THE EFFECTS OF BACTERIAL AND JASMONIC ACID TREATMENTS ON INSECTS OF CANOLA

by

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ABSTRACT

Two strains of plant growth-promoting rhizobacteria, *Pseudomonas chlororaphis* (PA23) and *Bacillus amyloliquefaciens* (BS6), can control some fungal diseases of canola through production of bacterial metabolites and through induced systemic resistance, which is initiated by the signalling molecule jasmonic acid. Direct application of jasmonic acid activates defence-related compounds and influences insect herbivory in canola. Field and laboratory studies investigated the effects of the two bacteria and of jasmonic acid on insects of canola. In the field there were no consistently significant effects of treatment on insects sampled by beat cloth or sweep net, level of flea beetle injury, canola yield or quality. In the laboratory, jasmonic acid significantly increased oviposition and decreased larval feeding in diamondback moth (*Plutella xylostella*) and slowed development and reduced reproduction in turnip aphid (*Lipaphis erysimi*). The effects of jasmonic acid on canola were systemic. Analysis of leaf tissue showed significant effects of treatment on defence-related compounds.

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Chapter 1

INTRODUCTION

Modern agriculture relies heavily on pesticides for the control of pathogens and invertebrate pests. Without effective control measures, it is estimated that yield losses due to pests would be more than double their present level (Oerke and Dehne 2004). However, the extensive use of existing pesticides has the potential to lead to the development of resistance, the loss of numerous pesticide options and more severe pest outbreaks (Bottrell and Adkisson 1977, Brattsten *et al.* 1986). These problems, as well as the growing concern over the safety of pesticides to humans and the environment, make alternative control measures very desirable.

In Canada, canola production reached 9.1 million tonnes in 2006 and is projected to reach 15 million tonnes by 2015 (Canola Council of Canada 2006). With increased production there has been an increase in diseases such as the blackleg pathogen (*Leptosphaeria maculans* Desmaz.) (Juska *et al.* 1997). In addition to increased disease pressure, the increase in canola production has provided a huge resource for insect herbivores. Successful management of these potential problems requires the development of alternative control strategies as part of an integrated pest management strategy.

Work done by Fernando *et al*. (2005, 2007) on the use of plant growth-promoting rhizobacteria (PGPR) in canola highlights the potential role bacteria can play in the control of fungal pathogens. Two PGPR species that are especially effective in reducing the incidence of disease by *Sclerotinia sclerotiorum* (Lib) De Bary in the field are *Pseudomonas chlororaphis* strain PA23 and *Bacillus amyloliquefaciens* strain BS6

(Fernando *et al.* 2007). Laboratory studies on canola and hot pepper show that treating plants with PA23 then inoculating them with a pathogen induces significantly higher levels of defence compounds compared with control plants (Nakkeeran *et al.* 2006, Fernando *et al.* 2007). Several of the induced defence compounds, including peroxidase, polyphenol oxidase and phenolic compounds, are involved not only in defence against plant pathogens, but also against insect herbivores (Duffey and Stout 1996). Following tissue damage by insect feeding, these compounds can react to form toxic or antinutritive compounds that can be important in plant defence against insect herbivores (Duffey and Felton 1991). Thus PGPR may be useful in the control of insect herbivores of canola.

For my Master's research I examined the effects of the PGPR strains *P. chlororaphis* PA23 and *B. amyloliquefaciens* BS6 on insects of canola. These PGPR may alter the feeding habits of herbivorous insects through changes in plant chemistry associated with induced resistance, or directly through the production of metabolites or volatile compounds. Jasmonic acid treatments were also investigated, as they are a means of activating the induced systemic resistance process, and so could elucidate mechanisms of any effects of PGPR treatments on insect herbivores. There were two main objectives of this research. The first was to conduct field experiments to study the effects of these treatments on insect herbivore population densities, focusing on some of the most important insect pests: diamondback moths, flea beetles, cabbage root maggots, bertha armyworms and lygus bugs. The second objective was to examine the effect of the treatments on specific aspects of the biology of the diamondback moth (*Plutella xylostella* L.) and turnip aphid (*Lipaphis erysimi* Kaltenbach) under controlled conditions in the laboratory.

Chapter 2

LITERATURE REVIEW

2.1 Canola

Rapeseed varieties containing low levels of erucic acid and glucosinolates were first developed in Canada at the University of Manitoba (Stefansson 1983). To promote newly developed varieties and prevent confusion with inedible forms of rapeseed, the Rapeseed Association (now the Canola Council of Canada) branded rapeseed with <5% erucic acid and <3 mg/g glucosinolates "canola" (Gray *et al.* 2006). The definition of canola has since been changed to cultural varieties (cv.) of *Brassica napus* (L.) and *B. rapa* (L.) (Brassicaceae) that contain <2% erucic acid and <30 μmoles/g glucosinolates (Canola Council of Canada 2006).

In Canada 9.1 million tonnes of canola were produced in 2006, and it is anticipated that production levels will nearly double by 2015 (Canola Council of Canada 2006). This increase in production will require an increase in agricultural land devoted to canola, or the accommodation of canola production on existing agricultural land through reduced rotational intervals. Either change will lead to a more homogenous landscape and a greater risk of pest outbreaks and disease epidemics.

2.2 Insects and pathogens of canola

Brassica napus, *B. rapa* and all other members of the plant family Brassicaceae contain glucosinolates, secondary plant compounds containing nitrogen and sulphur (Kjaer 1963, Feeny 1977). Glucosinolates hydrolyse in the presence of the enzyme

myrosinase to produce a number of potentially toxic products including isothiocyanates, also known as mustard oils (Chew 1988). Glucosinolates and myrosinase are stored separately in the plant (Louda and Mole 1991) and the formation and release of isothiocyanates occurs when plant tissue is damaged as well as at lower rates during normal catabolism (Schoonhoven *et al.* 1998). These hydrolysis products are toxic to insects, but insect specialists of Brassicaceae have adaptations to detoxify these compounds (Feeny 1977). Insects may use different enzymes including glycosidases and glutathione transferases in the breakdown of glucosinolates and their hydrolysis products into less toxic forms (Lindroth 1991).

Visual and chemical cues provide information required for host plant recognition by insects (Feeny 1991). In Brassicaceae, volatiles produced through glucosinolate hydrolysis are the main stimulants used by cruciferous insects in host plant recognition (Chew 1988), while visual cues, such as leaf shape and colour, are also important cues in host plant recognition for many insects (Thorsteinson 1960).

There are numerous insect herbivores that attack canola in the Canadian prairies (Lamb 1989). Chemical control may be required to prevent serious losses due to insects (Lamb and Turnock 1982, Bracken 1987), but for some insects, insecticidal control may not be effective (Soroka *et al.* 2004, Antwi *et al.* 2007). In addition to insect herbivores present in canola growing regions, there is also a threat of introduction of new insect herbivores. The cabbage seed pod weevil, *Ceutorhynchus obstrictus* (Marsham), and swede midge, *Contarinia nasturtii* (Kieffer), are serious invasive pests of canola that are currently found only in restricted parts of North America (Dosdall *et al.* 2002, Olfert *et al.* 2006). Modelling predicts that these insects will become more serious pests in the

future as they spread to other canola growing regions in Canada (Dosdall *et al.* 2002, Olfert *et al.* 2006). There is a desire to move away from dependence on chemical control measures, yet current insect herbivores can be difficult to control and new species may be introduced into agricultural areas, therefore, there is a need for alternative control strategies.

In the Canadian prairies there are also numerous bacterial, fungal and viral diseases that can cause yield losses in canola. Two important fungal pathogens, *Sclerotinia sclerotiorum* and *Leptosphaeria maculans* (Desmaz.), can be difficult to control with fungicides (Canola Council of Canada 2006), making alternative control measures desirable. Hundreds of plants species can be infected by *S. sclerotiorum*, causing a wide range of disease symptoms (Purdy 1979). In canola, *S. sclerotiorum* can cause leaf blight and stem rot, the latter being the more serious manifestation (Bardin and Huang 2001). This disease can cause economic losses in the Canadian prairies (Gugel and Morrall 1986), and fungicides used for control of *S. sclerotiorum* can be costly (del Rio *et al.* 2007) and highly variable in their efficacy (Bradley *et al.* 2006). Blackleg (*L. maculans*) is another important fungal pathogen found in most canola growing regions, including the Canadian prairies (Gugel and Petrie 1992). Blackleg can infect most parts of the plant, with most serious losses due to stem lesions or cankers (West *et al.* 2001). Control measures such as cultural practices (Guo *et al.* 2006) and the use of fungicides (Kharbanda 1992) can provide some control of Blackleg, but alternative control measures are needed (Kharbanda *et al.* 1999).

2.3 Induced plant defences

Plants defend themselves against herbivore or pathogen attacks in a variety of ways. Plant defences had long been thought of as constitutive, always present in the plant regardless of external influences (Karban and Baldwin 1997). Recently it has been discovered that plants may undergo changes in their defensive mechanisms in response to damage or stress; these changes are called induced responses (Karban and Myers 1989). Attack by herbivores and pathogens can induce plant responses that alter plant chemistry and render the plants unsuitable as hosts (Ryals *et al.* 1994, Stout *et al.* 2006).

Induced responses can also be activated in plants by a large number of biotic and abiotic elicitors that can induce plant defences in the absence of pathogen or herbivore attack (Oostendorp *et al.* 2001, Pieterse *et al.* 2001). Plant growth-promoting rhizobacteria (PGPR) and jasmonic acid are two elicitors that have the potential to control a number of plant diseases and insect herbivores, and these inducing agents will be discussed in detail. Wound-induced plant defences will also be discussed. A significant amount of research has been done on plant responses to wounding, and because they involve similar signalling pathways to those activated by PGPR and jasmonic acid, the induced plant responses are also similar (Gatehouse 2002). The use of PGPR and jasmonic acid elicitors in the control of insect herbivores and plant pathogens will be discussed.

2.3.1 Systemic acquired resistance

When a pathogen infects a plant a necrotic lesion frequently results. The lesion may be caused by the pathogen directly or by a hypersensitive response (HR) (Hammerschmidt 1999), in which the plant confines the pathogen by undergoing

programmed cell death in the area surrounding the infection site (Greenberg 1997). The necrotic lesion may result in the initiation of systemic acquired resistance (SAR) (Ryals *et al.* 1994), which confers long-term resistance against subsequent infection by numerous diseases, and has been known for almost 100 years (Chester 1933, Ryals *et al.* 1994). Following infection, salicylic acid production and accumulation occur locally and systemically (Gaffney *et al.* 1993), and is followed by the activation of the NPR1 gene and other genes that are involved in systemic acquired resistance regulation and pathogenesis-related (PR) protein production (Cao *et al.* 1997).

Although the mechanisms involved are not well understood, the enzymatic and antimicrobial activity of pathogenesis-related proteins can enhance resistance to subsequent pathogen attack by preventing pathogen development (Hammerschmidt 1999). The production and accumulation of pathogenesis-related proteins occur throughout the plant whenever systemic acquired resistance is induced by a pathogen, and, therefore, the presence of pathogenesis-related proteins indicates that systemic acquired resistance has been induced (van Loon 1997). In addition to, or instead of, the immediate activation of defence genes, plants may also undergo priming – the enhanced ability of plants to later induce defences in response to a subsequent attack by a pathogen or other pest (Conrath *et al.* 2002).

Although the systemic acquired resistance response has been observed for nearly a century, the mechanisms involved have only recently been elucidated. Ross (1961) infected portions of tobacco plants with tobacco mosaic virus, producing localized necrosis, and 7 days later inoculated uninfected plant parts with the virus. The previously infected plants produce smaller and less numerous lesions when challenged than do

control plants that were not previously infected. These results indicate a resistance response in the previously inoculated plants and that this response is systemic.

The exogenous application of salicylic acid can also induce resistance in tobacco against tobacco mosaic virus (White 1979), and similar genes are activated in plants treated with salicylic acid or inoculated with tobacco mosaic virus (Ward *et al.* 1991). Endogenous salicylic acid also increases significantly following tobacco mosaic virus infection in resistant tobacco cultivars (plants that produce both a hypersensitive response and systemic acquired resistance upon infection), but not in susceptible plants (Malamy *et al.* 1990), indicating that salicylic acid is required for resistance. These observations suggest that salicylic acid may be involved in systemic acquired resistance signalling.

Transgenic plants that do not produce or accumulate salicylic acid provide the most significant evidence in support of the hypothesis that salicylic acid is a signalling molecule for systemic acquired resistance. Gaffney *et al*. (1993) found that transgenic tobacco plants that lack salicylic acid do not exhibit systemic acquired resistance, and similar results have been found in other plant families (Rasmussen *et al.* 1991, Uknes *et al.* 1993).

2.3.2 Induced systemic resistance

Although systemic acquired resistance was first observed nearly a century ago, another form of non-constitutive resistance, induced systemic resistance (ISR), was recognized only about three decades ago (van Loon *et al.* 1998). While systemic acquired resistance is induced by pathogen infection, strains of specific rhizosphere bacteria called plant growth-promoting rhizobacteria (PGPR) can activate induced systemic resistance (Kloepper *et al.* 1992).

PGPR are symbiotic bacteria found in the rhizosphere that can provide plants with nutrients (Glick 1995), stimulate plant growth (Molla *et al.* 2001) in some plants, and can suppress pathogens in the soil (Suslow *et al.* 1979, Schroth and Hancock 1982). One way that PGPR are able to stimulate plant growth is through the production of plant hormones that stimulate development (Persello-Cartieaux *et al.* 2003). In the soil, these bacteria are able to suppress soil-borne plant pathogens through competition for nutrients (Kloepper *et al.* 1980, 1992) and through production of antibiotics (Fravel 1988).

Plant roots or surrounding soil can be inoculated with the bacteria to activate induced systemic resistance (van Loon *et al.* 1998). Induced systemic resistance can also be activated by applying bacteria to the above ground plant parts through pressure infiltration (Pieterse *et al.* 1996) in which plants are inoculated by infiltrating wounded leaves with a bacterial suspension (Swanson *et al.* 1988). Foliar applications of PGPR can also be used to activate induced systemic resistance (Fernando *et al.* 2007), but to show systemic induction rather than an antagonistic effect of the bacteria, laboratory experiments generally involve spatial separation with PGPR inoculation below soil and pathogen inoculation on leaves (Hoffland *et al.* 1995).

Induced systemic resistance is similar to pathogen-induced systemic acquired resistance, but it is not regulated by the signalling molecule salicylic acid (van Wees *et al.* 1997) and is not associated with the accumulation of pathogenesis-related proteins (Hoffland *et al.* 1995, Pieterse *et al.* 1996). Jasmonic acid and its derivatives (collectively termed jasmonates) are the signalling molecules involved in the octadecanoid pathway (Blechert *et al.* 1995), a pathway that regulates the production of defence related compounds associated with induced systemic resistance (Pieterse *et al.* 1998). Jasmonates

are common throughout the plant kingdom (Meyer *et al.* 1984), and are involved in plant development and senescence, as well as defence responses (Creelman and Mullet 1997). Following wounding (Creelman *et al.* 1992), insect feeding (Blechert *et al.* 1995), and induced systemic resistance (Pieterse *et al.* 1998), jasmonates are synthesized and can alter the expression of specific genes, in a similar manner to a stress-induced plant response (Mueller-Uri *et al.* 1988). Ethylene, another signalling molecule involved in induced systemic resistance, is activated downstream following the jasmonic acid response and sensitivity to both molecules is required for induced systemic resistance (Pieterse *et al.* 2001).

Salicylic acid can also be produced by some PGPR, and functions as an iron-chelating compound called a siderophore (Leeman *et al.* 1996). In some plants, the production of salicylic acid by PGPR can trigger induced systemic resistance (De Meyer *et al.* 1998), and if PR proteins are not activated the induced response is different from salicylic acid induced systemic acquired resistance (Hoffland *et al.* 1995). As with systemic acquired resistance, the gene NPR1 also regulates induced systemic resistance defence responses (van Loon *et al.* 1998), but different defence responses are activated by NPR1 depending on which pathway activates the gene upstream.

2.3.3 Wound-induced defences

Wound-induced responses are comparable to induced systemic resistance because jasmonic acid is also the signalling molecule in the wound-induced response to damage or insect feeding, although the pathways that are induced do not yield identical responses (Bostock 2005). Neither of these mechanisms of induced plant responses is very well understood, but the wound-induced model has been studied to a greater extent and can

provide important information about induced systemic resistance responses. As with systemic acquired resistance, priming can also occur in plants that exhibit induced systemic resistance or wound-induced responses (Pozo *et al.* 2005).

Induced responses through PGPR, insect feeding, wounding or other methods lead to an increase in the production of enzymes involved in jasmonic acid biosynthesis (Gatehouse 2002). This increase in enzymatic activity results in the activation of defence related genes such as those involved in the synthesis of defence proteins, volatiles and other secondary compounds (Gatehouse 2002). Induced responses in plants can also be activated by the direct application of chemical elicitors. For example, exogenous jasmonate application induces plant defences (Farmer and Ryan 1990, Blechert *et al.* 1995), and can be used to simulate plant responses to wounding (Thaler *et al.* 1996).

2.3.4 Differential induction with type of damage

Wound-induced responses in plants vary because induction can depend on the method of feeding or the presence of elicitors in oral secretions (Felton and Eichenseer 1999). Insect with biting and chewing mouthparts typically induce responses similar to induced systemic resistance that are dependent upon jasmonic acid as a signalling molecule, and may promote induced resistance against subsequent insect herbivores (Gatehouse 2002). Insects with piercing/sucking mouthparts tend to inflict limited tissue damage through feeding and are often able to evade the wound-induced defence response. These insects may activate defences similar to systemic acquired resistance responses that are dependent upon salicylic acid as a signalling molecule (Walling 2000). This induced plant response produces increased levels of pathogenesis related (PR)

proteins, enzymatic activity, and secondary metabolites, but only the latter are known to have negative effects on piercing/sucking insects (Walling 2000).

In many plant systems insect feeding can produce very different responses from those that follow mechanical damage. Feeding damage by insect herbivores can induce greater resistance to subsequent herbivory than occurs in mechanically damaged or undamaged plants (Agrawal 1998). In canola, mechanical wounding produces only a local response in plants, while insect herbivore damage produces a systemic response (Pontoppidan *et al.* 2003). The volatiles released in response to insect herbivore feeding may have a different composition to those released following mechanical damage (Agelopoulos and Keller 1994). Although it can be difficult to wound plants in a way that mimics insect feeding, the differences may be due to different elicitors found in insect saliva (Alborn *et al.* 1997). The type of salivary glands and the enzymes produced in oral secretions vary with different insect species, but little is known about the specific responses in most insect-plant interactions (Felton and Eichenseer 1999).

