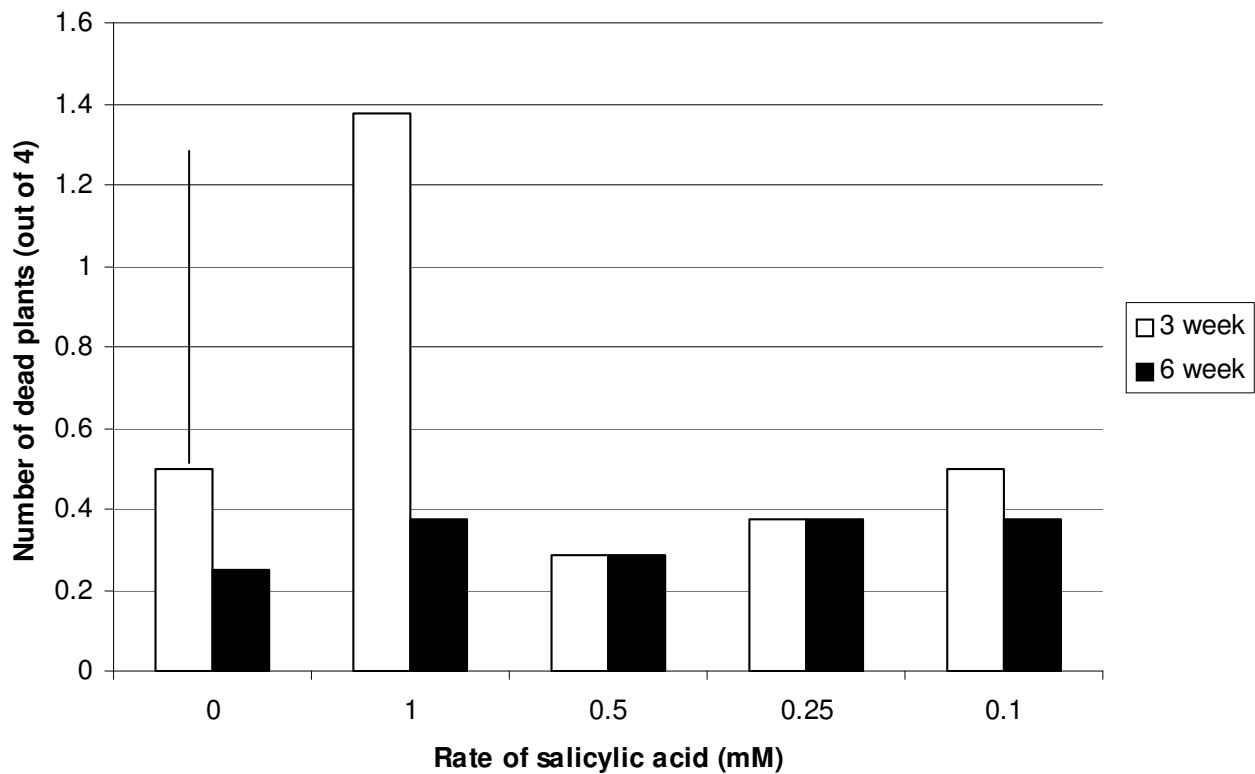
Figure 5.5. Mortality of broccoli cv. Marathon dipped for 15 minutes in SA at 3 weeks old (empty column) and cv. Tyson treated with SA at 6 weeks old (black column) and planted into the field. Plant death assessed at 6 weeks after transplanting. Bar represents l.s.d ($p<0.05$) of 0.8 dead plants per plot between 3 week old seedlings treated with different rates of SA. Differences between different rates of SA applied to 6 week old seedlings were not significant.
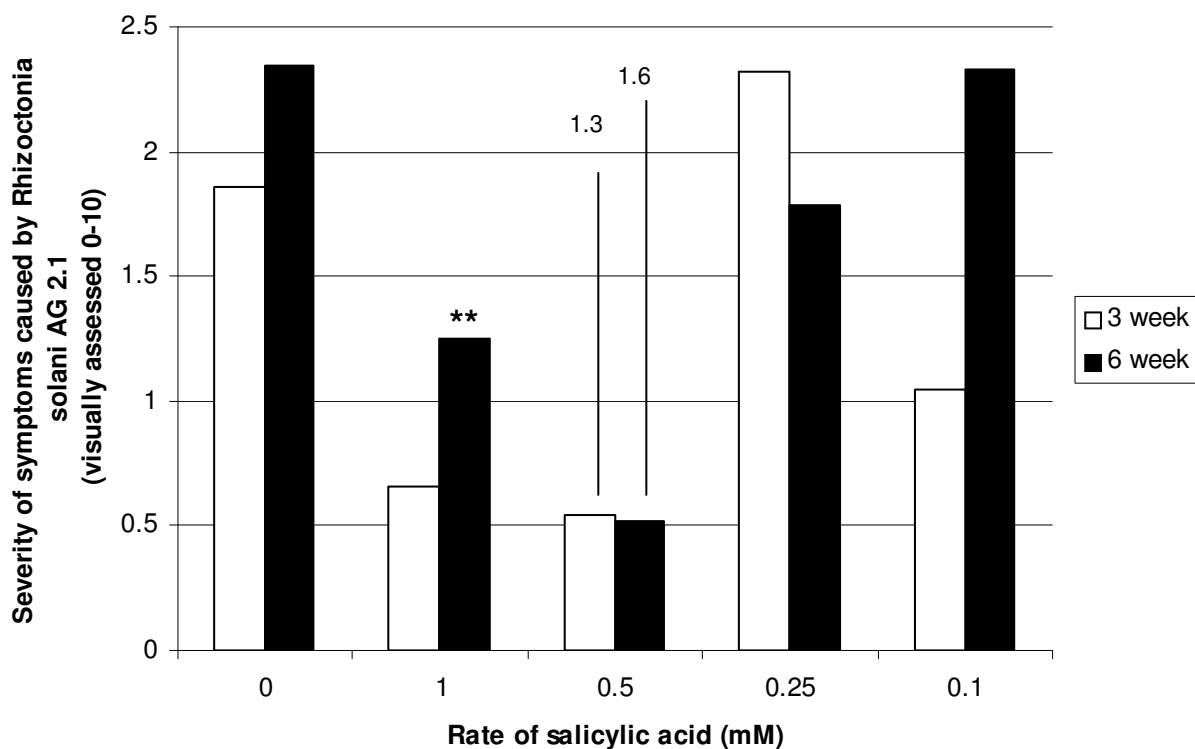
Figure 5.6. Severity of root symptoms caused by *Rhizoctonia solani* AG 2.1 on broccoli cv. Marathon treated with SA at 3 weeks old (empty column) and cv. Tyson treated with SA at 6 weeks old (black column). Bars represent l.s.ds between different rates of SA applied to 3 or 6 week old seedlings.