2.3.5 Activation of defence responses

Induced plant responses to PGPR, wounding or jasmonate elicitor application include the up-regulation of signals responsible for the expression of defence-related genes (Gatehouse 2002). Induced defences include low-molecular weight compounds called phytoalexins that exhibit antimicrobial properties and are produced by a plant following an infection, stress or damage (Kuć 1995), and high molecular weight proteinase inhibitors found in storage tissues in plants (Ryan 1990). Numerous phytoalexins can be induced in plants including phenolics (Karban and Myers 1989, Ongena *et al.* 2000), volatile compounds (Ryu *et al.* 2004, Arimura *et al.* 2005), and

oxidative enzymes like polyphenol oxidase and peroxidase (Constabel 1999). Both wound- and PGPR-induced plant responses and the effects on insects and pathogens will be discussed. There is a greater understanding of the wound-induced plant responses and the effects on insects, so these will be discussed in greater detail.

The production of phenolic compounds can result in higher levels of secondary plant metabolites as well as increased leaf toughness that may help deter herbivores (Karban and Myers 1989) and prevent pathogen infection (Nicholson and Hammerschmidt 1992). Local and systemic production of various enzymes, including polyphenol oxidase and peroxidase, occurs in plants in response to wounding (Duffey and Stout 1996) and induced systemic resistance by PGPR (van Loon and Bakker 2006). These enzymes can react with phenolics when plant tissue is wounded to form quinones that can negatively affect insect herbivores through their toxic nature, their ability to form toxic free radicals, and their interactions with proteins, which can decrease the nutritional content of plant material (Duffey and Felton 1991). Polyphenol oxidase and peroxidase also have antimicrobial effects that can help protect plants against disease (Tuzun 2001).

The production of herbivore-induced volatile compounds is common among plants, but the composition of volatiles varies markedly (Arimura *et al.* 2005). Volatiles can produce numerous insect responses, including the indirect defence (Dicke 1999) of attraction of insect parasitoids (Geervliet *et al.* 1994) and predators (Kessler and Baldwin 2001), or direct defence through deterrence of host-seeking herbivores (Arimura *et al.* 2005). Volatiles such as methyl jasmonate can also transfer signals to nearby undamaged plants (Gatehouse 2002).

Volatiles are also produced by PGPR, and some can control plant pathogens directly and indirectly. Some PGPR strains produce antifungal volatiles that can suppress pathogens on the surface of the plant (Fernando *et al.* 2005). Volatile production by PGPR can also trigger plant growth (Ryu *et al.* 2003) and activate plant defences through induced systemic resistance (Ryu *et al.* 2004).

One of the most important wound-induced responses for the control of insect herbivores is the production of proteinase inhibitors. Proteinase inhibitors have the potential to affect insect growth and development negatively by inhibiting proteolysis, the breakdown of protein, through competition for the binding sites of proteolytic digestive enzymes (Liener and Kakade 1980). Proteinase inhibitors can also interfere with the secretagogue control mechanism (Duffey and Stout 1996), which regulates enzyme production in insects (Chapman 1998). This interference causes the insect to increase production of digestive proteinases instead of body proteins (Lawrence and Koundal 2002). Both modes of action of proteinase inhibitors can result in decreased levels of amino acids and consequently less nitrogen available to the insect. Interference with nitrogen assimilation ultimately affects insect fitness, as nitrogen is a limiting factor in growth and development (Mattson 1980).

The action of proteinase inhibitors is generally specific against a single class of insect proteinases (Wolfson and Murdock 1990, Wolfson 1991). There are five classes of proteinases: aspartic, cysteine, serine, threonine and metalloproteinases (Barrett *et al.* 2004), and insect digestive proteinases of all classes except threonine proteinases have been detected (Lawrence and Koundal 2002). The high degree of specificity means that the induction of specific proteinase inhibitors will affect only those insects with

corresponding digestive proteinases. The diamondback moth (*Plutella xylostella*) uses serine-like proteinases and the crucifer flea beetle (*Phyllotreta cruciferae* Goeze) uses cysteine- and aspartic-like proteinases in digestion (Rymerson and Bodnaryk 1995). Rymerson and Bodnaryk (1995) found that the levels of proteinase inhibitors in untreated canola (*Brassica napus* and *B. rapa*) are not sufficient to provide resistance against these insects, but they noted that increasing the levels of proteinase inhibitors in canola could be a useful strategy to increase pest resistance.

2.3.6 Cross-Talk between Responses to Pathogens and Herbivores

The signalling pathways involved in systemic acquired resistance, induced systemic resistance and wound-induced responses are not entirely independent of each other and there is potential for cross-talk – positive or negative interactions between the signalling networks (Bostock 2005). These interactions – which are highly variable and depend on the insect, pathogen and plant involved – further complicate responses to pathogens, PGPR and wounding (Karban and Kuc 1999).

Wound-induced responses can induce resistance against pathogens and vice versa. Cotton plants (*Gossypium hirsutum* L.) exposed to feeding by spider mites (*Tetranychus urticae* Koch) have a lower incidence of infection with the fungal pathogen *Verticillium dahliae* (Kleb.) (Karban *et al.* 1987). Similarly, prior inoculation of cotton plants with *V. dahliae* results in significant reductions in the growth rate of mite populations on cotton plants (Karban *et al.* 1987). In tomato (*Lycopersicon esculentum* Mill.), feeding by larvae of the noctuid *Spodoptera exigua* (Hübner) induces resistance against the pathogen *Pseudomonas syringae* (van Hall) (Stout *et al.* 1998), and in Chinese cabbage (*Brassica rapa* L.), infection with the fungal pathogen *Alternaria brassicae* (Berk.) decreases the

fitness of the crucifer-specialist beetle, *Phaedon cochleariae* (Fabricius) (Rostás *et al.* 2002). Although the nature of these interactions is not well understood, resistance is assumed to be induced through the production of defence related compounds. This indicates that positive cross-talk, where induction of one pathway results in the activation of the other pathway, can occur in some interactions between plants, insects and pathogens.

There is also potential for negative cross-talk between the wound-induced (jasmonic acid-mediated) and pathogen-induced (salicylic acid-mediated) responses. An inverse relationship between salicylic acid mediated systemic acquired resistance and plant responses to insect feeding has been shown in the crucifer *Arabidopsis thaliana* (L.) (Cui *et al.* 2002). Compared with control plants, *A. thaliana* mutants with lower levels of salicylic acid-induced defence responses experience lower levels of feeding by the cabbage looper *Trichoplusia ni* (Hübner), and *A. thaliana* mutants with high levels of salicylic acid-induced defence responses experience higher levels of feeding (Cui *et al.* 2002). Studies on tomatoes have shown that jasmonate-induced resistance can significantly reduce insect populations (Thaler 1999a), but there is potential for negative cross-talk with systemic acquired resistance (Thaler *et al.* 2002a). When tomato plants are first treated with a synthetic salicylic acid analogue to induce resistance against pathogens (systemic acquired resistance) and then jasmonic acid treatments are applied, there is a reduction in the level of jasmonate-induced resistance (Thaler *et al.* 2002a). Thus, there can be negative cross-talk between the salicylic acid- and jasmonic acid-induced pathways, which must be taken into account when considering induced resistance as a control mechanism.

The interactions between induced plant responses to pathogens and insects are not well understood. The high degree of variability within and between plants species, as seen with both negative and positive cross-talk in tomatoes, indicates that generalizations cannot be made about interactions between the pathways. With further research into these areas and a greater understanding of plant responses, more general conclusions may be reached.

2.4 Potential for use in Agriculture

The activation of induced systemic resistance (ISR) by plant growth-promoting rhizobacteria (PGPR) could be used in agriculture to control plant pathogens and insect herbivores. The exogenous application of jasmonates has also been used to activate plant resistance. Although jasmonic acid is involved in plant defences against pathogens (Vijayan *et al.* 1998), most work on the exogenous application of jasmonates has been done for insect control, so that will be the focus of this review.

2.4.1 Potential for disease control by PGPR

Some PGPR species, particularly from the genus *Pseudomonas*, suppress disease through competition of the bacteria with pathogenic organisms and through the production of antibacterial compounds (Fravel 1988). In addition to this mechanism, many PGPR can suppress a wide range of diseases in a number of plant species through eliciting induced systemic resistance (van Loon *et al.* 1998). Some examples of PGPR-induced resistance to disease are highlighted in Table 2.1, which represents only a fraction of the research in this area. This list contains references cited in this thesis. A more complete list was compiled by van Loon *et al*. (1998). Because of the considerable

amount of research in this area on different plants, this section will only focus on work done on canola.

Sclerotinia sclerotiorum is a fungal pathogen that infects over 400 plant species, including many economically important crop plants (Boland and Hall 1994), and causes a wide range of deleterious disease symptoms (Purdy 1979). Fungicides are the main method used to control *S. sclerotiorum*, but they can be highly variable in their efficacy (Bradley *et al.* 2006). Because of this variability in control, and the potential for the development of fungicide resistance, alternative control strategies like induced systemic resistance are desirable. Fernando *et al*. (2005) screened 14 *Pseudomonas* isolates from canola and soybean for the potential to control *S. sclerotiorum*. Isolates were selected for further study based on their ability to produce antifungal volatiles and inhibit mycelial growth and sclerotial germination on agar plates. Several *Bacillus* spp. isolated from canola have also been screened for their ability to control *S. sclerotiorum* (Zhang 2004). Four isolates, strains *Pseudomonas chlororaphis* PA23, *Pseudomonas* sp. DF41, *Bacillus amyloliquefaciens* BS6 and *B. amyloliquefaciens* E16, provide significant disease suppression in greenhouse studies (Savchuck and Fernando 2004, Zhang 2004), and two of these strains (PA23 and BS6) control *S. sclerotiorum* in canola in the field (Fernando *et al.* 2007).

Pseudomonas chlororaphis PA23 and *B. amyloliquefaciens* BS6 can directly inhibit the growth of *S. sclerotiorum* through competition and the production of volatile compounds (Savchuck and Fernando 2004). These bacteria can significantly reduce the germination of *S. sclerotiorum* ascospores on the surface of canola compared with pathogen-inoculated controls (Fernando *et al.* 2007). The reduction of ascospore

germination on the surface of the plant is desirable for control of *S. sclerotiorum* in canola because high levels of disease are associated with high frequencies of petal infection (Gugel and Morrall 1986), and a reduction of germination on petals could reduce infection rates. In addition to the direct control of *S. sclerotiorum*, the application of PA23 followed by pathogen inoculation induces significantly higher levels of defence enzymes compared with canola inoculated only with pathogen or uninoculated controls (Fernando *et al.* 2007). In field trials, *P. chlororaphis* PA23 and *B. amyloliquefaciens* BS6 applications significantly reduced the level of stem rot caused by *S. sclerotiorum* (Fernando *et al.* 2007), despite significant reductions that occur in bacterial populations over time in the field (Zhang 2004). The activation of defence enzymes and the long-term disease resistance in field trials suggests that these bacteria induced systemic resistance in canola.

2.4.2 Potential for insect control by PGPR

PGPR-induced systemic resistance activates numerous defence responses that can suppress different pathogens, including bacteria, fungi and viruses (van Loon *et al.* 1998), and may also negatively affect insect herbivores. The few studies of the effects of induced systemic resistance on insect herbivores have mostly dealt with herbivorous insects that are also vectors of plant disease. Whiteflies, *Bemisia* spp., are vectors of the tomato mottle virus (ToMoV) (Schuster *et al.* 1996). Murphy *et al*. (2000) investigated control of the tomato mottle virus by three PGPR from the genus *Bacillus* that had previously been used in the control of cucumber mosaic virus (Zehnder *et al.* 2001). The PGPR were applied as seed treatments and/or powder amendments to soil in the greenhouse prior to movement of plants to the field. Although the results were variable,

some of the PGPR treatments significantly reduce the incidence of tomato mottle virus disease severity and increase yields. The treatments that had decreased disease severity also had lower densities of whitefly nymphs (Murphy *et al*. 2000), which may indicate that the treatments directly or indirectly affect the insect vectors. This study did not determine whether the results were due to increased pathogen or insect resistance, nor did the authors look at the direct effects of the PGPR or pathogen on insect behaviour, so more work is needed in this area.

Numerous diseases in cucumber can be controlled by PGPR (Liu *et al.* 1995a, Liu *et al.* 1995b), including cucurbit wilt (Zehnder *et al.* 1997a). Cucurbit wilt is a serious disease caused by the bacterium *Erwinia tracheiphila* (Smith) and transmitted by the striped cucumber beetle (*Acalymma vittata* Fabricus) and the spotted cucumber beetle (*Diabrotica undecimpunctata howardi* Barber) (Purcell 1982, Yao *et al.* 1996, Zehnder *et al.* 1997a). Cucumber beetles are thought to be attracted primarily to the compound cucurbitacin found in cucumber plants (Chambliss and Jones 1966, Ferguson *et al.* 1983).

To investigate the response of the disease vectors (cucumber beetles) to PGPR-induced plants, greenhouse studies were done on cucumber plants inoculated with the PGPR *Bacillus pumilus* strain INR-7 and *Flavomonas oryzihabitans* strain INR-5 as a seed treatment and soil drench (Zehnder *et al.* 1997a). Cucumber plants treated with PGPR have significantly lower insect feeding damage and wilt symptoms compared with untreated controls, and PGPR-treated plants show signs of wilt later than control plants (Zehnder *et al.* 1997a). Cucurbitacin concentrations are also significantly lower in PGPR-treated plants than the controls (Zehnder *et al.* 1997a). These results, and previous work that demonstrated a positive linear relationship between cucumber beetle density and

cucurbit wilt severity (Yao *et al.* 1996), suggest that the reduction in cucurbitacin reduces the attraction of the beetles, and, therefore reduces the feeding damage leading to lower disease severity.

In field studies, four PGPR, including the strains used in the greenhouse experiments, reduced the number of cucumber beetles in PGPR-treated plots compared with untreated controls on all sampling dates, except the last sampling date when beetle numbers were insignificant (Zehnder *et al.* 1997b). In no choice experiments in which beetles must feed on a single treatment, PGPR-treated plants have significantly lower levels of wilt than control plants (Zehnder *et al*. 1997b). These results demonstrate that PGPR reduce cucumber beetle populations due to reduced levels of cucurbitacin, but this does not confirm that the reduction leads to lower levels of wilt, as PGPR can control numerous pathogens in cucumber (Wei *et al.* 1991, Liu *et al.* 1995a), and this reason for the reduction in wilt cannot be excluded. Although the precise reason for the reduced disease incidence is not clear, the results demonstrates that PGPR have the potential to reduce plant compounds involved in insect attraction, and may have potential for herbivore control in other crop plants.

2.4.3 Potential for insect control by jasmonic acid

Exogenous application of jasmonates can activate genes involved in plant defence, demonstrating that jasmonates are an integral part of the defence signalling response in plants (Farmer and Ryan 1990, Gundlach *et al.* 1992). This plant response is often similar to induced systemic resistance because both require jasmonic acid as a signalling molecule, although the induced defence responses can be highly variable

(Bostock 2005). The exogenous application of jasmonic acid to activate plant defences has the potential to be an important tool in controlling insect herbivores.

The defence response of the model plant *Arabidopsis thaliana* has been studied extensively (Glazebrook 2001), and the use of transgenic *A. thaliana* plants has demonstrated that jasmonates are required in plant defences against insects (McConn *et al.* 1997). The exogenous application of jasmonates activates defence genes in *A. thaliana (*McConn *et al.* 1997, Stotz *et al.* 2000*)* and treated plants are more resistant to the generalist herbivores *Spodoptera littoralis* (Boisd.) (Stotz *et al.* 2002) and *S. exigua* (Hübner) (Cipollini *et al.* 2004). Jasmonate treated plants also attract significantly more parasitoids than untreated plants (Van Poecke and Dicke 2002). These studies demonstrate the role of jasmonate treatments in both direct and indirect plant defence against insect herbivores, but the potential for cross-talk must be considered. The activation of both systemic acquired resistance and induced systemic resistance can enhance defence against pathogens (van Wees *et al.* 2000), but the activation of systemic acquired resistance could compromise defences against insects (Cui *et al.* 2002).

Canola plants treated with jasmonic acid exhibit systemic increases in glucosinolate concentrations to levels significantly higher than those in wounded plants (Bodnaryk and Rymerson 1994) or untreated controls (Doughty *et al.* 1995). Compared to untreated controls, jasmonate-induced canola seedlings are fed upon less by the crucifer flea beetle (*P. cruciferae*) (Bodnaryk and Rymerson 1994). The authors found that jasmonate-induced seedlings are tougher, have lower protein content and have higher proteinase inhibitor levels than controls, which may explain the difference in flea beetle feeding. These results indicate an induced resistance response in the plants.

Defence compounds, including proteinase inhibitors and polyphenol oxidase, are systemically induced in tomato plants treated with jasmonic acid under greenhouse and field conditions (Thaler *et al.* 1996). Induction of proteinase inhibitors in tomato lowers the nutritional quality of plant material for insect herbivores by catabolizing amino acids in the insect midgut (Chen *et al.* 2005). Tomato plants treated with jasmonic acid have similar yields to untreated plants in the field, but plants are significantly less damaged by insect herbivores (Thaler 1999a). In field studies, Thaler *et al*. (2001) found significant reductions in beet armyworms (*S. exigua*) and western flower thrips (*Frankliniella occidentalis* Pergrande) on jasmonic acid treated tomato plants in both years of their study, and reductions in tobacco flea beetle (*Epitrix hirtipennis* Melsheimer) and potato aphid (*Macrosiphum euphorbiae* Thomas) populations on some of the treated plants. These results demonstrate the potential for jasmonic acid treatments to control insect herbivores with both types of mouthparts, although the extent to which individual species are affected is variable (Thaler *et al.* 2001). Jasmonic acid treatments also provide indirect defence for tomato plants through the enhanced production of volatile compounds that attract the parasitic wasp *Hyposoter exigua* (Viereck) that parasitizes lepidopteran pests (Thaler 1999b). There is also potential for negative cross-talk in tomato (Thaler *et al.* 2002a), so the potential for jasmonic acid treatments to affect defence against pathogen needs to be explored more fully.

2.5 Research Objectives

With the expansion of canola production in Canada, alternative pest control strategies are becoming increasingly important. Research on the use of PGPR on canola

and other plants indicates that these elicitors could be important in the control of plant pathogens, and may provide a useful alternative to chemical control measures (Fernando *et al.* 2004). Before PGPR treatments can be considered for use on a large scale, it is important to understand more fully the effects on insects. These treatments could affect insect behaviour making plants better defended against insect herbivores, or alternatively they could make plants more attractive to insects.

Jasmonic acid is another important elicitor of plant defences that has the potential to control insect herbivores. Research on tomato plants has shown that exogenous jasmonic acid treatments induce numerous defence-related compounds, protect plants against herbivorous pests (Thaler *et al.* 2001), and attract natural enemies (Thaler 1999b). Studies have also shown that jasmonic acid treatments can induce plant defences in canola (Bodnaryk and Rymerson 1994), and this may be an important method of insect control. It is of interest to explore the mechanism of insect response to PGPR through the use of jasmonic acid treatments, because of the role of jasmonic acid as a signalling molecule for induced systemic resistance.

The objective of this study was to investigate the effects of the bacterial strains *Pseudomonas chlororaphis* PA23 and *Bacillus amyloliquefaciens* BS6 and jasmonic acid treatments on insects of canola through field and laboratory studies. Field studies were carried out to investigate the effect of treatment on insect populations in canola through beat cloth, sweep net and root sampling. Laboratory studies were carried out to examine the effects of treatments on the biology of diamondback moth *P. xylostella* and the turnip aphid *Lipaphis erysimi* (Kaltenbach), two important pests of canola that exemplify insects with biting- chewing and piercing-sucking mouthparts respectively.

Table 2.1. Examples of systemic resistance induced by plant growth-promoting rhizobacteria.

Plant species	PGPR	Pathogen and symptoms	Reference
Arabidopsis thaliana (L.)	*Pseudomonas fluorescens* WCS417r	*Fusarium oxysporum* f. sp. r*aphani* (Kend. & Snvd.), vascular wilt	Pieterse *et al.* 1996
	Pseudomonas fluorescens WCS417r	*P. syringae* pv. *Tomato* (Okabe), bacterial speck	
	Bacillus pumilus SE34	Cucumber mosaic virus, necrotic lesions	Ryu *et al.* 2004
	Serattia marcescens 90-166		
Canola (*B.napus* L.)	*P. chlororaphis* PA23	*Sclerotinia sclerotiorum* (Lib.), stem	Fernando et al. *2007*
	DF-41 (*Pseudomonas*)		
	B. amyloliquefaciens BS6		Zhang 2004
	B. amyloliquefaciens E16		
Cucumber (Cucumis sativus L.)	*P. putida* 89B-27	*F. oxysporum* (Shclechtend.:Fr.) f. sp. Cucumerinum, vascular wilt	Liu *et al.* 1995a, 1995b
	S. marcesens 90-166	*P. syringae* pv. *Lachrymans* (Smith & Bryan), leaf spot	
Hot pepper (Capiscum annuum L.)	*P. fluorescens* Pf1	*Pythium aphanidermatum* (Edson), root rot	Ramamoorthy *et al.* 2002
	P. chlororaphis PA23		Nakkeeran *et al.* 2006
	B. subtilis BSCBE4		
Tomato (Lycopersicon esculentum L.)	*P. fluorescens* Pf1	*Pythium aphanidermatum* (Edson), root rot	Ramamoorthy *et al.* 2002

Chapter 3

FIELD STUDY ON THE EFFECTS OF *PSEUDOMONAS CHLORORAPHIS* STRAIN PA23, *BACILLUS AMYLOLIQUEFACIENS* STRAIN BS6 AND JASMONIC ACID ON INSECTS OF CANOLA

ABSTRACT

Pseudomonas chlororaphis strain PA23 and *Bacillus amyloliquefaciens* strain BS6 are two PGPR that have been shown to control some fungal diseases of canola. These bacteria are able to control diseases through multiple mechanisms including the activation of induced systemic resistance, which is initiated by the signalling molecule jasmonic acid. Field studies were performed to determine the effects of the two bacteria and of jasmonic acid on insects of canola. The treatments were applied as foliar sprays and applications were made at the cotyledon and bloom stage. The treatments were assessed for effects on populations of insect herbivores and beneficial species through beat cloth and sweep net sampling, and root maggot pupae were collected to investigate the effects on pupal parasitism. Plant samples were taken to examine the effects of treatment on flea beetle injury and seed samples were collected to examine the effects on yield and quality. There were no significant effects of treatment on injury by flea beetles, or on seed yield or quality. There were no significant effects of treatment on any of the insects sampled by sweep net or beat tray, except for flea beetles on one sampling date. There was a significant effect of treatment on cabbage maggot parasitism, with a significantly lower level of parasitoids among emerged insects when all treatments were combined and compared with the control.

3.1 Introduction

Canada in the largest producer of canola in the world, and most production in Canada occurs in the prairie provinces (Canola Council of Canada 2006). In the Canadian prairies there are numerous insects herbivores that feed on canola, and nearly all parts of the plant are susceptible to damage by one or more insect species (Lamb 1989). In the Canadian prairies diamondback moths (*Plutella xylostella* L.), bertha armyworms (*Mamestra configurata* Walker), flea beetles (*Phyllotreta* spp.), lygus bugs (*Lygus* spp.) and root maggots (*Delia* spp.) are some of the most important insect herbivores affecting canola (Lamb 1989). Larvae of the diamondback moth feed on leaf tissue (Harcourt 1957) and pesticide resistance is widespread, making them serious pests of Brassicaceae throughout the world (Talekar and Shelton 1993). In the Canadian prairies, diamondback moth generally does not cause serious damage, but there have been serious outbreaks in some years so monitoring is important (Dosdall *et al.* 2001). Larvae of another moth, the bertha armyworm, is a more serious pest in the Canadian prairies that can cause significant damage to canola pods during the final two instars (Bracken 1987). Outbreaks of the Bertha armyworm fluctuate, and long-term monitoring programs aid in predicting severity (Mason *et al.* 1998). The crucifer flea beetle (*Phyllotreta cruciferae* Goeze) and the striped flea beetle (*P. striolata* F.) are the most important flea beetles of canola in Manitoba (Burgess 1977, Lamb and Turnock 1982). These pests overwinter as adults (Burgess 1981) and can cause serious damage to canola cotyledons in spring (Burgess 1977). Lygus bugs feed on canola buds, flowers, pods and seeds (Lamb 1989), and in Manitoba three *Lygus* spp. are commonly found on canola – *Lygus lineolaris* (Palisot de Beauvois), *L. borealis* (Kelton), and *L. elisus* (Van Duzee) (Schwartz and Foottit 1992).

Annual seed losses due to lygus bug damage are estimated at 3-5% (Turnock *et al.* 1995). Larvae from several species of *Delia* (Diptera: Anthomyiidae) feed on the roots of canola (Dosdall *et al.* 1994), but the most significant of these is the cabbage maggot *D. radicum* L. (Turnock *et al.* 1992). In the Canadian prairies, Soroka *et al*. (2004) sampled nearly 3000 canola fields over a four year study and found that maggots were present in over 97% of all fields, with 96% of fields in Manitoba infested, up from 12% in the 1980s (Turnock *et al.* 1992).

Canola production in Canada is also limited by a number of diseases. Two of the most important diseases affecting canola in the Canadian prairies are *Sclerotinia sclerotiorum* (Lib.) and *Leptosphaeria maculans* (Desmaz.). These fungal pathogens are difficult to control with currently available fungicides (Canola Council of Canada 2006), and alternative control measures are desirable.

Plants are able to defend themselves against herbivorous pests through constitutive defences as well as through induction of defence compounds that can be activated in several ways. The use of elicitors to activate plant defences is an emerging area of pest control that could play an important role in canola production. In other crop species, plant diseases or insect herbivores can be negatively affected through the use of different elicitors, including biotic elicitors such as bacteria (van Loon *et al.* 1998) and fungi (De Meyer *et al.* 1998), and abiotic elicitors such as plant signalling molecules (White 1979, Thaler *et al.* 1996). Plant growth-promoting rhizobacteria (PGPR), examples of biotic elicitors, and jasmonic acid, an abiotic elicitor, may have the potential to control insect pests of canola through the activation of plant defence compounds.

The PGPR strains *Pseudomonas chlororaphis* (PA23) and *Bacillus amyloliquefaciens* (BS6) can provide effective control of the fungal pathogen *S. sclerotiorum* in canola in the laboratory and field (Savchuck and Fernando 2004, Zhang 2004, Fernando *et al.* 2007). In laboratory studies, PA23 and BS6 effectively colonize canola petals and suppress *S. sclerotiorum* when applied to canola plants prior to pathogen inoculation (Savchuck and Fernando 2004, Zhang 2004), and the application of PA23 followed by *S. sclerotiorum* activates induced systemic resistance-related compounds in canola (Fernando *et al.* 2007). Long-term disease suppression by BS6 in field studies suggests that this bacterial strain may also activate induced systemic resistance (Zhang 2004). Changes in plant chemistry associated with the activation of induced systemic resistance could affect the behaviour of insect herbivores of canola. These bacteria could also influence insect behaviour through the production of volatile compounds (Fernando *et al.* 2005).

Jasmonic acid is a signal molecule involved in the induced systemic resistance response of plants to PGPR (van Loon *et al.* 1998). It triggers the production of defence responses in plants, including the production of secondary metabolites (Gundlach *et al.* 1992). Exogenous applications of jasmonic acid stimulate the expression of plant defences (Farmer *et al.* 1992), and suppress insect herbivory of a number of plant species. For example, jasmonic acid treatments to canola seedlings induce plant defences resulting in reduced insect feeding damage (Bodnaryk and Rymerson 1994). In this study the effects on insect herbivores of treating canola with the PGPR strains PA23 and BS6, and with jasmonic acid were investigated in field experiments.

3.2 Methods

Field experiments were carried out at the University of Manitoba's Ian N. Morrison Research Farm, Carman, Manitoba. The field site was a 42 m x 26 m area divided into 20, 2 m x 6 m plots, with 2 m tilled buffer strips between plots to separate treatments and accommodate the seeder. A randomized complete block design was used in which four treatments were randomly allocated within each of the five replicate blocks (Fig. 3.1). In addition to the 20 experimental plots, there were 2 m strips of untreated buffer plots of canola planted around the entire field to prevent contamination with pesticides or other chemicals from other fields.

The canola was planted at a rate of 6 kg/ha and seeding depth of 2.25 cm on 15 May 2006 using a Noble® Drill. Due to weather conditions and the type of seeder used, there was a low level of initial emergence in the field, so a second field was planted adjacent to the existing field on 5 June 2006. The second field was identical in arrangement, seed source and seeding rate to the first field; however seeding was done with a double disc drill and seeding depth of 2.25 cm. The initial plots, planted on 15 May 2006, will be referred to as the 15 May field, and the second plots, planted 5 June 2006, will be referred to as the 5 June field.

On 29 June 2006 the herbicide Odyssey® (imazamox, imazethapyr) was applied to the 5 June field using a bicycle sprayer. The rate of application was 7 g product dissolved in 100 L of water per hectare. The buffers between plots were tilled on 5 July and 10 July to control the weeds.

3.2.1 Treatments

The two PGPR strains used in these experiments were obtained from existing cultures in Dilantha Fernando's laboratory at the University of Manitoba. The *Pseudomonas chlororaphis* strain PA23 was isolated from soybean root tips and *Bacillus amyloliquefaciens* strain BS6 was isolated from canola leaves (Savchuck and Fernando 2004, Zhang 2004). Bacterial stock cultures were maintained in the laboratory according to methods of Fernando *et al*. (2005). Fresh Luria Bertani Agar (LBA) plates were streaked with bacteria prior to use in the experiment. After 24 h, the newly plated bacteria was used to inoculate flasks containing autoclaved Luria Bertani (LB) broth (10 g Bacto™ tryptone (BD), 5 g Bacto™ yeast extract (BD), 5 g NaCl (Fisher), 1 L double distilled water). Inoculated flasks were placed in a shaking incubator maintained at 30˚C and 150 rpm. After 18 h, the bacterial cultures were removed from the incubator-shaker and their optical density was checked to ensure they were at the mid-log growth phase, approximately 10^8 cfu/ml (colony forming units) (Zhang 2004). The cultures were stored at 4˚C for up to 24 h prior to the time of the field application to allow time for sufficient quantities to be prepared. Jasmonic acid (Sigma-Aldrich, Oakville, Ontario) was stored at 5˚C until required for the experiments. Jasmonic acid was dissolved in methanol at a rate of 50 mg/ml methanol. The jasmonic acid solution was then diluted with distilled water to reach a final concentration of 0.5 millimolar jasmonic acid. Control plots were treated with water. One drop/L of tween® 20 (polyoxyethylene glycol sorbitan monolaurate, Sigma-Aldrich) was added to each of the treatments.

The first treatment application was made on 8 June 2006 to the 15 May field. The bacterial treatments were sprayed at a rate of 250 x 10^8 cfu per m^2 and the jasmonic acid

solution was sprayed at a rate of 0.125 mM per m^2. Control plots were sprayed with equivalent amounts of water (3 L). The bacterial, jasmonic acid and control treatments were applied to the plants using a backpack sprayer. Overnight the area received 5 mm of unexpected rainfall.

The second application of treatments was made on 19 July 2006 to the 5 June field at the 30–50% bloom stage. Plants were sprayed to run off using a backpack sprayer, requiring approximately 6 L of each treatment per plot. The two bacterial treatments were sprayed at a rate of 250 x 10^8 cfu per m^2. The 0.5 millimolar solution of jasmonic acid was sprayed to runoff (6 L per plot). Control plants were sprayed to runoff with 6 L of water. Plants were treated in the early evening and there was no rain in the 24 h following application.

3.2.2 Flea beetle injury

The first treatment application was sprayed on plants in the 15 May field at the cotyledon stage at about the time of flea beetle attack. Because of the uneven emergence in the 15 May field, pre- and post-treatment samples were taken of plants at the two growth stages present: cotyledon stage and first true leaf stage. Immediately before treatment on 8 June, 10 plants at each stage were removed from each plot, and on 14 June a further 10 plants of each stages were removed from each plot. All samples were placed in plastic bags and stored at 5˚C until processing could be done.

Feeding injury by flea beetles was assessed using the 11 point scale of Palaniswamy *et al*. (1998), in which a rating of 0 indicates no injury, 1 represents 1-10% injury of leaf area damaged and the scale is linear to 10 which indicates 100% of the leaf destroyed (Palaniswamy *et al.* 1998). For each plot, injury to pre- and post-treatment

samples of cotyledons and of true leaf samples were rated and recorded separately. The effect of treatment on mean post-treatment injury ratings was subjected to analysis of variance. A repeated measures analysis was used to assess the effect of treatments on the temporal changes between the pre- and post-treatment samples.

3.2.3 Beat cloth and sweep net samples

Beat cloth and sweep net samples were taken in the 5 June field that received treatment on 19 July. On 26 July 2006 and 9 August 2006 beat cloth samples were taken in each plot. Random numbers were used to select six 1 m sections around the outside of each plot. At each location a 1 m x 0.5 m cloth was placed along the edge of the plot and 1 m length of canola plants from the first outside row was bent over the cloth and beaten vigorously 10 times with the handle of a sweep net, as described by Kogan and Pitre (1980). Plants were then pushed back and the more mobile insects were aspirated from the cloth. The contents of the aspirators and remaining insects on the cloth were placed together in plastic bags, which were sealed and put on ice in coolers for transport to the laboratory where they were frozen.

On the same dates as beat cloth sampling, each plot was also sampled with a sweep net (50 cm radius) using the lazy-8 method (Kogan and Pitre 1980), with the top of the net held just below the top of the canola. The plots were swept while moving forward between the plots, with 5 complete figure eights made on each side of the plot. The contents of the net were sealed in plastic bags, and transported and stored in the same way as the beat cloth samples.

Of the insects collected, those of most interest, diamondback moths (*P. xylostella*), bertha armyworm (*M. configurata*), flea beetles (*Phyllotreta cruciferae*, *P.*

striolata), lygus bugs (*Lygus lineolaris*, *L. borealis*, *L. elisus*) and cabbage root maggots (*D. radicum*), were identified to species except *D. radicum*. Because of the difficulty in properly identifying *D. radicum*, all flies from the family Anthomyidae were recorded together. Two other lepidopteran species, the imported cabbageworm (*Pieris rapae* L.) and the zebra caterpillar (*Melanchra picta* Harris) that do not generally cause economic damage but are often found in canola (Canola Council of Canada 2006), were identified to species. Several species of thrips (Order: Thysanoptera) are found on canola in the prairies, and some may cause economic damage (Burgess and Weegar 1988), but due to the large number of thrips collected these insects were only identified to order. Total numbers of aphids were recorded as adult and nymphs, winged or mummies, but none of these were identified beyond family (Aphididae) because of the difficultly of identifying specimens after freezing. All other insects were identified to order or family, and those that were present in sufficient numbers were analyzed, including lacewings (Family: Chrysopidae), lady beetles (Family: Coccinelidae), heleomyzid flies (Family: Heleomyzidae), parasitic wasps (Order:Hymenoptera), and bees (Superfamily:Apoidea). All insects were identified using taxonomic keys (Kelton 1980, Foottit and Richards 1993, Triplehorn and Johnson 2004) and when possible, compared with specimens from the J.B. Wallis Museum of Entomology, Department of Entomology, University of Manitoba.

The data from each sampling date were log transformed before analysis of variance to investigate the effect of treatment. For those insects with sufficient numbers on both sampling dates, a repeated measures analysis was also done to investigate the effect of treatment on the pattern of change between sample dates.

3.2.4 Cabbage maggot sampling

Root samples for cabbage maggots, *D. radicum*, were taken during crop growth in the 5 June field on 27 July and 11 August. The final sampling date was 6 September 2006. Root samples were taken along the edge of each plot at 6 randomly selected sites. Using a random number table, the sampler took a random number of steps and selected the closest plant to pull for the root sample. Plants were cut above the location of the cotyledons, placed in sealable plastic bags, and stored at 5˚C until processing could be done. Root damage was rated on a scale of 0–4, according to the methods described by Dosdall *et al*. (1994). Larvae were dissected from the roots and identified according to Brooks (1951). On 6 September, one week after canola harvest, further root samples were taken. These roots were rated in the field. There were no larvae present at this time, so larval numbers were not recorded.

The data from each sampling date were subjected to analysis of variance to investigate the effect of treatment on the number of cabbage root maggot larvae and the level of damage. A repeated measures analysis was used to detect treatment effects on temporal patterns.