**Note that since there were a significant number of deaths in the 3 week old seedlings dipped in 1 mM SA the numbers presented here for 1 mM SA are the average of the surviving plants per plot.

5.2.2 Gene expression studies

Six week old broccoli seedlings cv. Tyson obtained from the nursery for field trial studies were treated with 4 rates of SA (0.1, 0.25. 0.5 and 1 mM) and concurrently analysed for changes in gene expression in root and leaves 24 hr after treatment. RNA extracted from the roots of these older seedlings was not of good quality and hence gene expression studies were conducted using leaf samples only.

In plants treated with 1 mM SA the expression of PR1 gene was very high in all 5 replicates ranging from 17-1181 folds (Table 5.3). However, there was no increase in the expression of PR2 and chitinase gene. There was an increase in the expression of PAL gene (between 2-16 fold) which indicated an increase in SA level in leaves. In older seedlings no phytoxicity was observed at this rate.

Table 5.3 Gene expression changes measured in leaves of six week old broccoli seedlings cv. Tyson 24 hr after treatment with SA (0.1, 0.25, 0.5 and 1 mM)

Rates of SA (mM)	Gene expression (fold change)			
	PR1	PR2	Chitinase	PAL
1	17-1181	Not induced	Not induced	2-16
0.5	3-565	8-24	4-8	3-19
0.25	Not induced	1.4-4.8	2-4	1.4-4
0.1	Not induced	3.4-5.6	2-5	2.4-4

The expression of all four genes increased in plants treated with 0.5 mM SA. PR1 gene was not induced in either of the lower rate SA treatments (0.1 and 0.25 mM). However, PR2, chitinase and PAL genes showed a consistent increase in expression level at these lower rates. From these results it appears that 0.5 mM may be the optimum rate of SA treatment to be used for future studies using older seedlings although there is a lot of variation between replicates. At this rate SA caused mild symptoms of phytotoxicity on younger (3 week old) cv. Marathon seedlings (Figs 5.2 & 5.3).

In each of the field experiments two cultivars of broccoli were used. Cultivar Marathon had been used previously in optimising pot trials and gene expression studies. It is a known clubroot susceptible variety and has been widely used for research purposes. It was included for consistency and to ensure that the results could be compared with earlier trials. Cultivar Marathon is no longer used commercially though. The older (6 week old) seedlings were sourced from a commercial nursery. The varieties used cvs. Atomic and Tyson were selected as seasonally appropriate current commercial varieties. Since the 3 week and 6 week old seedlings were different cultivars, genetic differences in their susceptibility to phytotoxicity cannot be ruled out.

In this experiment plants treated with SA were transplanted 24 hr after treatment. Future experiments should evaluated multiple planting times (ie. 48 and72 hr after treatment) since HPLC

analysis (chapter 4) indicates that SAR may take 2-3 days post SA treatment to fully establish in the plant and that plants left for 72 h after SA treatment might have increased resistance to *P. brassicae*.

5.3 Conclusions

Field trials conducted using *P. brassicae* were inconclusive due to the extreme severity of disease and problems with residual herbicide damage. From the *Rhizoctonia* trials and gene expression studies it is evident that the maximum rate of SA that can be applied as a 15 minute dip is 0.5 mM. At this rate symptom of phytotoxicity were only slight in the young (3 week old) seedlings and were not evident in the commercially produced (6 week old) seedlings. All four plant defence related genes were upregulated at 0.5 mM SA and symptoms caused by *Rhizoctonia solani* AG2.1 on broccoli were significantly reduced.

Evaluating SAR in other vegetable brassica crops

6. Evaluating SAR in other vegetable brassica crops

Experiments were conducted using cabbage and cauliflower plants to determine whether SA induction of SAR is specific to broccoli or can be applied more widely to other *Brassica* vegetables. Distinct differences were observed between the responses of broccoli tested previously and the other *Brassica* vegetables. Cabbage and cauliflower were more susceptible to clubroot than broccoli. In particular the variety of cabbage used was highly susceptible. Many of the anticipated changes in the expression of key genes known to be associated with SAR and observed previously in broccoli following treatment with 0.1 mM SA were not observed in cabbage or cauliflower. When higher rates (0.5 and 1.0 mM SA) were used in the cauliflower trial PR-1 and chitinase genes were consistently induced but PR-2, which was previously upregulated 60 fold in broccoli was not induced. At these higher rates symptoms of phytotoxicity were evident. These symptoms were so severe in plants treated with 1 mM SA that they failed to recover and remained stunted for the duration of the experiment. The gene expression studies indicate that SA rates between 0.25 and 0.5 mM are likely to induce a SAR response without causing significant problems of phytotoxicity but, to date a significant reduction in symptoms of clubroot disease has not been observed in any Brassica vegetable species other than broccoli.

6.1 Materials and methods

6.1.1 Cabbage plant growth, inoculation and SA treatment

Cabbage cv. Sugarloaf seedlings were grown in a controlled environment room as described for broccoli (3.1.1). Trays containing three week old seedlings (1 tray each) were dipped for 15 minutes in 0.1 mM SA solution (pH 7) once (single dip, 24 hrs before transplanting) or three times (triple dip, 72, 48 and 24 hrs before transplanting). Control trays were dipped in water for 15 minutes. At transplanting 6 cabbage plants from treated trays and two control plants were uprooted and processed as described previously for RNA extraction (3.1.3). Expression of key defence genes PR-1, PR-2 and chitinase was measured in roots and leaves. Treated and untreated plants were transplanted into pots containing vegetable seed raising mixture (Biogro, Bayswater, VIC) and immediately inoculated with 200 µL of *P. brassicae* spore suspension (10^4, 10^5 and 10^6 spores/mL). The trials were designed as completely randomised blocks consisting of 10 replicates of each treatment.