At the time of root sampling on 6 September, soil was collected from a 3 cm radius around the root to a maximum depth of 6 cm. The soil was placed in a tub of water and broken up so that floating puparia could be collected. Additional sampling was done 12 September to collect a sufficient puparia to estimate rates of parasitism. On this occasion, at locations around the edges of the plots, roots were pulled up and they and the surrounding soil were placed in white trays to look for puparia. For each plot, 30–40 puparia were collected. Puparia were brought back to the laboratory and each was placed

in a vial containing a 1:1 mixture of moist sand and vermiculite and put in an incubator at 16:8 h L:D and 20˚C. Vials were checked twice a week for emergence and the emerged species were recorded. This was done for 6 weeks, at which time no more insects were emerging, and the remaining pupae were assumed to have entered diapause. The vials were placed at 1°C for 12 weeks then returned to 20°C, and the vials were checked for emergence for the next 10 weeks until insects were no longer emerging. The remaining puparia were dissected and the contents were recorded. Dissections were performed on 30 March and 2 April 2007, approximately 3 weeks after insects had stopped emerging. Insects that did not emerge but could be identified were recorded, as well as unidentified insect remains.

The effect of treatment on the frequency of emergence was analyzed by a two-way contingency table, to see if the data for unemerged insects could be excluded from further analysis. The pupal parasitism data was then analyzed by a two-way contingency table to examine the effects of treatment of the level of parasitism and on the frequency of insect emergence. All data were analyzed by two-way contingency tables using the likelihood ratio Chi-square ($LR\chi^2$).

3.2.5 Yield and seed quality

To measure yield, a 1 m^2 area of canola was harvested from each plot 31 August 2006 using hand sickles. Harvested plants were placed in burlap or flour sacks, which were hung up under cover to dry until 14 September when the seeds were collected using a belt thresher. The cleaned seed was weighed for each treatment to compare yield. A FOSS NIRSystems model 6500 near-infrared reflectance scanning monochromator was used to analyze seeds for oil, protein and glucosinolate content using standardized

methods calibrated for canola. The effects of treatment on seed yield and quality were investigated by analysis of variance.

3.3 Results

3.3.1 Flea beetle injury

Due to uneven emergence, the first treatments were applied to canola plants that were at cotyledon and true leaf stages and had already sustained some flea beetle damage. Injury ratings for the cotyledon stage and first true leaf stage were compared separately (Fig. 3.2 and 3.3). No significant treatment effects were found for the level of flea beetle damage before treatment for cotyledons ($F=1.52$, $df=3,12$, $P=0.261$), true leaf ($F=0.28$, $df=3,12$, $P=0.842$) or combined plant samples ($F=0.42$, $df=3,32$, $P=0.738$) or after treatment for cotyledons ($F=0.79$, $df=3,12$, $P=0.525$), true leaf ($F=0.85$, $df=3,12$, $P=0.494$) or combined plant samples ($F=1.10$, $df=3,32$, $P=0.365$). A repeated measures analysis also showed no treatment effects on overall injury ratings for the cotyledon data ($F=0.37$, $df=3,12$, $P=0.779$), first true leaf data ($F=0.41$, $df=3,12$, $P=0.748$) or the combined data ($F=0.43$, $df=3,32$, $P=0.733$), or on the pattern of temporal change for the for the cotyledon data ($F=2.25$, $df=3,12$, $P=0.135$), first true leaf data ($F=0.45$, $df=3,12$, $P=0.720$) or the combined data ($F=0.83$, $df=3,32$, $P=0.485$).

3.3.2 Beat cloth and sweep net samples

From the beat cloth samples, aphids (Family: Aphididae), diamondback moths (*P. xylostella*), imported cabbageworms (*P. rapae*), flea beetles (*P. cruciferae*, *P. striolata*), lacewings (Family: Chrysopidae), lady beetles (Family: Coccinelidae), lygus bugs (*L. lineolaris, L. elisus*, *L. borealis*), thrips (Order: Thysanoptera) and zebra caterpillars (*M.*

picta) were present in sufficient numbers for statistical analysis on one or both sampling days (Table 3.1). From the sweep net samples aphids, diamondback moths, Diptera (Families: Anthomyiidae, Heleomyzidae) flea beetles, parasitic wasps (Order:Hymenoptera), bees (Superfamily:Apoidea) lacewings, lady beetles, and lygus bugs were present in sufficient numbers for statistical analysis on one or both sampling days (Table 3.2). All life stages of the insects were combined for analyses.

There were no significant treatment effects for any of the insects sampled on either sampling date for either beat cloth or sweep net samples (Tables 3.1 and 3.2), with the exception of flea beetles sampled by beat cloth on 26 July. A Dunnett's test was performed following the analysis of variance of the flea beetles for 26 July and none of the treatments were found to be significantly different from the controls.

There were also no significant effects of treatment on the overall number of insects collected for both sampling dates or on the pattern of change between the sample dates for either beat cloth or sweep net samples (Tables 3.3 and 3.4), with the exception of flea beetles from the beat cloth sampling in which there was a significant effect of treatment on the overall number of insects.

3.3.3 Cabbage maggot sampling

When each sample date was analyzed separately, there were no significant effects of treatment on the number of larvae (July: $F=2.08$, $df=3,12$, $P=0.155$; August: $F=0.15$, $df=3,12$, $P=0.930$). When a repeated measures analysis was performed, there were no significant effects of treatment on numbers of larvae overall ($F=0.26$, $df=3,12$, $P=0.852$) or on the pattern of temporal change ($F=1.27$, $df=3,12$, $P=0.328$) (Fig. 3.4). There were also no significant effects of treatment on the level of root damage when each sample

date was analyzed by sampling date (July: F=1.28, df=3,12, P=0.327; August: F=0.54, df=3,12, P=0.661; September: F=0.49, df=3,12, P=0.698). When a repeated measures analysis was performed, there were no significant effects of treatment on overall root damage (F=0.67, df=3,12, P=0.587) or on the pattern of temporal change (F=0.87, df=6,24, P=0.534) (Fig. 3.5).

The insects that emerged from the cabbage maggot puparia were *D. radicum*, *Aleochara bilineata* (Gyllenhal), and *Trybliographa rapae* (Westwood) (Table 3.5). When the puparia were dissected after emergence ceased, the insects were identified as not emerged if the puparia contained insects that developed but failed to emerge, or unidentified remains if no insect could be identified. There were no parasitoids identified during puparial dissection, only *D. radicum* (89.9%) and unidentified remains (10.1%). The effect of treatment on the frequency of emergence was analyzed by comparing the *D. radicum* emerged with those identified through dissection, and there were no significant effects ($LR\chi^2$=6.319, df=3, P=0.097). The total number of all emerged and not emerged insects were analysed by a two-way contingency table and there were no significant effects of treatment on the frequency of emergence ($LR\chi^2$=5.224, df=3, P=0.156), so data on unemerged individuals were excluded from further analyses of the level of parasitism.

In the emerged insects, the level of parasitism by *T. rapae* was too low for analysis (Table 3.5), so only the frequency of *A. bilineata* parasitism was analyzed. Although there was no significant overall treatment effect on the frequency of *A. bilineata* compared with *D. radicum* ($LR\chi^2$=4.927, df=3, P=0.177), there was a consistently lower percentage of *A. bilineata* among emerged insects in all treatments

than in untreated controls (Table 3.5), and this effect was significant ($LR\chi^2=4.842$, df=1, P=0.028).

3.3.4 Yield and seed quality

There were no significant effects of treatment on the seed yield (g/m^2) (F=2.65, df=3,12, P=0.096) (Fig. 3.6), glucosinolate content (F=2.85, d=3,12, P=0.082) (Fig. 3.7) or on the level of oil (F=1.30, d=3,12, P=0.318), or protein (F=1.35, d=3,12, P=0.306) (Fig. 3.8). Based on results gained in laboratory experiments (see chapter 4) it was of interest to do a contrast between the control and jasmonic acid treatments for the seed yield and glucosinolate content. There were no significant effects of a contrast between the control and jasmonic acid for seed yield (F=3.45, df=1,12, P=0.088) or glucosinolate content (F=3.98, df=1,12, P=0.069), although 43.3% of the yield variability and 46.6% of the glucosinolate variability in the original analysis were attributed to jasmonic acid.

3.4 Discussion

The spring weather conditions and the type of seeder used in planting the canola resulted in a low level of initial emergence in the 15 May field followed by a second wave of emergence. These problems lead to the plots containing fewer canola plants than expected, and plants were at different stages of development. The application of the first treatments was planned for when cotyledons first appeared, but was delayed to ensure that there would be enough plants in all plots for treatment and sampling. Because of this delay significant flea beetle damage occurred before treatment. In addition to these problems, there was also 5 mm of rain shortly after the treatments were applied that could have reduced their effect.

Adult flea beetles emerge in the spring and feed on cotyledons and first true leaves of canola, seriously damaging or even killing seedlings (Burgess 1977). Plants that are not killed may exhibit stunting, delayed or uneven maturity, and lower yields (Lamb 1984). Granular or foliar insecticides are often used to help control flea beetle injury on seedlings, but insecticide treated plants can still be significantly damaged by flea beetles (Lamb 1984, Antwi *et al.* 2007).

From the repeated measures analysis of the cotyledon data, there were no significant effects on the pattern of temporal change, but there was an apparent decline in the injury ratings on the second sampling date for the true leaf samples. This difference may be attributed to plant growth and reduced feeding, as flea beetles feed more on the newly emerged cotyledons.

In laboratory studies, treatment of canola seedlings with jasmonic acid reduces feeding rates by *P. cruciferae* by approximately half compared with untreated controls (Bodnaryk and Rymerson 1994). This response is attributed to the systemic induction of defences leading to greater leaf toughness, higher proteinase inhibitor levels, and reduced protein content (Bodnaryk and Rymerson 1994). Wounding and insect feeding can also activate defences in canola cotyledons. Mechanically wounded cotyledons receive 40% less feeding damage compared with unwounded controls (Bodnaryk 1992). When populations are high, flea beetle can cause serious injury to plants even if they are treated with insecticides. The ability of these insects to overcome toxic treatments suggests that the activation of non-toxic plant defences through wounding or jasmonic acid application is not likely to have a significant effect on flea beetle populations in the field.

Based on previous studies on canola and other plants, it was predicted that jasmonic acid treatments would have an effect on insect herbivores or natural enemy populations. In tomato plants, jasmonic acid treatments can directly reduce herbivory by increasing plant defences that act on numerous insects (Thaler *et al.* 2001) and indirectly through the activation of volatile compounds that increase attraction of natural enemies of lepidopteran larvae (Thaler 1999b). In field studies jasmonic acid treated tomato plants have increased levels of polyphenol oxidase and proteinase inhibitors (Thaler *et al.* 1996), compounds that are involved in plant defence against insects (Duffey and Stout 1996).

In laboratory studies on canola, jasmonic acid treatments can increase proteinase inhibitor levels, as well as other defences and glucosinolate levels (Bodnaryk and Rymerson 1994). Although increased glucosinolate levels do not determine the level of resistance to insect attack (Bodnaryk and Palaniswamy 1990, Birch *et al.* 1992), other defence compounds may also be induced, but there were still no significant effects on any of the insects sampled in this study with the exception of flea beetles sampled on 26 July. On this date there were very few flea beetles present in the field and the significance of the treatment effect did not persist to the second sample date. This significance is attributed to random chance, as there were no other significant results from the large number of tests performed on the beat cloth and sweep net samples, and it is expected that there would be some type 1 error.

The 5 June field emerged evenly and was used for the remainder of the field season. At the time of cotyledon emergence in the 5 June field there were no flea beetles present, so treatments were not made at this time. In previous field studies, PA23 and

BS6 treatments provided the greatest level of control for *S. sclerotiorum* when plants received two bacterial applications, applied at 30% and 50% bloom (Fernando *et al.* 2007). Plants from the field study were not investigated for levels of defence compounds, but laboratory studies indicate a peak and decline in defences approximately 4–6 days after inoculation with PA23 and *S. sclerotiorum* (Fernando *et al.* 2007). The single application of treatment could be a reason why no treatment effects were seen in my field study.

There were no significant treatment effects on the level root damage by *D. radicum* larvae for any of the sample dates and no effect of treatments on the temporal pattern, yet the data appears to show a trend of greater damage ratings on the second sample date than the first or third (Fig. 3.5). The plots were sampled in the same manner on all collection dates, but this difference could be attributed to the visual ratings done in the field instead of the laboratory on the final sampling date. Larval damage increases throughout the summer, then reaches a relatively constant level near the end of the season (Holliday 2002), which explains the differences seen between the first and second sampling dates.

In the Canadian prairies, *T. rapae* and *A. bilineata* are the most important natural enemies of *D. radicum* (Hemachandra *et al*. 2007). In this study the level of parasitism by *T. rapae* was too low for statistical analysis, so the effect of treatment on *A. bilineata* was analyzed. *Alechochara bilineata* use infochemicals (volatile organic compounds) from *D. radicum* host plants to locate prey, and are significantly more attracted to infested plants over uninfested plants (Royer and Boivin 1999). Dimethyl disulfide (DMDS), is the most abundant volatile compound emitted by *D. radicum*-infested canola roots through the

breakdown of glucosinolates, and is presumed to be the main volatile released from other Brassicaceae as well (Ferry *et al.* 2007). Although dimethyl disulfide is highly attractive to *A. bilineata* at high doses, it is not as attractive as infested canola roots (Ferry *et al.* 2007), which is likely due to a high degree of specificity of plant volatiles required for host location by *A. bilineata* (Riley *et al.* 2007).

The percent parasitism data shows a significant increase in *A. bilineata* parasitism in the control plots (untreated) compared with all other treatments combined (treated). This could indicate that the process of treatment results in a decrease in parasitism by *A. bilineata*. This decrease could be due to an increased avoidance of treated plants by *A. bilineata* or an increased attraction to control plots.

Root feeding by *D. radicum* can induce systemic plant responses that can help defend plants against subsequent herbivory on aboveground plant parts (van Dam *et al.* 2005). Treatment with jasmonic acid can induce local and systemic plant responses similar to *D. radicum* feeding (van Dam and Raaijmakers 2006) including an increase in aromatic glucosinolates (van Dam *et al.* 2004). There were no significant effects of treatment on the number of *D. radicum* larvae in the roots or on the larval damage ratings, so volatile release due to larval feeding damage does not explain this difference in *A. bilineata* parasitism. It is possible that induced responses to PGPR or jasmonic acid lead to changes in the volatile blend emitted by the plants, which could have an effect host location by *A. bilineata*.

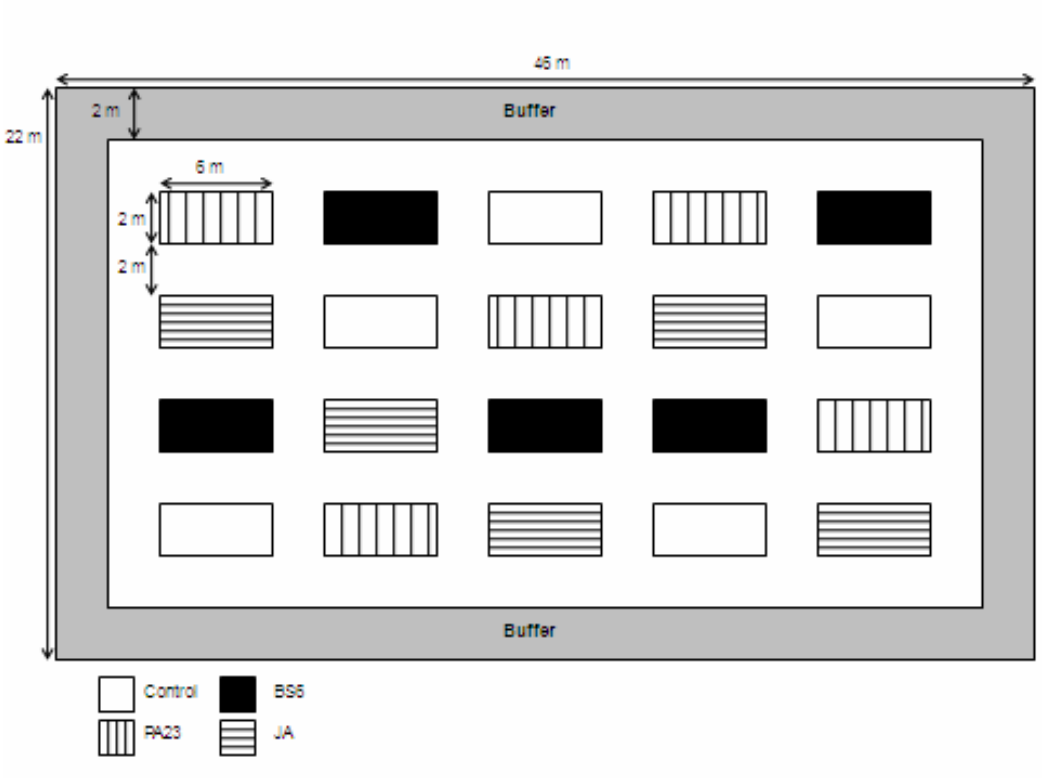

Figure 3.1. Field layout (randomized complete block design) used for fields seeded 15 May and 5 June 2006.

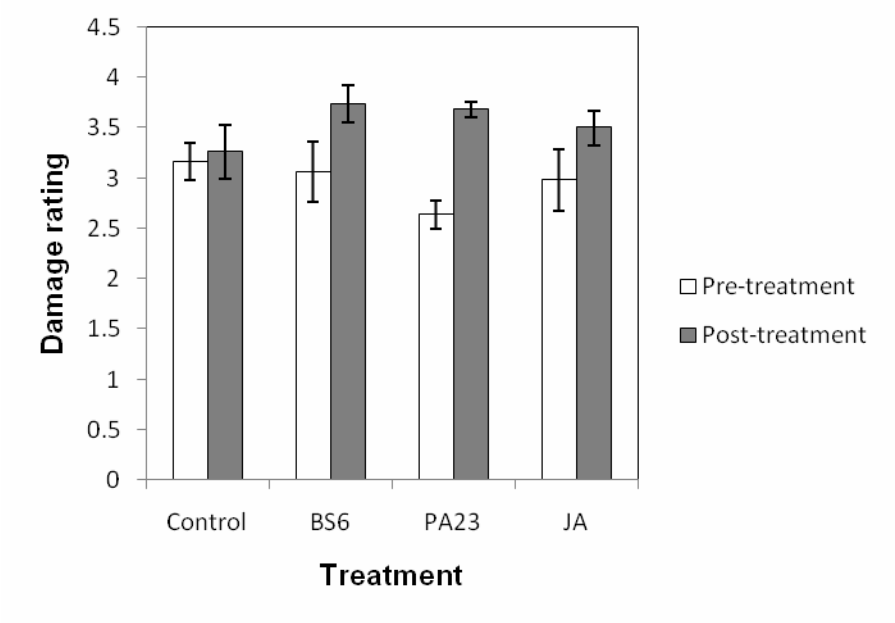

Figure 3.2. Mean (±SEM) flea beetle injury ratings (N=5) for 10 plant-samples at cotyledon stage prior to application of treatments (8 June, pre-treatment) and one week following treatment (15 June, post-treatment).

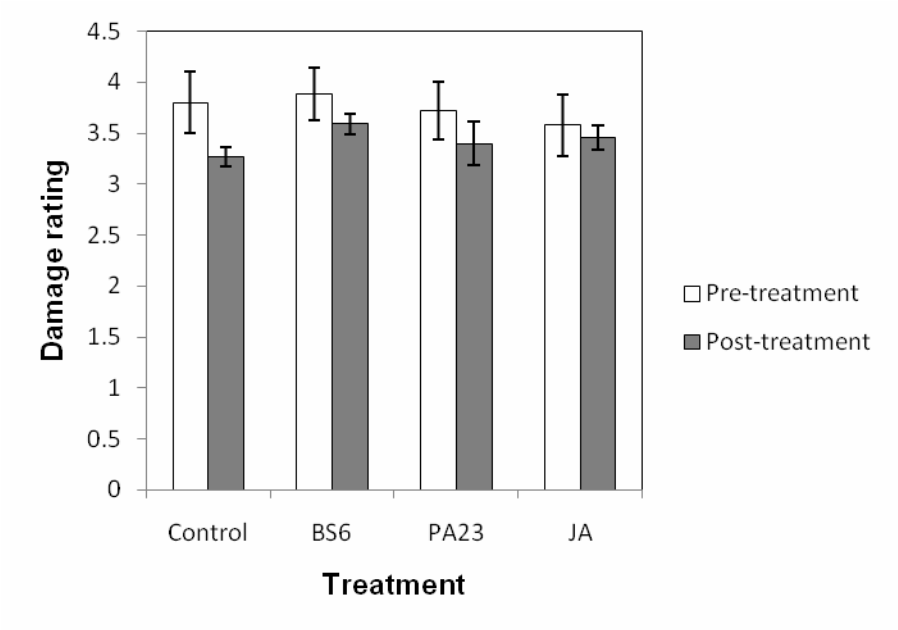

Figure 3.3. Mean (±SEM) flea beetle injury ratings (N=5) for 10 plant-samples at true leaf stage prior to application of treatments (8 June, pre-treatment) and one week following treatment (15 June, post-treatment).

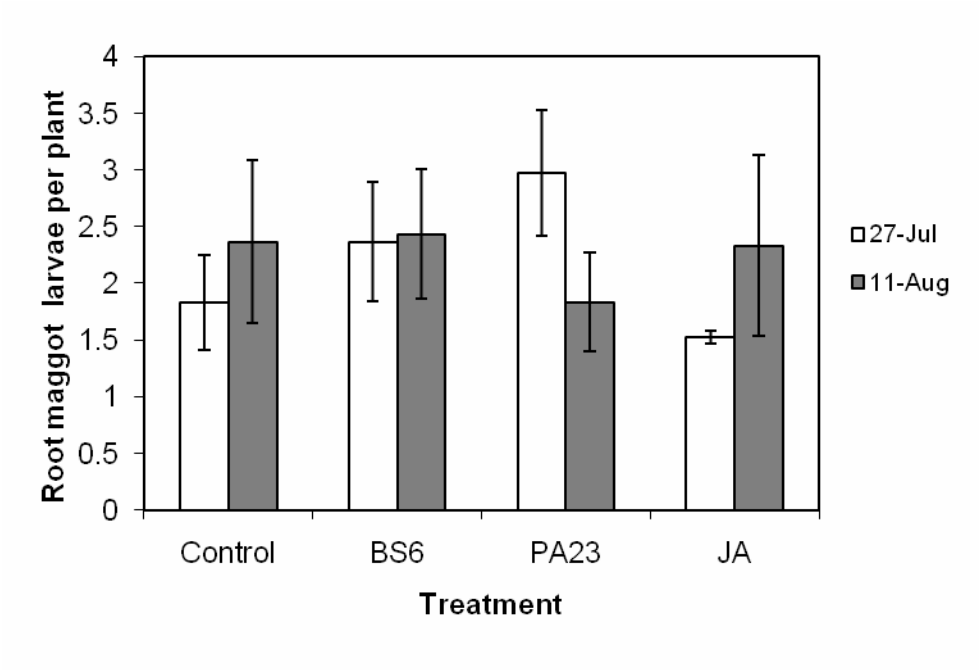

Figure 3.4. Mean (±SEM) number of root maggot larvae per plant sampled on 27 July and 11 August 2006 (N=5).

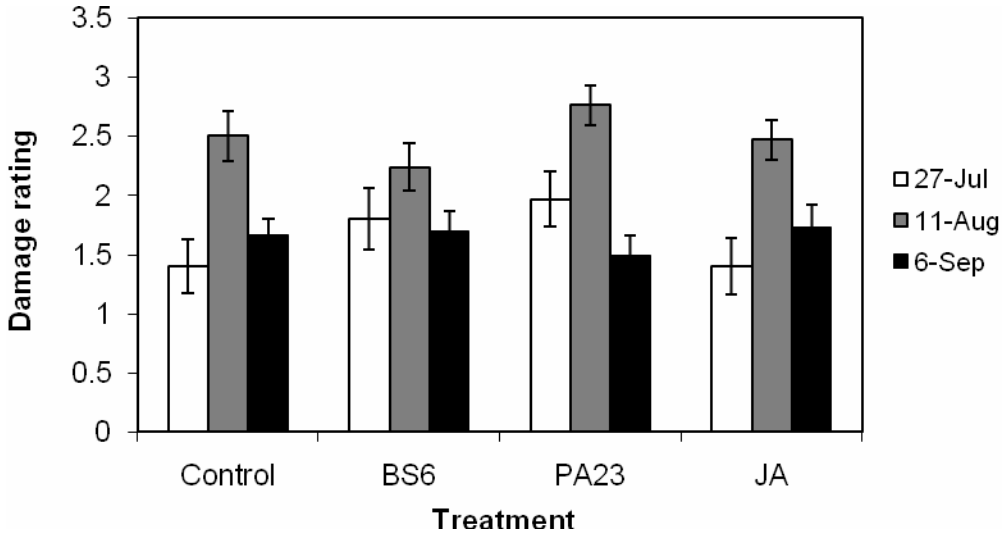

Figure 3.5. Mean (±SEM) root maggot damage rating (N=5) for samples collected on 27 July, 11 August and 6 September 2006. Root damage was rated on a scale of 0-4 according to Dosdall *et al.* (1994).

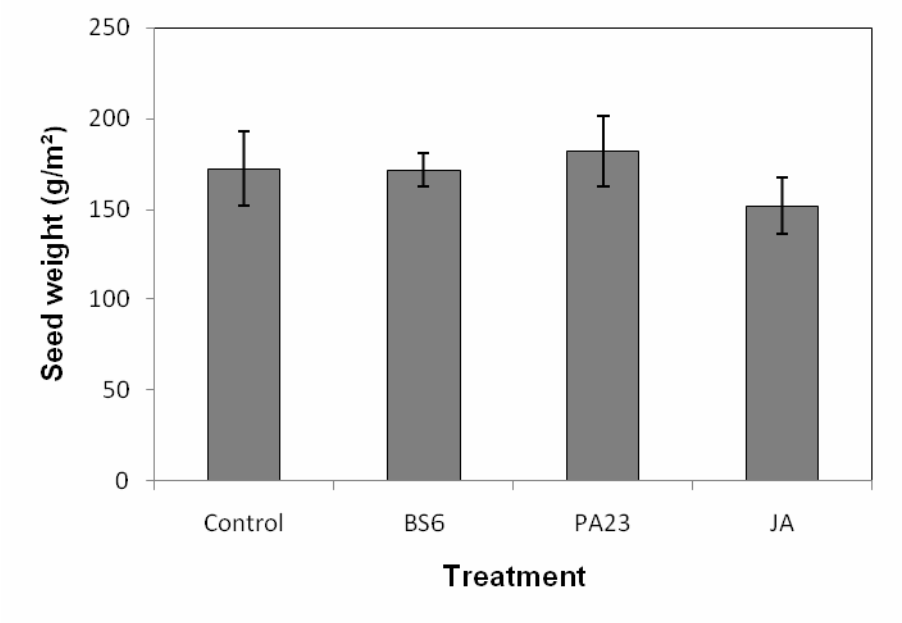

Figure 3.6. Mean (±SEM) seed weight of canola for each treatment harvested 31 August 2006 (N=5).

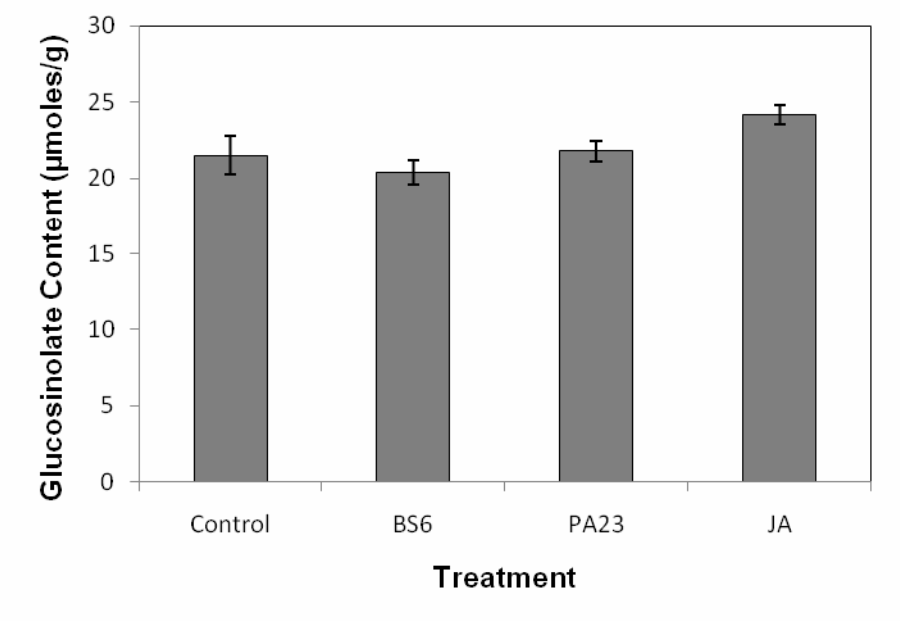

Figure 3.7. Mean (±SEM) glucosinolate content (µmoles/g) of canola seeds harvested 31 August 2006 (N=5).

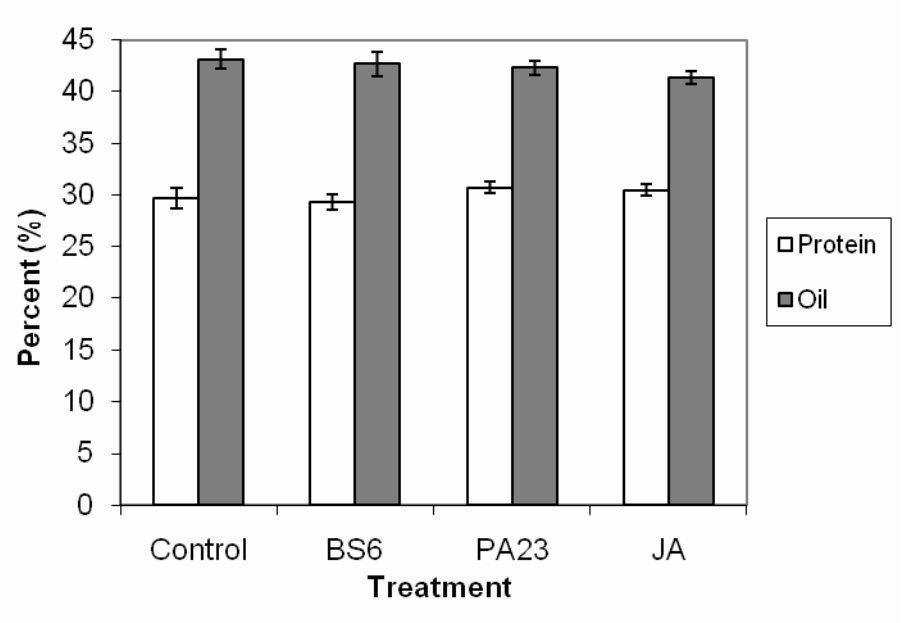

Figure 3.8. Percent (%) protein and oil (dry weight) of canola seeds harvested 31 August 2006 (N=5).

Table 3.1. Mean number of insects (± SEM) from beat cloth samples collected 26 July and 9 August 2006 (N=5, df=3,12).

		Treatment					
Sample Date	Insect Sampled	Control	BS6	PA23	JA	F ratio	*P*
26-Jul	Aphididae	31.0±4.8	39.2±7.6	29.0±6.2	32.2±6.7	0.29	0.833
	Chrysopidae	0.2±0.2	0.8±0.4	1.0±0.3	0.4±0.2	0.08	0.968
	Coccinelidae	2.4±0.7	4.0±1.4	3.0±0.9	2.0±0.5	0.98	0.433
	Flea beetles	1.6±0.5	1.4±0.5	1.2±0.7	2.2±0.9	6.95	0.031
	Lygus bugs	33.6±6.6	42.6±14.1	38.0±1.1	40.8±10.4	0.20	0.895
	Melanchra picta	0.4±0.4	0±0.0	0±0.0	0.2±0.2	*	*
	Plutella xylostella	54.4±6.4	69±4.5	49.2±6.6	62.0±5.4	1.92	0.180
	Pieris rapae	20.8±3.7	27±3.5	27.0±4.1	25.4±1.3	1.18	0.358
	Thysanoptera	683.4±79.4	612±94.1	659.8±96.3	639±50.9	0.15	0.927
9-Aug	Aphididae	17.2±9.3	15.6±7.2	16.8±10.6	16.4±11.2	0.49	0.697
	Chrysopidae	5.6±0.5	9.2±1.6	8.6±2.3	7.0±1.6	0.89	0.472
	Coccinelidae	15.2±2.2	12.6±2.0	13.8±1.5	19.8±2.5	2.75	0.089
	Flea beetles	33.6±6.0	28.8±2.5	26.6±4.1	26.2±4.3	0.93	0.456
	Lygus bugs	430.0±114.	338±31.8	395.0±65.8	321.0±38.1	0.77	0.532
	Melanchra picta	11.4±3.6	9.2±1.5	11.6±1.9	12.2±2.8	0.98	0.438
	Plutella xylostella	3.0±1.1	2.8±1.1	2.6±0.8	3.0±0.8	0.18	0.908
	Pieris rapae	3.4±1.5	4.2±2.1	2.2±0.9	4.8±1.6	1.03	0.428
	Thysanoptera	6.4±4.7	3.0±1.0	2.8±0.8	1.8±1.1	0.43	0.736

* indicates that sample size was insufficient for statistical analysis

Table 3.2. Mean number of insects (± SEM) from sweep net samples collected 26 July and 9 August 2006 (N=5 df=3,12).

		Treatment					
Sample Date	Insect Sampled	Control	BS6	PA23	JA	F ratio	P
26-Jul	Anthomyidae	14.8±1.7	7.6±3.2	12.4±2.2	11.8±2.9	0.85	0.500
	Aphididae	23.2±5.5	15.6±8.2	17.8±6.0	16.4±4.4	0.56	0.649
	Apoidea	5.4±2.0	2.6±1.0	4.2±0.6	3.6±0.5	0.64	0.605
	Chrysopidae	13.6±7.2	12.4±2.9	17.4±16.5	16.0±5.4	0.28	0.840
	Coccinelidae	3.2±0.7	4.6±1.4	3.4±0.5	3.2±0.9	0.67	0.589
	Plutella xylostella	43.6±3.8	43.2±5.8	37.0±9.6	62.4±9.6	2.38	0.120
	Flea beetles	2.2±1.1	1.2±0.5	1.2±0.6	1.6±0.4	0.24	0.866
	Heleomyzidae	6.2±4.3	5.2±2.4	6.2±3.4	8.6±5.2	0.81	0.526
	Lygus bugs	68.8±1.9	82.2±7.1	69.2±1.3	80.0±16.3	0.70	0.566
	Parasitic Hymenoptera	8.6±3.4	6.8± 2.4	9.2±2.2	7.6±2.9	0.54	0.665
9-Aug	Anthomyidae	6.8±2.8	9.6±2.0	8.2±1.5	11.0±3.9	1.01	0.415
	Aphididae	0.4±0.2	0.2±0.2	0.2±0.2	0.2±0.2	*	*
	Apoidea	0±0.0	0±0.0	0.6±0.2	0±0.0	*	*
	Chrysopidae	20.6±3.9	27.8±10.2	18.6±5.9	19.0±4.1	0.40	0.752
	Coccinelidae	13.0±1.5	15±3.5	12.2±2.6	12.2±1.9	0.24	0.867
	Plutella xylostella	0.2±0.2	0±0.0	0±0.0	0.6±0.4	*	*
	Flea beetles	301.6±28.0	307±30.7	279.4±26.3	301.8±35.8	0.14	0.936
	Heleomyzidae	29.6±6.4	46.4±9.2	31.4±8.4	46.6±10.7	0.84	0.491
	Lygus bugs	151.2±9.6	166±15.0	156.6±31.2	133.2±10.7	0.79	0.517
	Parasitic Hymenoptera	13.0±1.6	21.0±3.2	27.0±7.0	18.6±7.7	1.20	0.343

* indicates that sample size was insufficient for statistical analysis

Table 3.3. Results from repeated measures analyses for the effect of treatment on the overall numbers of insects collected from both sampling dates (between subjects) and the effect of treatment on patterns of temporal change (within subjects) for beat cloth samples (df=3,12).