One uninoculated plant from each treatment (+SA) and untreated control (-SA) was transplanted into pots for each replicate block to check for phytoxicity. Disease was assessed six weeks after inoculation by examining all the plants within a treatment group. The plants were removed from soil, roots washed with water and visually assessed for symptoms of root galling using a 0-9 severity scale where 0 = no disease and 9 = severe disease, no healthy roots (as described previously 3.1.2). Statistical analyses were performed as described in section 3.1.4.

6.1.2 Cauliflower plant growth, inoculation and SA treatment

Cauliflower cv All Year Round seedlings were grown in a controlled environment room as described for broccoli (3.1.1). Trays containing three week old seedlings (1 tray each) were dipped in SA solution (single 15 minute dip in 0.1, 0.25, 0.5 or 1 mM SA (pH 7) 24 hours before transplanting in pots). Control trays were dipped in water. At transplanting 6 cauliflower plants from each treatment group and two control plants were uprooted and processed as described

previously for RNA extraction (3.1.3). Expression of key defence genes PR-1, PR-2, PAL and chitinase was measured in leaves.

Visual assessment of symptoms of phytoxicity was conducted 24 hrs post treatment immediately before transplanting into pots containing vegetable seed raising mixture (Biogro, Bayswater VIC). Transplanted seedlings were inoculated with 200 µL of *P. brassicae* spore suspension (10^4, 10^5 and 10^6 spores/mL). One uninoculated plant from each treatment (+SA) and untreated control (-SA) was transplanted into pots for each replicate block to check for phytoxicity. The trials were designed as completely randomised blocks consisting of 10 replicates of each treatment.

Disease was assessed four weeks after inoculation by examining all the plants within a treatment group. The plants were removed from soil, roots washed with water and visually assessed using a 0-9 severity scale where 0 = no disease and 9 = severe disease, no healthy roots (as described previously 3.1.2). Statistical analyses were performed as described in section 3.1.4.

6.2 Results and Discussion

6.2.1 Cabbage

Disease was severe in the cabbage plants even at the lowest rate of inoculum. The cabbage used in this study was found to be more susceptible to clubroot than the broccoli used previously and there was no significant reduction in disease from either of the SA treatments.

Studies of the expression of key genes showed distinct differences between the broccoli studied previously and the cabbage plants used in this trial. The cabbage plants showed high constitutive expression of PR-1 gene in untreated control leaves compared with broccoli. Induction of PR-1 occurred only in roots and not in leaves even 72 hour after SA treatment (Table 6.1). The PR-2 gene induction was also lower in cabbage leaves (2.9 fold change) compared with broccoli (60 fold change). The chitinase gene was not induced in either roots or leaves of cabbage after single or triple dip treatment. Many of the anticipated changes in the expression of these key genes known to be associated with SAR and observed previously in broccoli were not observed in cabbage (Table 6.1). It is possible that 0.1 mM SA treatment 72 hour before transplanting was insufficient for the induction of SAR in cabbage plants. Further evaluation of higher rates of SA (0.25 – 0.5 mM) 72 hours prior to transplanting is required to confirm whether SAR induction is possible in cabbage and to determine any effects on disease. It is possible that different genes are induced in cabbage or that cabbage is not as amenable to SAR induction as broccoli. Higher rates of SA (up to 1 mM) were used in the subsequent cauliflower trial (6.2.2).

Table 6.1. Fold changes in gene expression in cabbage seedling roots and leaves 72 h after triple SA treatment of cabbage seedlings compared to broccoli seedlings treated in the same way in a previous study (3.3.3).

Gene	Cabbage root	Cabbage leaf	Broccoli root	Broccoli leaf
PR-1	11.5 fold change	Not induced	38 fold change	4 fold change
PR-2	8.9 fold change	2.9 fold change	Not induced	60 fold change
Chitinase	Not induced	Not induced	Not induced	23 fold change

6.2.2 Cauliflower

Symptoms of phytotoxicity similar to those observed previously in the broccoli field trials (chapter 5) were observed in cauliflower following a single 15 minute dip in SA solution at 0.5 and 1 mM (Figure 6.1). Plants were assessed for disease severity after 4 weeks.

There was no significant effect of any of the four SA treatments on disease severity even at the lowest rate of inoculum. Plants treated with 1 mM SA failed to recover from the symptoms of phytotoxicity observed 24 hours after treatment. These plants were severely stunted showing a reduction in both root and shoot growth (Figure 6.2). Even at the highest rate of SA (1 mM) a range of symptoms of disease were observed at the highest inoculum concentration (10^6 spores/ml, Fig. 6.3). Plants treated with lower rates of SA recovered from any initial symptoms of phytotoxicity and exhibited normal root and shoot growth.

Changes in the expression of key defence related genes from within a treatment group were not consistent in all the replicates tested. At the lower rates (0.1 and 0.25 mM) the four genes tested (PR1, PR2, chitinase and PAL) were not induced at all or induced in some of the replicates with a very low fold change. At the higher rates (0.5 and 1 mM) PR1 and chitinase genes were consistently induced in all the replicates, whereas PR2 and PAL were not induced in any of the 5 replicates (Table 6.2).
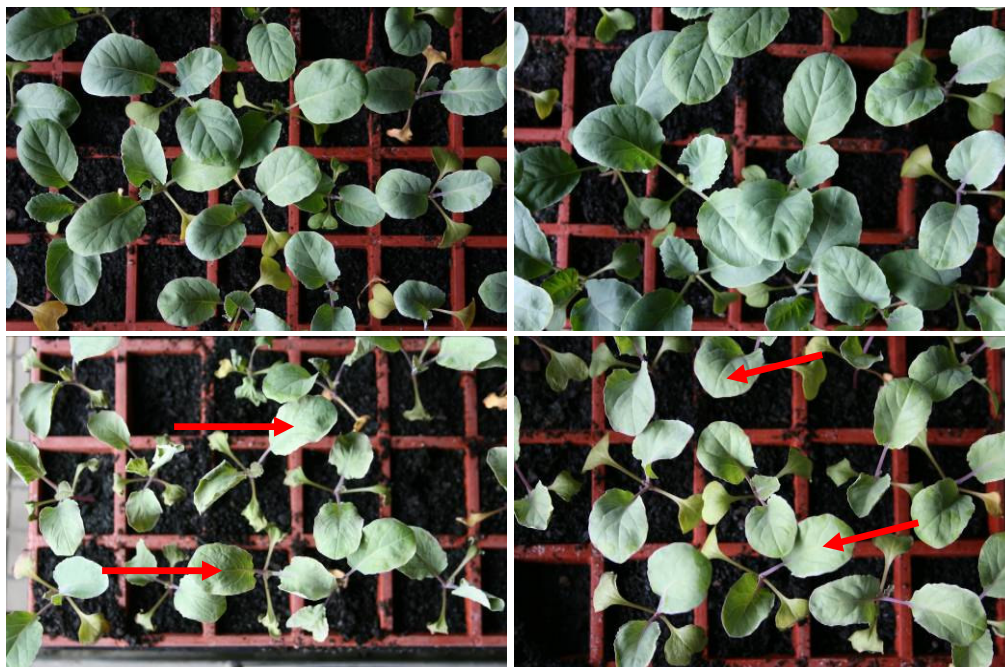
Figure 6.1. Three weeks old cauliflower seedlings 24 hrs post treatment (15 min dip in 0.1, 0.25, 0.5 and 1 mM SA) clockwise from top left. Note the phytotoxicity in 0.5 (mild) and 1 mM (severe) treated plants (arrows).