	Between subjects		Within subjects	
Insect Sampled	F ratio	*P*	F ratio	*P*
Aphididae	0.43	0.734	0.36	0.785
Coccinelidae	0.46	0.720	2.99	0.082
Flea beetles	6.55	0.035	3.73	0.095
Lygus bugs	0.04	0.989	1.07	0.398
Plutella xylostella	0.05	0.984	0.69	0.581
Pieris rapae	0.95	0.463	1.11	0.399
Thysanoptera	0.44	0.729	0.39	0.767

Table 3.4. Results from repeated measures analyses for the effect of treatment on the overall numbers of insects collected from both sampling dates (between subjects) and the effect of treatment on patterns of temporal change (within subjects) for sweep net samples (df=3,12).

	Between subjects		Within subjects	
Insect Sampled	F ratio	*P*	F ratio	*P*
Anthomyidae	0.60	0.628	0.98	0.440
Chrysopidae	0.30	0.824	0.44	0.728
Coccinelidae	1.22	0.349	3.81	0.769
Flea beetles	1.30	0.319	1.31	0.317
Heleomyzidae	0.59	0.641	1.45	0.309
Lygus bugs	1.15	0.368	0.29	0.830
Parasitic Hymenoptera	1.45	0.277	0.57	0.648

Table 3.5. Species composition of insects emerged from *Delia* radicum puparia in each treatment.

		Species composition (%)		
Treatment	N	*D. radicum*	*A. bilineata*	*T. rapae*
Control	56	46.4	53.6	0
BS6	67	59.7	35.8	4.5
PA23	58	60.3	32.8	6.9
JA	51	58.8	35.3	5.9

Chapter 4

LABORATORY STUDIES ON THE EFFECTS OF *PSEUDOMONAS CHLORORAPHIS* STRAIN PA23, *BACILLUS AMYLOLIQUEFACIENS* STRAIN BS6 AND JASMONIC ACID ON THE DIAMONDBACK MOTH (*PLUTELLA XYLOSTELLA*) AND THE TURNIP APHID (*LIPAPHIS ERYSIMI*)

ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) and jasmonic acid are elicitors of induced plant defences. Laboratory studies were carried out using canola (*Brassica napus*) to determine effects of two PGPR, *Pseudomonas chlororaphis* strain PA23 and *Bacillus amyloliquefaciens* strain BS6, and jasmonic acid on oviposition and larval feeding and growth rate of the diamondback moth (*Plutella xylostella*). Additional larval feeding and growth rate experiments were performed to investigate the effects of treatment with PA23, PA23+pathogen inoculation and jasmonic acid on *P. xylostella*. Experiments were also carried out to investigate the effects of PA23, PA23+ pathogen inoculation and jasmonic acid on development time and reproduction of the turnip aphid (*Lipaphis erysimi*). Jasmonic acid increased oviposition and decreased larval feeding and growth rate of *P. xylostella*, and increased development time and reduced reproduction of *L. erysimi*. Bacterial treatments had no effect. Leaf tissue analysis found significant effects of treatment on peroxidase and phenolic activity and on glucosinolate levels. The significant effects were attributed to the jasmonic acid treatment, which had a systemic effect on canola plants. The effects of the interaction between treatment, time and insect feeding were also investigated.

4.1 Introduction

Canola refers to rapeseed that has reduced levels of erucic acid and glucosinolates to meet industry standards (< 2% erucic and < 30μmoles/g glucosinolates) (Gray *et al.* 2006). Canola is the most economically important crop in Canada next to wheat (Canola Council of Canada 2006). Annual seed production is estimated to be 6.2 million tonnes, and the industry contributes nearly $14 billion to the Canadian economy (Canola Council of Canada 2006).

There are numerous bacterial, fungal and viral diseases that can affect canola at all growth stages. Two of the most economically important diseases are the fungal pathogens *Sclerotinia sclerotiorum* (Lib.) and *Leptosphaeria maculans* (Desmaz.). *Sclerotinia sclerotiorum* can infect hundreds of plant species and is responsible for stem rot in canola (Boland and Hall 1994). This fungal pathogen is very difficult and expensive to control with fungicides (del Rio *et al.* 2007), and alternative control measures are desirable. Blackleg, *L. maculans,* is a fungal pathogen that can cause economic losses in canola growing regions of Australia, Canada and Europe (Fitt *et al.* 2006). There are several different isolates of the blackleg pathogen that vary in aggressiveness, and the prevalence of more aggressive strains may be increasing in Western Canada (Chen and Fernando 2006). Although there are resistant canola varieties currently available for the common strains of blackleg, the apparent shift in prevalence of aggressive strains makes research into new methods of control necessary.

The activation of plant defences through the use of different elicitors may be an important method of pest control in canola. Plant growth-promoting rhizobacteria (PGPR) are elicitors of plant defences, which are regulated by the signalling molecule

jasmonic acid (Pieterse *et al.* 1998). Two strains of PGPR, *Pseudomonas chlororaphis* strain PA23 and *Bacillus amyloliquefaciens* strain BS6, have been used to control pathogens in canola (*Brassica napus* L.) in laboratory and field studies (Zhang 2004, Fernando *et al.* 2007). Strain PA23 is able to colonize canola plant surfaces and inhibit germination of *S. sclerotiorum* ascospores (Savchuck and Fernando 2004). The inhibition of the pathogen on the surface of the plant is attributed to the production of antifungal volatile compounds (Fernando *et al.* 2005). In field studies, long-term disease suppression has been observed when bacterial populations are no longer present on plant surfaces (Zhang 2004). This long-term disease suppression is attributed to the induction of systemic resistance, resulting in increased levels of defence compounds, including β-1,3-glucanase and chitinase (Fernando *et al.* 2007), which are involved in plant defence against pathogens (Ward *et al.* 1991).

In addition to diseases, canola is also attacked by numerous insect herbivores. Economically important insects include flea beetles (*Phyllotreta* spp.), diamondback moth (*Plutella xylostella* L.), bertha armyworm (*Mamestra configurata* Walker), lygus bugs (*Lygus* spp.), root maggots (*Delia* spp.), and aphids (*Brevicoryne brassicae* L. and *Lipaphis erysimi* Kaltenbach) (Lamb 1989). Insects recognize host plants through the detection of visual and chemical cues (Feeny 1991). Volatile compounds produced through the hydrolysis of glucosinolates are the most important recognition cues for specialist insects of canola and other Brassicaceae (Chew 1988), although other plant compounds can also be important in host plant recognition by specialist herbivores and their natural enemies.

The diamondback moth, *P. xylostella*, uses visual and chemical cues in host-plant location and acceptance (Couty *et al.* 2006). Host plant volatiles attract *P. xylostella* adults (Palaniswamy *et al.* 1986), but oviposition also requires contact stimuli (Justus and Mitchell 1996). Oviposition is stimulated by contact with glucosinolates (Reed *et al.* 1989), and can also be the result of non-polar stimulants (Hughes *et al.* 1997). Ovipostion can also be affected by other factors including leaf waxiness (Justus *et al.* 2000), demonstrating the complexity of *P. xylostella* oviposition. Numerous factors can determine larval food recognition and relative growth rate in *P. xylostella*. Thorsteinson (1953) found that artificial diet and non-host plants treated with glucosinolates stimulate *P. xylostella* larvae to feed. Although glucosinolates stimulate feeding, high concentrations of glucosinolates in plants can reduce the relative growth rate of *P. xylostella* larvae (Li *et al.* 2000). Other compounds also stimulate larval feeding, including various flavanoids (van Loon *et al.* 2002) and leaf waxes (Eigenbrode *et al.* 1991) and manipulating these factors may affect acceptance of host plants (van Loon *et al.* 2002) and larval relative growth rate (Eigenbrode 1998).

Aphids also use a variety of visual and chemical cues to locate host-plants (Pickett *et al.* 1992). The turnip aphid *L. erysimi* uses volatile isothiocyanates released by Brassicaceae for host plant location (Nottingham *et al.* 1991). Once aphids are on the plant surface, potential host plants are identified through the stepwise acceptance or rejection of a variety of stimuli detected by walking and probing the surface, then during penetration of the plant to reach the phloem sieve elements (Klingauf 1987). For *L. erysimi*, glucosinolates are phagostimulants that can stimulate probing, and may also be involved in sieve element location (Nault and Styer 1972). Other factors, including

pigments that determine host plant colour (Yue and Liu 2000, Rana 2005), may be important in host-plant acceptance by *L. erysimi*. Aphid development and reproductive rate are affected by the nutritional quality of host plants. Fecundity of *L. erysimi* is greatest for aphids feeding on *Brassica* spp. with high protein content (Malik 1989). Plants containing high levels of total glucosinolates can negatively affect fecundity of *L. erysimi*, but individual glucosinolates can have variable effects on fecundity (Malik *et al.* 1983).

Insects with biting and chewing mouthparts, such as lepidopteran larvae that feed on plant tissues, can cause significant wounding damage and removal of foliage, while insects with piercing-sucking mouthparts, such as aphids and whiteflies that feed on phloem, can cause wilting and reduced plant growth, but inflict limited wounding to plant tissue. This difference in feeding can expose them to different plant defences (Walling 2000), and the insects may respond differently to plant treatments. Biting and chewing insects wound plants and activate the jasmonic acid signalling pathway (Blechert *et al.* 1995) that can also be activated by other methods of mechanical wounding (Karban and Baldwin 1997). Insect-induced plant defences generally refer to this type of feeding damage unless otherwise stated. Piercing-sucking insects can activate both the salicylic acid and jasmonic acid pathways (Moran and Thompson 2001). The activation of the jasmonic acid pathway regulates the activation of plant defences against biting and chewing insects (Blechert *et al.* 1995, Gatehouse 2002), but it is not well understood how the activation of plant defences associated with the salicylic acid or jasmonic acid pathway affects piercing-sucking insects (Walling 2000).

The objective of this research was to investigate the effects of *Pseudomonas chlororaphis* strain PA23, *Bacillus amyloliquefaciens* strain BS6, and jasmonic acid on *P. xylostella* oviposition preference and larval feeding and growth rates, and on *L. erysimi* development and reproduction on canola. These insects were chosen because of their importance as insect pests of canola (Talekar and Shelton 1993, Gu *et al.* 2007), and because they exemplify the two different methods of insect feeding.

4.2 Methods

Laboratory experiments were done by exposing insects to canola plants grown in a standard way. The plants were treated with one of the four spray treatments: *Pseudomonas chlororaphis* strain PA23, *Bacillus amyloliquefaciens* strain BS6, jasmonic acid, or distilled water control. Studies were done on *P. xylostella* to investigate the effect of treatment on oviposition preference of female moths and feeding and growth rates of larvae. Additional experiments with larvae were carried out to test the effect of a challenge by pathogen inoculum, to investigate whether the plants responses were systemic and to assay plants for defence-related chemicals. For *L. erysimi*, studies were done on the effects of treatments on development and reproductive rate in the presence of pathogen inoculation.

4.2.1 Plants

One or two seeds of *B. napus* cv. Westar were planted approximately 1 cm deep in soil cups or plastic seedling containers filled with MetroMix®. The containers were watered regularly, and after emergence plants were reduced to one per container. About two weeks later, healthy plants were transplanted into 12.7 cm diameter pots. Seedlings

were planted in a mixture of soil, sand and peat (2:2:1). Plants were fertilized immediately after transplanting with an aqueous solution of 3.75 g/L of 20-20-20 (N-P-K) until soil was saturated. Plants were kept in a growth chamber at 16:8 h L:D at 21 ± 1˚C and 50–60% relative humidity and were watered regularly until required for experiments.

4.2.2 Treatments

4.2.2.1 Spray preparation

Pseudomonas chlororaphis strain PA23 and *Bacillus amyloliquefaciens* strain BS6 were obtained from existing cultures in Dilantha Fernando's laboratory at the University of Manitoba and maintained in the laboratory according to methods used by Fernando *et al*. (2005).

To prepare the bacteria for the experiments, fresh Luria Bertani Agar (LBA) plates were streaked with bacteria, and following a 24 h incubation at room temperature, the bacterial colonies were used to inoculate flasks containing autoclaved Luria Bertani (LB) broth (10 g Bacto™ tryptone (BD), 5 g Bacto™ yeast extract (BD), 5 g NaCl (Fisher), 1000 mL distilled water). Flasks were placed in a shaking incubator at 30˚C and 150 rpm for 18 h. The optical density was then checked to ensure bacterial cultures were at the mid-log growth phase, approximately 10^8 cfu/ml (colony forming units) (Zhang 2004). The cultures were then centrifuged at 3500 rpm for 20 minutes in a MSE Minor bench centrifuge. The pellet was resuspended by vortexing in sufficient distilled water to produce a concentration of 10^8 cfu/ml.

The resuspended cultures were placed in spray bottles with one drop of Tween 20 (Sigma-Aldrich) and 4.2 ml/L methanol. Because the jasmonic acid treatment required

methanol as a solvent, 4.2 ml/L methanol was added to the bacterial spray formulations so that it comprised an equivalent amount of methanol as that added to jasmonic acid treatments. Treatments were applied to the plants within approximately 30 min of spray formula preparation.

Prior to beginning the experiments, trials were run to ensure that the methanol would not be detrimental to the bacteria. Using the methods described above, test tubes containing 10 ml of Luria Bertani broth were inoculated with either *P. chlororaphis* strain PA23 and *B. amyloliquefaciens* strain BS6. Methanol was added to half of the test tubes at a rate of 4.2ml/L, and tubes were placed in a shaking incubator for 18 h after which time the optical density was checked to ensure bacterial cultures reached the mid-log growth phase. There were two test tubes for each treatment and the experiment was repeated twice.

Jasmonic acid (Sigma-Aldrich) was stored at 5˚C until needed. To prepare the spray solution, 100 mg of jasmonic acid was dissolved in 2 ml of methanol and diluted with distilled water to which 1 drop of Tween 20 was added. The final concentration was 1 mM/L jasmonic acid and 4.2 ml/L methanol in the spray mixture. Control treatments consisted of distilled water, 1 drop of Tween 20 and 4.2ml/L methanol.

4.2.2.2 Spray application

When the canola plants were at the 4–5 leaf stage, plants were selected for use in experiments. Unhealthy plants or those with broken leaves or stems were not included in the experiments. Plants were separated into groups based on similar size and appearance and plants were randomly assigned one of the treatments by placing treatment labels upside down on the laboratory bench and randomly selecting one for each of the plants,

so that each group of plants contained equal numbers of each treatment. The plants were labelled with the given treatments as well as a replicate number.

Plants were placed in a fume hood one treatment at a time to be sprayed. Each plant was sprayed 6 times with a spray bottle containing the specific treatment solution so that each plant received approximately 5.4 ml of the spray solution. Plants were left on the bench to dry for 2–4 h then returned to the growth chamber.

4.2.2.3 PA23+Pathogen Inoculation

The pathogen used in the larval and aphid experiments was blackleg (*Leptosphaeria maculans* Desmaz.), isolate 89-3 (PG-2). It was collected in Melfort, SK in 1989, and each year was passaged through the susceptible canola cultivar Westar to retain its virulence. Pycnidia were grown on V8 agar plates amended with 0.1 g/L streptomycin sulphate (Sigma-Aldrich) to prevent bacterial growth. Plates with high levels of pycnidia were flushed with sterilized distilled water and scraped with a microscope slide. The liquid was filtered through Miracloth (Calbiochem®) into a 10 ml test tube to remove the pycnidia and allow the pycnidiospores through. For each test tube 2–3 plates were used to gather sufficient inoculum. Test tubes were centrifuged at 3500 rpm for 15 minutes in a MSE Minor bench centrifuge, and the supernatant was removed with a pipette, leaving approximately 2 ml that was used to resuspend the spores. The spore suspension was stored in microcentrifuge tubes at -20°C until required for the experiments.

The pathogen inoculum was made up to a concentration of 1.5–2.5 x 10^7 pycnidiospores per ml. The concentration was measured by adding 2–3 drops of the concentrated spore solution to a test tube and distilled water was added to make a final volume of 10 ml. Spore counts were made using a haemocytometer, and concentrations

were adjusted as needed. After reaching the correct concentration, the required volume of solution was made up and placed in a spray bottle on ice.

Pathogen inoculation was done 24 h after bacterial treatments. Plants receiving the PA23+pathogen treatment were wounded with a pair of forceps by making 5 pin-size holes on leaves one and two to provide entrance wounds for the pathogen. Immediately following wounding the plants were treated with the spray formulation of the blackleg pathogen in the fume hood using the procedure previously described. All plants were left out on the laboratory bench for 2–4 h to allow the inoculated plants to dry, and then plants were moved back to the growth chamber. Plants were sprayed within approximately 30 minutes of making up the inoculum.

4.3 Studies with *Plutella xylostella*

Diamondback moths (*P. xylostella*) were obtained from an existing culture at Agriculture and Agri-Food Canada (Saskatoon Research Centre) for the oviposition experiment and the first set of larval feeding and growth rate experiments. For the remaining laboratory experiments, *P. xylostella* larvae were collected on 24 September 2006 from volunteer canola growing at The Point at the University of Manitoba, Fort Garry Campus. The insects were reared in the laboratory for five months prior to use in experiments. Voucher specimens were deposited in the J.B. Wallis Museum at the University of Manitoba's Department of Entomology. The different larval instars were identified through measurements of 250 larval head capsule widths done using a dissecting microscope (4X) with 10X magnification eye pieces with a micrometer.

Insects cultures were maintained in 30 x 30 x 30 cm wood and mesh sleeve cages containing potted canola plants. Pupae were added to cages and adult moths were left to

lay eggs for several days at which time the adults were removed and larvae were left develop. When canola plants were severely damaged by larval feeding, plants were removed and new plants were added to the cage. Larvae were transferred as needed to the new plants using a nylon paintbrush or leaves were removed and placed on top of new leaves for 24 h to allow larvae to move to the new plants. Pupae were collected using soft forceps, placed in Petri dishes and placed in cages with new plants, or kept at 5ºC as a backup to the culture to ensure survival of the population. Insects were reared in a growth chamber on canola (*B. napus* cv. Westar) prior to use in experiments. The growth chamber was at 16:8 h L:D at 21 ± 1˚C, and 50–60% relative humidity.

4.3.1 Oviposition preference

Effects of treatments on diamondback moth oviposition preference were investigated in choice experiments in which ovipositing female moths could choose among four plants, one from each treatment. Plants were at the 4–5 leaf stage at the time of treatment and the four treatments were PA23, BS6, jasmonic acid and control. The diamondback moth culture was checked daily and pupae were transferred to empty cages. After 24 h, remaining pupae were removed from the cage, and emerged adults were left for another 24 h to allow for mating which usually occurs at dusk on the day of emergence (Harcourt 1957).

The experiment was done in three sets of 10 replicates. Three mated females were transferred using a mouth aspirator to cages containing the four treated plants. After 24 h the adult moths were removed from the cages and plants were placed at 5ºC until observations on numbers and location of eggs laid were made the same day. Initial observations were made with the unaided eye, and leaves were then examined under a

dissecting microscope (10X) to confirm numbers. Eggs were removed as they were discovered during microscopic observations to prevent counting eggs twice.

4.3.2 Larval feeding and growth rate

The effects of treatments on larval feeding and growth rate were investigated using third instar larvae that had moulted from second instar within the previous 24 h. One larva was transferred using a nylon paintbrush (size 00) to the fourth fully expanded leaf from the bottom of a plant that had been treated 24 h previous with either PA23, BS6, jasmonic acid or solvent control. A perforated plastic bag (approximately 10–15 cm^2) was attached to the leaf with masking tape around the petiole to prevent the insect from escaping. The larva was left to feed for 48 h, at which time the larva was removed and wrapped individually in aluminium foil, labelled and placed in the freezer. After 24 h, the larvae were removed and dried in an oven at 60˚C for 72 h, before weighing to obtain final dry weight of each larva. Initial dry weights of larvae fed on treated leaves could not be measured directly so were estimated using newly-moulted third instar larvae from the same pool as those fed on treated leaves. At the same time as the leaf feeding began, 5–10 larvae from that pool were wrapped in labelled, pre-weighed aluminium foil and placed in the freezer. After 24 h they were removed and dried in an oven at 60˚C for 72 h. Individual final dry weights of larvae from feeding treatments and initial dry weights were determined using a Fisher Scientific accu-124D dual range scale with a precision of 0.001 mg. From these weights the relative growth rate of each larva in the feeding treatments was calculated using the formula:

$$RGR = e^{\frac{\log_e W_f - \log_e W_i}{2}} - 1$$

Where W_t = final larval dry weight and W_i = initial larval dry, as outlined by Hoffmann and Poorter (2002).

Photos were taken of each leaf prior to the addition of the larva, and again following feeding. All photos were taken with a digital camera on a camera stand at the same height, with a blue background to provide contrast and a ruler placed next to the leaf for scale. Assess®, an image analysis program, was used to measure the amount of leaf area consumed by each larva. Leaf area consumed by the larva was measured directly from the second image, except when feeding was at the leaf margin the initial image was used for comparison.

4.3.3 Test of systemic influences

To investigate whether the jasmonic acid treatments affected diamondback moth larvae through surface residues or systemic effects mediated by the plants, the relative growth rate study was repeated, but with a modified method of delivering the treatments. Only jasmonic acid and control treatments were applied in this study and plants were sprayed with one of these treatments as before, except that either the third or fourth leaf was enclosed in a polyethylene bag during the spraying to prevent this leaf from being sprayed. One newly moulted third instar larvae was added to each of the third and fourth leaves of each plant 24 h after spray treatment. To isolate the larva, each of the leaves was covered with a mesh nylon bag (approximately 10–15 cm^2) with a metal clip placed over foam around the petiole to prevent the insect from escaping without putting excessive pressure on the leaf. Larvae were left to feed for 48 hours. As before, final dry weight was obtained and relative growth rate was calculated using initial dry weights from larvae selected from the same pool as the fed larvae.

4.3.4 Changes in plant chemistry

Additional larval experiments were carried out with *P. xylostella* larvae to investigate the relationship between the effects of treatments on insects and the changes in leaf chemistry associated with defence responses. The treatments used in this experiment were PA23, PA23+pathogen, jasmonic acid and control. These treatments were used with and without the addition of insect feeding to characterize the effects of treatments alone, as well as synergistic or other interactive effects from the addition of insect feeding. To examine the effects of the treatments over time, leaf tissue was taken by destructive sampling at time 0, 24 and 48 h. For each of these eight treatments and three sampling times, the samples were analyzed for three defence-related compounds.

The PA23, jasmonic acid and control treatments were applied as previously described, followed 24 h later by the application of a pathogen inoculation to those plants receiving the PA23+pathogen treatment. The plants were returned to the laboratory 24 h after the pathogen inoculation, and larvae were applied to those plants receiving insect feeding. Initial dry weights were calculated for larvae selected from the same pool as the fed larvae. On the experimental plants, one newly moulted third instar larva was confined to the third fully expanded leaf from the bottom of each plant in a mesh nylon bag as previously described. The larva was left to feed for 24 or 48 h, depending on the feeding treatment duration, and then frozen for 24 h, before drying for 72 h in an oven at 60˚C. Final dry weight was obtained and relative growth rate was calculated as before.

Leaf samples were taken at times 0, 24, and 48 h relative to the time of application of the insects and at corresponding times in the treatments without insects. Samples at 0 h relative to the feeding initiation were taken 48 h after application of the

PA23, jasmonic acid and control treatments and 24 h after pathogen inoculation to the PA23+pathogen treatment. The fourth leaves of four plants from the same treatment and time of sampling were pooled together for extraction and analysis. The leaves were removed with scissors and immediately ground with a pestle in a mortar containing liquid nitrogen; the extract was then placed in a 50 ml centrifuge tube and stored at -86˚C until analysis.

4.3.5 Peroxidase Activity

Peroxidase activity was measured using methods modified from Ramamoorthy *et al*. (2002). One gram of frozen plant tissue extract was mixed with 1 ml of 0.1 M phosphate buffer pH 7.0 in a cooled mortar. The homogenate was centrifuged at 15000 g for 15 min at 4º C using a Fisher Scientific accuSpin™ MicroR benchtop centrifuge with a rotor diameter of 8.5 cm; 1 ml of the supernatant and 1.5 ml pyrogallol were transferred to a disposable cuvette and placed in an Ultrospec™ 2100 pro, UV-visible spectrophotometer. The reaction was started when 0.5 ml of 1% hydrogen peroxide was added. Absorbance at 420 nm was measured at 30 s interval for 3 min. A boiled enzyme preparation from a control leaf sample served as a blank. The enzyme activity was expressed as change in absorbance per min per mg of leaf tissue (fresh weight) ($\Delta OD/min^{-1}mg^{-1}$).

4.3.6 Phenol Concentration

Phenol was measured using methods modified from Ramamoorthy *et al*. (2002). One gram of frozen plant tissue was mixed with 10 ml of 80% methanol using a cooled mortar. The homogenate was centrifuged at 15000 g for 20 min using a Fisher Scientific accuSpin™ MicroR benchtop centrifuge with a rotor diameter of 8.5 cm; 1ml of the

supernatant was placed in a hot water bath at 70ºC for 15 min to prevent further enzyme activity. The supernatant was removed from the hot water bath, and 200 µL of supernatant was added to 5 ml distilled water, followed by the addition of 250 µL of Folin-Ciocalteau reagent. After 30 min incubation at room temperature, 1 ml of saturated aqueous sodium carbonate and 1 ml of distilled water were added, and the mixture vortexed and then incubated for 1 h. Following incubation, 1 ml was transferred to a disposable cuvette and placed in an Ultrospec™ 2100 pro, UV-visible spectrophotometer where the absorbance was measured at 725 nm. Catechol was used as the standard. Phenol was expressed as µg catechol per gram of plant tissue (fresh weight).

4.3.7 Glucosinolates

Glucosinolate extraction methods were modified from Kraling (1990) and Branca *et al*. (2002). Between 100–200 mg of frozen leaf extract was weighed out in a liquid nitrogen-cooled mortar. The leaf tissue was then stored in 1.5 ml micro-centrifuge tubes in liquid nitrogen. Tubes were removed from the liquid nitrogen and 400 µl of 100% methanol, 70 µl double distilled water (ddH_2O), 50 µl internal standard (5 mM aqueous sinigrin solution) and 10 µl barium acetate (0.3 M aqueous solution) were added and the mixture incubated at 80˚C for 15 min before centrifuging at 3200 rpm for 15 minutes using a Thermo Scientific Microlite IEC centrifuge with a rotor radius of 8.35 cm, which was used for all subsequent centrifugation. The supernatant was transferred to a new tube and centrifuged again for 10 min. The supernatant was transferred to a new tube with 250 µl of sephadex solution (1 g DEAE Sephadex [Sigma-Aldrich] and 15 ml ddH_2O made up 24 h in advance and stored at 4˚C). Tubes were inverted several times to mix the contents thoroughly, allowed to settle at room temperature for 5 min, then centrifuged at

3200 rpm for 1 min. The supernatant was discarded and 1 ml of 70% methanol was added to the pellet, before vortexing followed by centrifugation at 3200 rpm for 1 min. The supernatant was discarded; 1 ml ddH_2O was added to the pellet and tubes again vortexed then centrifuged for 1 min. The supernatant was discarded.

Previously, a purified sulfatase solution was prepared by mixing 350 mg sulfatase (from *Helix pomatia*, Sigma-Aldrich) with 15 ml dH_2O and 15 ml 100% ethanol. This was centrifuged at 5000 rpm for 20 min. The supernatant was combined with 45 ml of 100% ethanol and centrifuged at 4000 rpm for 15 min. The supernatant was discarded and the pellet was dissolved in 12.5 ml ddH_2O and divided between 1 ml microcentrifuge tubes that were stored at -20˚C for later use. Ten µl of purified sulfatase solution was added to the pellet to digest the glucosinolates during overnight incubation (14–15 h) at room temperature.

After incubation, 200 µl of ddH_2O was added and the mixture was vortexed then centrifuged at 3200 rpm for 1 min. The supernatant was transferred to a new tube and set aside. To the pellet was added 200 µl of 70% methanol, before vortexing followed by centrifugation at 3200 rpm for 1 min. If there was any remaining Sephadex in the supernatant, tubes were centrifuged for another minute. The supernatant was combined with that set aside in the previous step and used in the high-performance liquid chromatography (HPLC).

For HPLC, a Waters 2695 Separation module auto-sampler equipped with a Waters 996 photodiode array detector (PDA) was used, with a 250 x 4 mm LiChroCART column of 5 µm pore size. A gradient elution was used for the mobile phase. The column temperature was 30˚C and samples in waiting were kept at 10˚C. The HPLC

chromatograms produced for the desulfoglucosinolates from each sample were compared with known profiles from the cultivar "Linetta" as done by Branca *et al*. (2002) and work done by Dr. Genyi Li at the University of Manitoba (unpublished data).

4.4 Studies with *Lipaphis erysimi*

Turnip aphids (*L. erysimi*) were collected on 24 September 2006 from volunteer canola growing at The Point at the University of Manitoba, Fort Garry Campus. The aphid colony was a single clone that was selected on the basis of its high levels of fecundity in the laboratory. Slides of alatae and apterae aphids were made using modified methods of Richards (1964). Taxonomic keys were used to identify aphids to genus (Foottit and Richards 1993) and species (Blackman and Eastop 1984). Voucher specimens were deposited in the J.B. Wallis Museum at the University of Manitoba's Department of Entomology.

The aphid colony was maintained on canola (*B. napus* cv. Westar) in a growth chamber at 16:8 h L:D at 21 ± 1˚C, and 50–60% relative humidity prior to use in experiments. Aphids were transferred using a nylon paintbrush to new canola plants every 5–7 days. Potted canola plants were then covered with plastic dome cages and placed inside 30 x 30 x 30 cm wood and mesh cages to prevent insects from escaping.

4.4.1 Development and reproduction

The effects of four treatments on *L. erysimi* experiments development and reproductive rate were examined. The four treatments were PA23, PA23+pathogen inoculation, jasmonic acid and control. Plants were treated with PA23, jasmonic acid or control treatments, and 24 h later all plants were moved to the laboratory and those

PA23-treated plants to receive pathogen inoculation were treated. Aphids were applied to the plants 24 h after the pathogen inoculation and 48 h after the initial treatments were made.

Apterous adult aphids that had recently reached maturity were used in the experiment. Aphids in the final nymphal instar and newly moulted adults from the culture were transferred to new plants. These plants were inspected 24–48 h later and adults with 1–5 offspring were selected for use in the experiment. Using a nylon paintbrush (size 00), one aphid was applied to the third fully expanded leaf of each of the treated plants. Mesh nylon bags (approximately 10–15 cm^2) were placed over the leaf and secured with strips of foam and metal hair clips to isolate the aphid on the third leaf.

Plants were maintained in a growth chamber (16:8 h L:D at 21 ± 1˚C, and 50–60% relative humidity) and observations were made 24, 36 and 48 h after aphid introduction to monitor the number of young produced. If less than 5 nymphs were produced in the 48 h period, the replicate was abandoned. When 5 to 10 nymphs were present, the adult was removed and the nymphs were left to develop. When the nymphs were nearing adulthood, observations were made every 12 h. When the first aphid began to reproduce, the remaining nymphs were removed and the single aphid was left to reproduce. The elapsed time from the application of the first aphid to the isolation of one reproducing aphid was recorded as “days to first young”. When the nymphs of the isolated reproducing aphid were nearing adulthood, observations were again made every 12 h and the experiment was terminated when the first nymph produced offspring. The number of nymphs produced by the isolated aphid and the time elapsed from isolation of

that aphid until first reproduction by her offspring ("days to second young") were recorded.

The effect of treatment on the observed nymph production, development time (days to first young, days to second young, total days), and an estimate of intrinsic rate of increase were assessed by analysis of variance. Intrinsic rate of increase was calculated as

$$r_m = \frac{0.738(\log_e M_d)}{d}$$

where M_d is the number of offspring produced during the days to second young (d) (Wyatt and White 1977).

4.5 Statistical Analyses

The effect of methanol on bacterial growth was analyzed by analysis of variance using a repeated measures analysis. The effect of treatment on *P. xylostella* oviposition was analyzed by analysis of variance. This experiment investigated the effect of treatment on the total number of eggs laid, the distribution of eggs on the leaves (cotyledons, leaves 1 and 2, leaves 3 and 4, leaves 5 and 6), and location of eggs on the top, bottom or petiole of leaves and the interactions between these factors were investigated.

The amount of leaf area consumed by larvae, the relative growth rate and the biomass conversion efficiency were subjected to analysis of variance to investigate the effect of treatment on larval feeding and growth rate. The test for systemic influences was investigated by repeated measures analysis in which the plants were the subjects and the within subjects factor was the leaves as samples from two leaves on the same plant cannot be considered to be independent. The effect of treatment on changes in peroxidase

activity, phenol concentration and glucosinolate levels was investigated by analysis of variance.

A means model was used to analyse all changes in plant chemistry, because there was only one set of data for the time 0 h, while times 24 and 48 h had data for both with and without insects. For each compound investigated, an overall model for all the data and all effects and interactions was done, as well as means models for the effect of treatment, time, insect and the interactions between these effects. The overall model was used to obtain the overall error degrees of freedom and sum of squares used in the subsequent analyses. The results from the overall model are not meaningful because of the shared data points at time 0 h.

For the changes in plant chemistry experiment, the effect on relative growth rate of the larvae was also analyzed by analysis of variance for those treatments in which larvae fed on plants for 48 h. For the studies on *L. erysimi*, analysis of variance was used to examine the effect of treatment on nymph production, development time and the intrinsic rate of increase.

All statistical analyses were performed using Systat® statistical software (SYSTAT 2004). A randomized complete block design was used for all experiments, and the block (replicate) was retained in the model. Levene's test and assessment by graphical analysis were performed on the data to determine whether transformations were necessary. When significant treatment effects were detected, a two-tailed Dunnett's test was performed (α=0.05) to detect significant differences of treatment means from the control.

4.6 Results

4.6.1 Effects of methanol on bacterial growth

The optimal temperatures recommended to obtain the mid-log growth phase for PA23 and BS6 bacterial cultures are 28 and 30ºC respectively. The incubator was set at 30ºC to ensure that BS6 would reach this phase without negatively affecting PA23, and this lead to PA23 cultures exceeding the mid-log growth phase (approximately 10^8 cfu/ml). To account for the difference in optical density, the interaction between the methanol and the bacterial culture was analyzed by analysis of variance using a repeated measures analysis. There was a significant difference in the effect of treatment on optical density, as seen by the significant overall effect of treatment ($F=34.65$, $df=1,12$, $P=<0.001$), but there was no significant effects of methanol on optical density ($F=0.03$, $df=1,12$, $P=0.856$) and no significant effect of the interaction between bacterial strain and methanol ($F=0.99$, $df=1,12$, $P=0.339$) on optical density.

4.6.2 Studies with *Plutella xylostella*

4.6.2.1 Oviposition preference

The oviposition experiment was conducted in 30 replicates on three separate dates (runs); data for 29 of the 30 replicates were analysed as no eggs were laid in one replicate. Initial analyses using the untransformed data showed heteroscedasticity, so data were log transformed. The replicates were used as the blocking factor. The cotyledon data was analysed separately from the leaf data. The average number of eggs laid on leaves and cotyledons for each treatment is shown in Fig. 4.1, and the number of eggs laid on the bottom, petiole or top of leaves 1–2, 3–4 and 5–6 is shown in Fig. 4.2.

A repeated measures analysis was performed on the transformed leaf data, and there was a significant overall effect of treatment on oviposition ($F=4.41$, $df=3,84$, $P=0.006$). There were also significant effects of treatment on the leaf number ($F=4.22$, $df=6,168$, $P<0.001$), position ($F=2.26$, $df=6,168$, $P=0.017$) and the interaction between treatment, leaf number and position ($F=2.46$, $df=12,336$, $P<0.01$). Analysis of variance was performed on the cotyledon data using a means model because there were many missing data points and the data were erratic. There was no effect of treatment on oviposition preference ($F=0.89$, $df=3,92$, $P=0.452$) or when a contrast was done between the control and jasmonic acid treatments ($F<0.01$, $df=1,92$, $P=0.968$).

4.6.2.2 Effects on larval feeding and relative growth rate

The ratio of median head capsule width between successive instars was constant, and there were four distinct larval instars identified (Fig. 4.3). Based on data from Fig. 4.3, the larvae used in the growth rate experiments had head capsule widths ranging from 0.36–0.45 mm.