Figure 6.2. Effect of SA treatment (0.1, 0.25, 0.5 and 1 mM SA) on the control uninoculated cauliflower plants (assessed 4 weeks after treatment) compared with the control untreated plant (C). Note the stunted growth in 1 mM treated plants showing a reduction in both root and shoot growth compared with 0.1 mM treated plant.
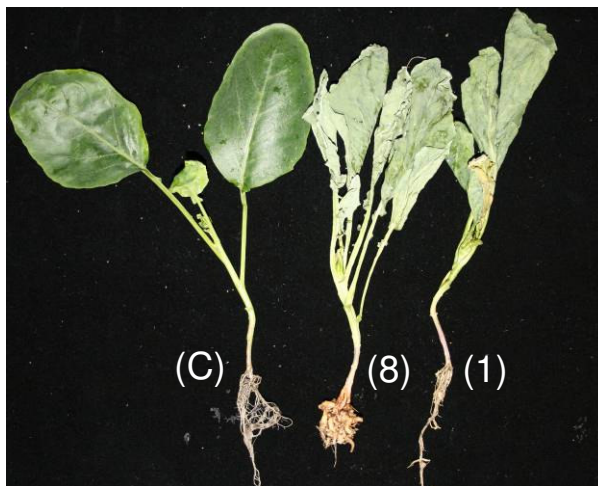
Figure 6.3. Range of root galling symptoms on SA treated (1 mM SA) cauliflower plants 4 weeks after inoculation with *P. brassicae*. Disease severity as observed with the highest rate of inoculum used (10^6 spores/mL). Numbers in brackets refer to the clubroot root gall severity rating assessed visually on a 1-9 scale where 1 = no root galling, 9 = severe root galling, no healthy roots. (C) is the control (1 mM SA treated), uninoculated plant.

Table 6.2. Gene expression changes measured in leaves of three week old cauliflower seedlings 24 hr after treatment with SA (0.1, 0.25, 0.5 and 1 mM)

Rates of SA (mM)	Gene expression (fold change)[*]			
	PR1	PR2	Chitinase	PAL
1	20-105	Not induced	5-41	Not induced
0.5	2-159	Not induced	0.5-31	Not induced
0.25	0.4-1.7	0.6-5.2	0.4-1.6	0.4-3.8
0.1	0.2-15	0-3.4	0.2-5.5	0.7-1.7

[*]The range of fold change reported here is for 5 replicates

6.3 Conclusions

It is clear from this work that there are distinct differences between broccoli and the other *Brassica* vegetables tested. Cabbage and cauliflower were more susceptible to clubroot than broccoli. In particular the variety of cabbage used was highly susceptible. Many of the anticipated changes in the expression of key genes known to be associated with SAR and observed previously in broccoli following treatment with 0.1 mM SA were not observed in cabbage or cauliflower. When higher rates (0.5 and 1.0 mM SA) were used in the cauliflower trial PR-1 and chitinase genes were consistently induced but PR-2, which was up-regulated 60 fold in broccoli was not induced. At these higher rates symptoms of phytotoxicity were evident. These symptoms were so severe in plants treated with 1 mM SA that they failed to recover and remained stunted for the duration of the experiment. The gene expression studies indicate that SA rates between 0.25 and 0.5 mM are likely to induce a SAR response without causing significant problems of phytotoxicity but, to date a significant reduction in symptoms of clubroot disease has not been observed in any *Brassica* vegetable species other than broccoli.

Evaluation of SAR in other pathosystems

7. Evaluation of SAR in other pathosystems

In order to determine whether the disease suppressive effect of SAR induction is specific only against *Plasmodiophora brassicae* (clubroot) or applies more widely against other broccoli pathogens, experiments using a foliar pathogen *Albugo candida* (white blister) were established. Rates between 0.2 and 1 mM were phytotoxic in pot and hydroponic trials. A hydroponic assay system was developed to allow consistent application of low rates of SA independent of soil water status. White blister symptom development in trials was inconsistent with pustules developing on true leaves in some trials while only on the cotyledons in other trials. In all hydroponic trials, SA application resulted in reduced white blister severity but this was only statistically significant for the first two trials. These results indicate that there is potential for SA induction of SAR to be used to manage multiple pathogens of broccoli but further work under higher disease pressure is recommended to confirm these findings.

7.1 Materials and methods

7.1.1 Plant material

Broccoli (cv. Marathon) was grown from seed in vegetable seed raising mix (Biogro, Bayswater VIC). Initial trials were performed on seedlings grown in pots (10 cm diameter, 4 plants per pot). In later trials, plants were grown in hydroponics in 2.5 L plastic tubs with 4 plants per tub in LP50 pots filled with perlite. Nutrient mixture ('Greendream' Flairform WA) was added to the tubs at a rate of 5 mL/L.

7.1.2 Treatment

A 10 mM stock Salicylic acid (SA) solution was prepared by dissolving SA in hot deionised water with stirring. Solutions of 1 mM and 0.2 mM were prepared by dilution in deionised water for soil drench treatments in pot experiments or in nutrient solution for hydroponic experiments. For pot experiments, pots were placed in a tray of SA solution (approximately 3 cm depth) for 15 minutes. For hydroponic experiments, plants were exposed to SA containing solutions for 4 days before the solutions were replaced with fresh nutrient solutions containing no SA.

7.1.3 Inoculum

Initial experiments were performed with *Albugo candida* inoculum collected from broccoli grown in Werribee South (Vic). Zoosporangia containing zoospores (white powdery masses) were removed from plants and were stored frozen at -18ºC for several months before use. This inoculum proved to be non-viable. Another isolate of white blister was collected from Boneo (Vic). Zoosporangia were maintained on seedlings in the glasshouse to ensure their viability for use in all subsequent experiments. Initially zoosporangia were collected by rupturing the epidermis of white blister pustules and scraping sporangia from the pustules over a beaker containing chilled distilled water. An improved method for inoculum preparation was subsequently developed. This involved rupturing the epidermis of white blister pustules with a scalpel and then shaking them in a jar filled

with deionised water to suspend the zoosporangia. For both inoculum preparation methods, the suspension was mixed thoroughly and the concentration of zoosporangia adjusted to 1 x 10^5/mL. The zoosporangia suspension was incubated at 14ºC to allow zoospore release. When motile zoospores could be observed (3-4 hours) the suspension was applied to broccoli leaves using a hand held spray gun. Plants were then covered with plastic bags to maintain leaf wetness and incubated for 12 hours. After 12 hours, the plastic bags were removed and plants were incubated in the glasshouse until symptoms developed (typically 8-10 days).

7.1.4 Disease assessment

The percentage leaf area covered with white blister pustules was estimated by taking digital photographs of leaves, converting images to grayscale and then measuring areas infected and total area (Imagetool, UTHSCSA USA).

7.1.5 Statistical analyses

The statistical software GenStat (12th edition) was used for statistical analysis in all the experiments reported. Analysis of Variance of angular transformed data was used to determine the effect of SA treatment.

7.2 Results and Discussion

Pot Trial 1

SA at 1 mM was highly phytotoxic and treated plants showed symptoms within minutes (Fig. 7.1). SA at 0.25 mM was less phytotoxic while no phytotoxicity was observed at 0.1 mM. No reduction in white blister was observed at the end of the incubation period (no white blister was recorded on plants treated with 1 mM SA but the leaves had dessicated) (Fig. 7.2). It was subsequently decided to use lower doses of SA in hydroponic culture to allow consistent uptake of SA at a lower concentration over a period of time.


Figure 7.1. Symptoms of phytotoxicity on plants treated with 1 mM SA (24 hrs post-treatment) in pot experiment.
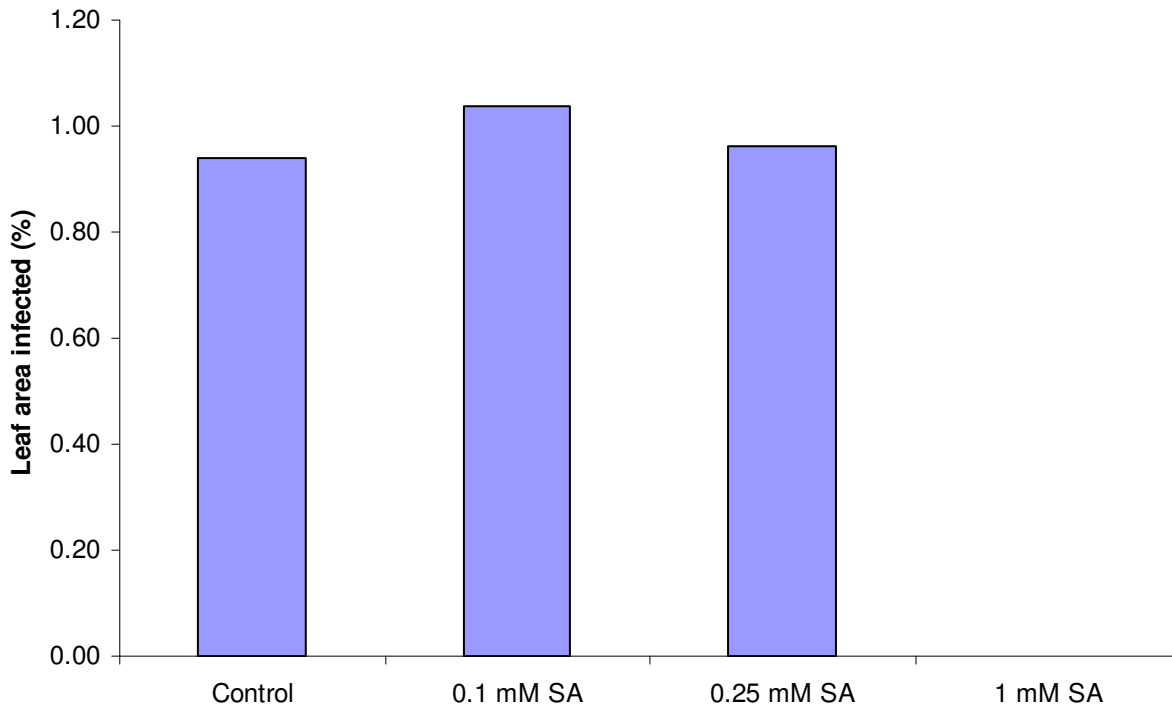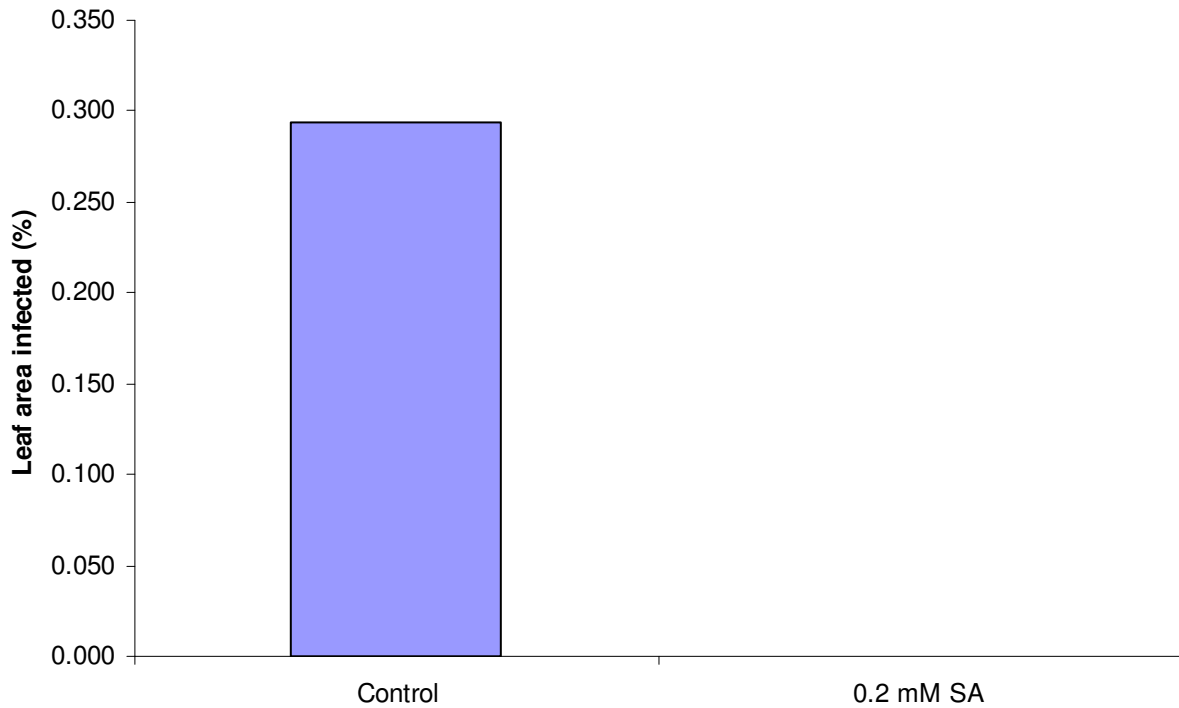
Figure 7.2. White blister development on broccoli seedlings in pot experiment 1 (note, 1 mM SA was phytotoxic with leaves dessicated therefore no white blister could be observed).

Hydroponic trial 1

In the hydroponic system, 1 mM SA was still highly phytotoxic but much lower levels of phytotoxicity occurred at 0.2 mM SA although growth was still reduced relative to the control treatment (Fig. 7.3). White blister development in this trial was low with only two replicates developing disease in the control treatment. There was no white blister development in the 0.2 mM SA treatment (Fig. 7.4).
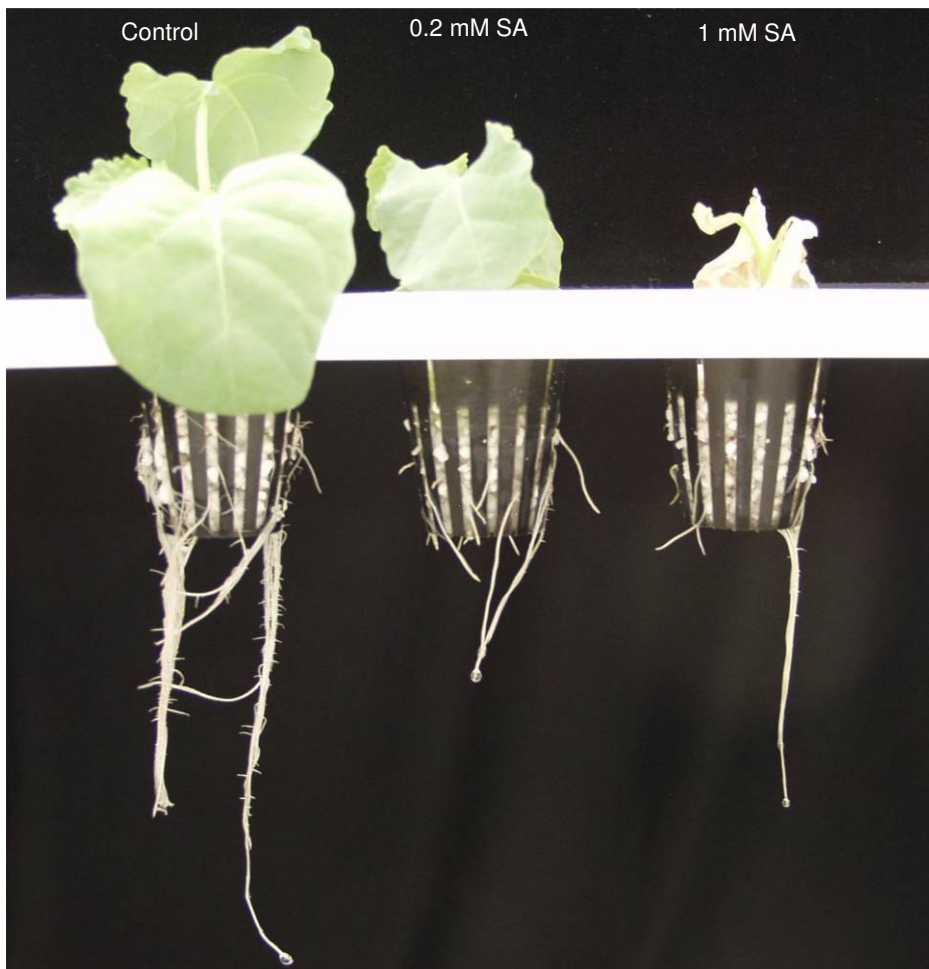
Figure 7.3. The effect of SA concentration on the growth of broccoli in hydroponics (at end of experiment, approx 2 weeks post treatment).
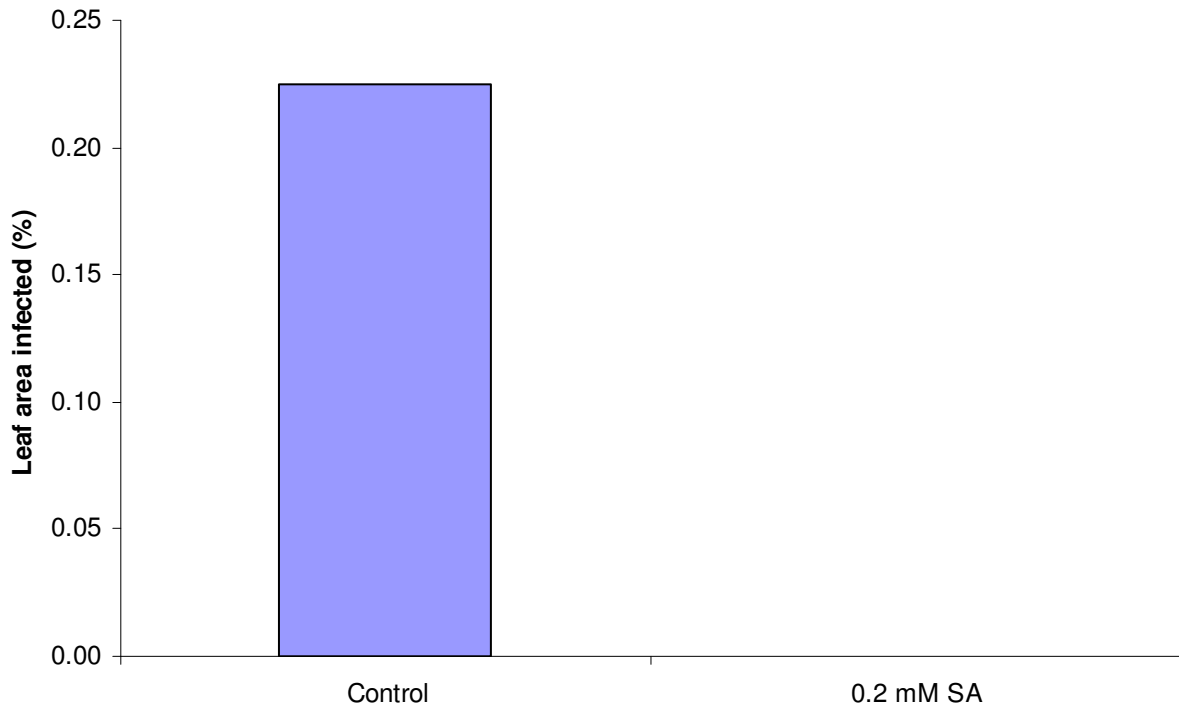
Figure 7.4. Hydroponic Trial 1. Effect of SA on white blister infection of broccoli grown in hydroponics. This difference was significant (analysis of angular transformed data) p=0.018.

Hydroponic trial 2

White blister development in this trial was low on the untreated control with disease symptoms only present on cotyledons but not on the true leaves. There was no white blister development in the 0.2 mM SA treatment (Fig 7.5).
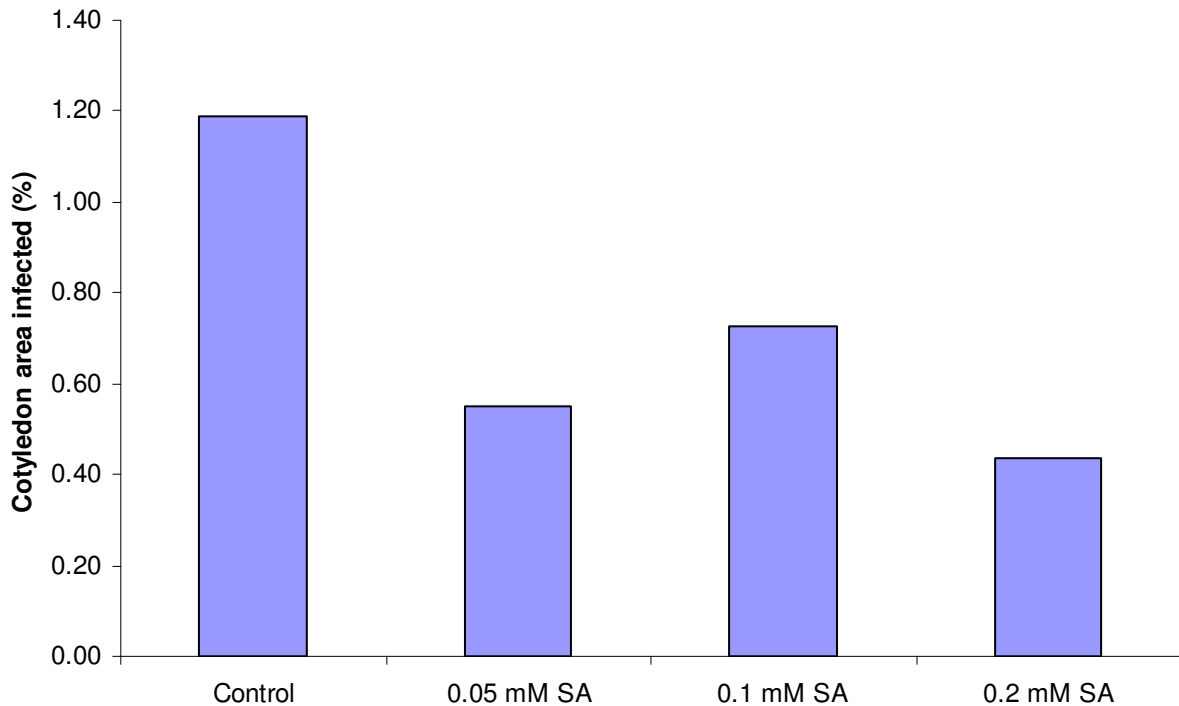
Figure 7.5. Hydroponic Trial 2. Effect of SA on white blister infection of broccoli grown in hydroponics (cotyledons only, visual assessment). This difference was significant (analysis of angular transformed data) p=0.03.

Hydroponic trial 3

White blister development in this trial was low with disease symptoms only present on cotyledons but not on the true leaves. White blister pustules developed on all treatments but none of the treatments were significantly different to the untreated control (p=0.557) (Fig. 7.6).

Figure 7.6. Hydroponic Trial 3. Effect of SA on white blister infection of broccoli grown in hydroponics (cotyledons only, visual assessment).

7.3 Conclusion

Rates of SA between 0.2 and 1 mM were phytotoxic in pot and hydroponic trials. A hydroponic assay system was developed to allow consistent application of low rates of SA independently of soil water status. White blister symptom development in trials was inconsistent with pustules developing on true leaves in some trials while only on the cotyledons in other trials. In all hydroponic trials, 0.2 mM SA application resulted in reduced white blister severity but this was only statistically significant for the first two trials. These results indicate that there is potential for SA induction of SAR to be used to manage multiple pathogens infecting broccoli. Further studies under conditions more conducive to severe disease would be beneficial.

Technology transfer

This chapter details the communication activities and outcomes for the project. A range of communication strategies were employed including workshops, field days, presentations and displays at industry conferences and events, industry articles and scientific papers. Since the project was innovative and developmental the focus of technology transfer activities was not on delivering a completed disease control strategy to industry or the scientific community, rather, activities were directed towards delivering the concept of systemic acquired resistance to industry, demonstrating progress and providing an avenue for industry and scientific feedback.

Scientific papers

Agarwal A, Kaul V, Faggian R and Cahill DM (2009). Development and use of a model system to monitor clubroot disease progression with an Australian field population of *P. brassicae*. *Australasian Plant Pathology* **38,** 120-127.

Agarwal A, Kaul V, Faggian R, Ludwig-Mueller J and Cahill DM. Gene expression changes during the primary phase of the interaction between *Arabidopsis thaliana* and *Plasmodiophora brassicae*. (submitted to 'Molecular Plant Microbe Interactions')

Presentations at scientific conferences

The research has been presented internationally at a workshop associated with the 9th International Plant Pathology congress held in Torino, Italy during August 2008.

Agarwal A, Kaul V, Faggian R and Cahill D (2008). Host-pathogen interactions in compatible Arabidopsis roots with an Australian field population of *P. brassicae*. (Poster). Plasmodiophorid pathogens and related organisms workshop, 9th International Plant Pathology Congress (Torino Italy, August 2008).

Caroline Donald presented a summary of Australian clubroot research 'Managing plasmodiophorid pathogens on Australian vegetable farms' at the Plasmodiophorid pathogens and related organisms workshop, 9th International Plant Pathology Congress (Torino Italy, August 2008). This oral presentation included discussion of the poster by Agarwal *et. al* (details above) and a summary of progress towards SAR induction in broccoli. This presentation led to a 2009 visit by Prof. Jutta Ludwig-Müller (Technical University Dresden, Germany) which resulted ongoing collaboration between the two research groups.

In addition the research has been presented at conferences nationally
- Arati Agarwal presented a conference paper at the Australasian Plant Pathology Society APPS, 2009 conference held in Newcastle (29th Sept-1st Oct, 2009).

Agarwal A, Kaul V, Faggian R, Cahill DM (2009). 'Gene expression changes during host-pathogen interaction between *Arabidopsis thaliana* and *Plasmodiophora brassicae'*, in conference proceedings page 65.

- Caroline Donald presented a conference paper at the Australasian Plant Pathology Society APPS, 2009 conference held in Newcastle (29th Sept-1st Oct, 2009).
  Agarwal A, Donald EC, Faggian R, Cahill DM, Lovelock D, Porter IJ (2009). 'Systemic acquired resistance-a new addition to the IPM toolbox?' in conference proceedings page 68.
- A poster was presented by the PhD student David Lovelock at the Australasian Plant Pathology Society APPS, 2009 conference held in Newcastle (29th Sept-1st Oct, 2009).
  Lovelock D, Agarwal A, Donald EC, Porter IJ, Faggian R, Cahill DM (2009). 'Development of techniques to measure SAR induction in broccoli for clubroot disease resistance', in conference proceedings page 173.
- A poster was presented by the PhD student David Lovelock at the Australasian Soilborne Diseases Symposium ASDS, 2010 conference held on Sunshine Coast (9th -11th Aug, 2010).
  Lovelock D, Agarwal A, Donald EC, Porter IJ and Cahill DM (2010). 'Monitoring root and leaf salicylic acid to optimise induction of systemic acquired resistance in broccoli' (submitted).

Promotional/information articles submitted to industry publications

An updated one page subprogram flyer was prepared and included in a program booklet 'Vegetable IPM disease program an overview'. This booklet was used to promote the vegetable pathology program at the Vegetable Industry conference and expo during May 2009 and subsequently at field days and workshops.

An article was published in Vegetables Australia
'Systemic acquired resistance: boosting nature's defences' Vegetables Australia 5.4 (Jan/Feb 2010) pp46-47.

An article is being prepared for 'Brassica IPM' a newsletter distributed nationally to vegetable brassica growers. This newsletter will be distributed in September 2010.

Steering/advisory committee

It was anticipated that an IPM coordinator would be in place for the pathology program during January 2008 to oversee communication and extension activities of the HAL pathology programs including steering committee activities. This appointment was delayed until April 2008 awaiting the outcome of a number of vegetable industry reviews. Researchers were advised at the November program workshop (Melbourne on 27th-28th November 2008) that no appointment to the IPM coordinator position had been made nor had a pathology program steering committee been established. This situation continued throughout 2009 with the position tendered but not filled. In consultation with Leanne Wilson (former program manager) it was decided that since the project is strongly innovative and the research developmental, project leaders should seek an appropriate scientific network to provide relevant technical and scientific advice. These networks were established through the laboratory of Prof. Jutta Ludwig-Müller (Technical University, Dresden). This laboratory specialises in plant defence responses in particular in response to clubroot disease and is currently working to induce plant defence in *Arabidopsis* and Chinese cabbage. Their

advice and assistance in the development of molecular and biochemical methods to monitor changes in gene expression and plant tissue SA has been invaluable. More recently the team has been approached by the laboratory of Dr Simon Bullman (New Zealand Institute for Plant and Food). Discussions have been initiated regarding the development of a collaborative relationship with this laboratory. In addition several fruitful discussions ensued following presentations made by the project team at the Australasian Plant Pathology Conference (Newcastle 29th Sept-1st Oct 2009). In particular discussions with Robin MacDiarmid (New Zealand Institute for Plant and Food), Adrienne Hardham (Australian National University) and David Guest (Sydney University) were productive.

Presentations at industry workshops and events

A number of presentations were made at industry workshops and field days. These provided a means to introduce the concept of systemic acquired resistance to growers and to seek feedback particularly on the commercial suitability of methods of application of SA that were being developed. Most of these events were held in conjunction with soilborne disease subprograms 2.1 and 2.2.

Field days
National Vegetable Expo Display - 7th-8th May 2009.
Lindenow VIC field day - 10th June 2009
Clyde VIC field day - 19th June 2009

Workshops
Granite Belt QLD – 1st July 2008
Lockyer Valley QLD – 2nd July 2008
Cranbourne VIC – 3rd July 2008
Darwin NT – 23rd/24th September 2008
Devonport TAS workshop – 4th August 2010
Gympie QLD workshop – 11th August 2010
Gatton QLD workshop – 12th August 2010
Lindenow VIC workshop – 18th August 2010
Cranbourne VIC workshop – 19th August 2010

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