For the amount of leaf area consumed, initial analyses using the untransformed data showed heteroscedasticity, so data were log transformed. There were significant effects of treatment on the amount of leaf area consumed ($F=3.47$, $df=3,113$, $P=0.019$) by *P. xylostella* larvae, and a Dunnett's test ($\alpha=0.05$) showed that only the jasmonic acid treatment differed from the control. Larvae on the jasmonic acid treated plants consumed less foliage (Fig. 4.4). There were also significant treatment effects on the relative growth rate of the larvae ($F=6.39$, $df=3,109$, $P<0.001$), and a Dunnett's test showed that only the jasmonic acid treatment differed significantly from the control. Relative growth rate was lowest in the jasmonic acid treatment (Fig. 4.5). There were no significant effects of

treatment on the number of feeding sites (F=2.49, df=3,115, P=0.064); however, there was a significant effect of treatment when a contrast between the control and jasmonic acid was performed (F=7.21, df=1,115, P=0.008) (Fig. 4.6). There were no significant treatment effects on biomass conversion efficiency (F=0.19, df=3,104, P=0.897) which was analyzed as larval weight gain per amount of leaf area consumed (mg/cm^2) (Fig. 4.7).

4.6.2.3 Test for systemic influences

The relative growth rates of larvae from the test for systemic effects of treatment was analyzed by analysis of variance using a repeated measures test to account for the similarity in responses in leaves on the same plant. There were significant overall effects of treatment (F=10.76, df=1,50, P=0.002), with lower relative growth rates for larvae on the jasmonic acid treated plants, as was expected from the previous experiment. The relative growth rate of larvae feeding on sprayed leaves and unsprayed leaves was 0.936 (±0.068) and 0.924 (±0.067) for controls and 0.668 (±0.047) and 0.647 (±0.053) for jasmonic acid. There were no significant effects of the interaction between treatment and the leaf fed upon (F=0.01, df=1,50, P=0.937) or between the leaf treated (either 3^{rd} or 4^{th}) and the leaf fed upon (F=0.82, df=1,50, P=0.369). There were also no effects of the three way interaction between treatment, leaf treated, and leaf fed upon (F=0.01, df=1,50, P=0.912), indicating that that there were no significant differences in the relative growth rate of larvae on leaf 3 or 4 regardless of which leaf was treated.

4.6.2.4 Changes in plant chemistry

At the site of pathogen inoculation there was evidence of disease for all PA23+pathogen treated plants, indicating that the plants were successfully inoculated

with *L. maculans*. The data for larval relative growth rate were assessed for normality by graphical analysis and a Levene's test was not significant, so untransformed data was used. The effect of treatment on larval relative growth rate was nearly significant ($F=2.92$, $df=3,19$, $P=0.061$), and showed trends similar to those seen in the previous experiments with lower relative growth rates for jasmonic acid treatments relative to the control (Fig. 4.8). A contrast between the control and jasmonic acid treatments was performed and there was a significant effect of treatment ($F=7.63$, $df=1,19$, $P=0.012$).

4.6.2.5 Peroxidase activity

For the peroxidase analysis the overall model was significant, as well as the treatment effect (pooled over time and insect) (Tables 4.1 and 4.2). There was a significant treatment effect when jasmonic acid was contrasted with control ($F=22.75$, $df=1,58$, $P<0.001$) and the residual treatment effects were not significant ($F=3.08$, $df=2,58$, $P=0.534$). The peroxidase activity was greatest in the jasmonic acid treatments overall, but the means do not indicate that there are any consistent effects of time on jasmonic acid or any of the treatments.

4.6.2.6 Phenol concentration

For the phenol analysis the overall model was significant, as well as the treatment effect (pooled over time and insect), the time effect (pooled over treatment and insect), and the interaction between treatment and insect (pooled over 24 and 48 h) (Tables 4.3 and 4.4). There was a significant treatment effect when jasmonic acid was contrasted with control ($F=61.50$, $df=1,64$, $P<0.001$) and the residual treatment effects were also significant ($F=4.63$, $df=2,64$, $P=0.013$), but 86.9% of the variability in the original analysis was attributed to the contrast between control and jasmonic acid. The interaction

between treatment and insect (pooled over time) was not significant when jasmonic acid was contrasted with control (F=2.48, df=1,64, P=0.120) and the interaction between treatment and time (pooled over insect) was nearly significant effect when jasmonic acid was contrasted with control (F=3.04, df=2,64, P=0.055). To investigate this further, the interaction between treatment and insect was examined at 24 and 48 h separately. The means model for the interaction between treatment and time was significant at 24 h (F=20.43, df=3,64, P=<0.001) but not at 48 h (F=2.23, df=3,64, P=0.092). When jasmonic acid was contrasted with control, the interaction between treatment and time was significant at 24 h (F=15.93, df=1,64, P=<0.001) and at 48 h (F=4.17, df=1,64, P=0.045).

4.6.1.7 Glucosinolates

Through HPLC, glucobrassicin, neoglucobrassicin, 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin were identified, although 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin were present in only five or fewer replicates and were not sufficient for analysis. No standards were available for these glucosinolates, so results from the chromatograms were expressed as ratios of peak area to the area of the peak for the standard sinigrin, 50µl of which was added to each sample. Where no peaks were identified the ratios were zero.

For the glucobrassicin analysis the overall model was significant, as well as the treatment effect (pooled over time and insect), the insect effect (pooled over treatment and time) and the interaction between treatment and insect (pooled over time 24 and 48 h) (Tables 4.5 and 4.6). There was a significant treatment effect when jasmonic acid was contrasted with control (F=210.62, df=1,65, P<0.001) and the residual treatment effects

were also significant (F=48.03, df=2,65, P<0.001), but 68.7% of the variability in the original analysis was attributed to the contrast of control with jasmonic acid. The interaction between treatment and insect was significant when jasmonic acid was contrasted with control at 24 h (F=3.11, df=1,65, P=0.032) and 48 h (F=10.38, df=1,65, P<0.001), and the residual treatment effects were significant only at 48 h (F=5.38, df=2,65, P=0.007). The interaction between treatment and time was not significant when pooled over treatments with insects (F=1.90, df=6,65, P=0.094) or with no insects (F=1.06, df=6,65, P=0.396), or when a contrast was done between jasmonic acid and control with no insects (F=1.76, df=2,65, P=0.180), but the interaction was significant for a contrast between jasmonic acid and control with insects (F=3.47, df=2,65, P=0.037).

For the neoglucobrassicin analysis the overall model was significant, as well as the treatment effect (pooled over time and insect), the insect effect (pooled over treatment and time), the interaction between treatment and time (pooled over insect) and the interaction between treatment and insect (pooled over time 24 and 48 h) (Tables 4.7 and 4.8). There was a significant treatment effect when jasmonic acid was contrasted with control (F=163.80, df=1,65, P<0.001) and the residual treatment effects were also significant (F=39.06, df=2,65, P<0.001), and 67.7% of the variability in the original analysis was attributed to the contrast of control with jasmonic acid. The interaction between treatment and insect was significant when jasmonic acid was contrasted with control at 24 h (F=5.36, df=1,65, P=0.024) and 48 h (F=16.11, df=1,65, P<0.001), and the residual treatment effects were significant only at 48 h (F=4.11, df=2,65, P=0.021). The interaction between treatment and time was not significant when pooled over treatments with insects (F=2.06, df=6,65, P=0.068) or with no insects (F=2.23, df=6,65,

P=0.051), but the interaction was significant when a contrast was done between jasmonic acid and control both with insects (F=4.11, df=2,65, P=0.021) and with no insects (F=3.97, df=2,65, P=0.023).

The mean ratios of glucobrassicin to sinigrin and neoglucobrassicin to sinigrin were close to zero for all treatments, except for jasmonic acid treatments (Tables 4.5 and 4.7). Using the same initial values (0 h) for jasmonic acid and jasmonic acid with insect feeding, similar trends were seen for both glucosinolates over time (Figs. 4.9 and 4.10). For glucobrassicin the jasmonic acid treatment shows a decline over time, while the jasmonic acid with insect feeding shows a slight decline at 24 h, followed by a large increase at 48 h. For neoglucobrassicin, the jasmonic acid treatment shows a decline at 24 and a small increase at 48 h, while the jasmonic acid treatment with insect feeding shows a slight decline at 24 h, followed by a large increase at 48 h as seen in glucobrassicin.

4.6.2 Studies with *Lipaphis erysimi*

4.6.2.1 Development and reproduction

The results for the aphid experiment and analyses are shown in Table 4.9. There were significant effects of treatment on nymph production, intrinsic rate of increase, and all measures of development time except days to first young. Where the analysis was significant a Dunnett's test showed that only jasmonic acid treatments differed significantly from the controls. In the jasmonic acid treatment, development was slower, fewer offspring were produced and the rate of population growth was lower than in the control.

4.7 Discussion

In the oviposition study, cotyledons and leaves were analysed separately because the cotyledon data were erratic and there were many replicates that had no data, due to senescence of cotyledons. From the oviposition data for leaves, most eggs were laid overall on the jasmonic acid treated plants. For all treatments, the greatest numbers of eggs were laid on the top of leaves, and leaves 1 and 2 had greatest numbers of eggs over all. When *P. xylostella* is provided with cauliflower leaves, there are no significant differences in location of egg laying, but the moths appear to prefer to oviposit on leaves with greater surface contours (Syed and Abro 2003). This was not addressed directly in the analysis, but my observations indicated that eggs were often found on the top of leaves in depressions.

The distinct larval instars were identified prior to the start of the experiment by measuring head capsule width. Dyar (1890) found that head capsule width can be used to identify distinct instars for larvae of Lepidoptera. This corresponds with other research showing that *P. xylostella* has four larval instars of similar size to these results (Ecole *et al.* 1999).

In the larval feeding and growth rate study, the amount of leaf area consumed by larvae was represented as an area rather than leaf weight, although the latter would be more desirable as it would allow direct assessment of biomass conversion efficiency (MacKay and Lamb 1996). Preliminary studies showed no significant relationship between leaf length and/or width and dry weight that could have been used to provide a non-destructive method of estimating initial leaf mass. Although using area makes the assumption that leaf thickness and cellular structure were not influenced by the

treatments, similar studies on the effects of jasmonic acid on larval feeding have found that area and weight of the plant tissue consumed are highly correlated (Avdiushko *et al.* 1997), justifying the use of leaf area consumed to investigate the effects of the treatments.

Relative to the control treatments, leaf area consumption and relative growth rate in the jasmonic acid treatments were both reduced by 27%. These reductions were associated with a tendency for larvae to initiate feeding on more sites on the leaf. As there were no significant treatment effects on the efficiency of biomass conversion, the reduction in larval growth rate can be attributed to the reduced larval feeding.

In the investigation of whether jasmonic acid treatment affects *P. xylostella* growth rate through localized or systemic effects, the results support the theory that jasmonic acid treatments produced a systemic effect. If the effect had been localized, rather than systemic, there would be a decrease in the relative growth rate of insects feeding on the treated leaves, but not for those feeding on untreated leaves in the jasmonic acid-treated plants. These results confirmed the earlier findings that the jasmonic acid treatments inhibit larval growth. The absence of significant differences in growth between larvae feeding on treated and untreated leaves on the same plant indicates that the mechanism is systemic and plant-mediated.

Leaf tissue was analyzed to further elucidate mechanisms affecting the response of *P. xylostella* to treatments. Although the response of larval growth to treatments was not significant, this can be attributed to the reduced replication, as the focus of this experiment was on characterizing the temporal change of defence chemistry in response to treatments. Direct comparison between jasmonic acid and control was justified based

on the results of previous experiments, and jasmonic acid was found to be significantly different from the control.

For the peroxidase analysis the significant effect of treatment was attributed to the jasmonic acid treatment, which had the highest levels of peroxidase activity. There were no significant effects of time, insects, or any interactions between time, insect and treatment. For the phenol concentration, the jasmonic acid treatment accounted for most of the significant effect of treatment, with the greatest phenol concentration occurring in the jasmonic acid treated plants. Although the overall interaction between treatment and insect was significant when the interaction was analysed at the two times separately, only jasmonic acid was significant at both 24 and 48 h. Although the overall phenol concentration was greatest in the jasmonic acid treatment, the high levels of phenol seen at 24 h in the PA23 and PA23+pathogen treatments with insect feeding, are responsible for the significant residual treatment effects as well as the significant interaction between treatment and time at 24 h but not at 48 h.

The major glucosinolates found in leaves of *B. napus* are progoitrin ([R] 2-hydroxy-3-butenyl-glucosinolate), gluconapin (3-butenyl-), glucobrassicanapin (4-pentenyl-), glucobrassicin (3-indoylmethyl-), neo-glucobrassicin (N-methoxy-3-indoylmethyl-) gluconasturtiin (2-phenylethyl-), gluconapoleiferin (2-hydroxy-4pentenyl-), 4-hydroxyglucobrassicin (4-hydroxy-3-indoylmethyl-), and 4-methoxyglucobrassicin (4-methoxy-3-indoylmethyl-) (Sang *et al.* 1984, Kiddle *et al.* 2001). Only glucobrassicin and neoglucobrassicin were detected at levels sufficient for analysis. There were very low levels detected in all of the treatments, except the jasmonic acid treatments that had the greatest levels of both glucosinolates. The main difference between the analyses was the

significant overall interaction between treatment and time for neoglucobrassicin not seen in the glucobrassicin analysis. When the analysis of the interaction between treatment and time was divided into with and without insects, the interaction was significant for both analyses in neoglucobrassicin, but only with insects for glucobrassicin. With insect feeding, both glucosinolates declined slightly from 0 to 24 h, then increased at 48 h, but without insect feeding neoglucobrassicin increased at 48 h, while glucobrassicin declined. This difference probably accounts for the dissimilar interactions between treatment and time for the two glucosinolates.

Glucobrassicin levels have previously been shown to increase for approximately 3 d following jasmonic acid application, followed by a decline (Bodnaryk 1992, 1994), which is similar to the response seen in the present study for glucobrassicin and neoglucobrassicin. Canola plants treated with jasmonic acid followed by mechanical wounding, have significantly greater levels of glucobrassicin compared with plants treated only with jasmonic acid, and although the levels decrease after approximately 3 d, they remain higher than plants treated only with jasmonic acid for at least 14 d (Bodnaryk 1992). The addition of insect feeding produces similar responses to mechanical wounding, but since wounding cannot exactly replicate insect feeding, differences in plant responses may account for the apparent decline in glucosinolates, followed by an increase. If further observations had been made after 48 h, the glucosinolate levels in the jasmonic acid treatment with insect feeding may have also decreased as was seen in mechanically wounded plants.

Exogenous jasmonic acid treatments activate defence-related genes in canola (Sarosh and Meijer 2007), yet *P. xylostella* oviposition was found to increase on jasmonic

acid treated plants in choice experiments. One possible explanation for these results is that the jasmonic acid treatments may have resulted in greater levels of volatile chemicals. *Plutella xylostella* females oviposit significantly more frequently on plants damaged by larval feeding than on undamaged plants (Shiojiri and Takabayashi 2003), and prefer plants damaged by *Pieris rapae* over conspecifics (Shiojiri *et al.* 2001). These responses suggest that highly specific volatiles are released by damaged plants, and females are responding to these chemical attractants. Because jasmonic acid treatments can activate induced responses in plants that are similar to wounding (Thaler *et al.* 1996, Moore *et al.* 2003b), the oviposition preference seen in the present study could be in response to the induction of highly attractive, plant-mediated volatiles.

Induced plant responses to jasmonic acid can be highly variable even in closely related Brassicaceae species, as shown by the response of *P. xylostella* to Chinese cabbage (*Brassica campestris* L.) and common cabbage (*B. oleracea* L.) treated with jasmonic acid (Lu *et al.* 2004). Adult females prefer to oviposit on Chinese cabbage more than common cabbage in untreated choice experiments, but when jasmonic acid treatments are applied, diamondback moths increase oviposition on common cabbage and decrease oviposition on Chinese cabbage, indicating that the treatments induce different defence responses in the plants (Lu *et al*. 2004). These studies highlight the complicated nature of induced plant defences and the extreme variation in response seen in one insect species.

My results indicate that the effects of jasmonic acid on *P. xylostella* larvae are responses to systemic changes in plant chemistry rather than to residues on the leaves, and are in accord with research showing that jasmonic acid does not have direct effects

on insects. The development of the cabbage looper (*Trichoplusia ni* Hübner) and tobacco hornworm (*Manduca sexta* L.) is not significantly affected when larvae are reared on artificial diet containing various amounts of jasmonic acid (0.001–15 µl methyl jasmonate) (Avdiushko *et al.* 1997). Treatment of the fungus gnat (*Bradysia impatiens* Johannsen) with jasmonic acid spray treatments (0.01%) does not significantly affect mortality compared with spraying with water (McConn *et al.* 1997). Relative to control treatments, there are no effects on survivorship or growth of beet armyworm (*Spodoptera* exigua Hübner) sprayed directly with jasmonic acid (0.5 mM or 1.5 mM) treatments and then allowed to feed on untreated leaves (Thaler *et al.* 2001).

Increased peroxidase activity in plants can be induced following jasmonate application (Schenk *et al.* 2000) or insect feeding (Moore *et al.* 2003b), and can help defend the plant against future insect attacks (Moore *et al.* 2003a). The present study found inconsistent effects of exogenous jasmonic acid treatments on the induction of peroxidase which has also been shown in other plant species. Thaler *et al.* (1996) investigated the response of several defence compounds in tomato plants to jasmonic acid and found that the activity of all compounds tended to increase with increasing concentrations of jasmonic acid (0.1–10 mM), except for peroxidase activity which was lower than the control treatments except at the highest concentrations. In wild mustard, 0.5 mM jasmonic acid treatments increase peroxidase activity, but the response is only marginally significant (Cipollini and Sipe 2001).

Peroxidase activity can also increase in plants through PGPR-mediated ISR (Ramamoorthy *et al.* 2002) and increased peroxidase activity can help defend plants against pathogens (Peng and Kuc 1992, Passardi *et al.* 2004). Based on previous studies

involving PA23 it was expected that peroxidase activity would be elevated in PA23+pathogen treatments (Ramamoorthy *et al.* 2002, Fernando *et al.* 2007). In my studies, there was an increase in peroxidase activity in the PA23 treatment with insect feeding at 24 h, although it declined after 48 h. There was no increase in peroxidase activity in the other PA23 or PA23+pathogen treatments, with or without insect feeding.

Phenolic compounds are present in all plants as preformed compounds that can serve as plant defences against herbivores and pathogens (Vermerris and Nicholson 2006). Phenolic compounds can also be induced in plants following stress (Vermerris and Nicholson 2006). Phenol can be important in plant defence against insect herbivores, for example, peroxidases oxidize phenolic compounds resulting in the production of quinones and free radicals that can oxidize the lipids and proteins in insect tissues (Bi *et al.* 1997a).

Exogenous jasmonic acid application increases phenolics in other plant species, but most studies do not consider the effects of treatment with and without insect feeding. In field studies where plants are not protected from insect feeding, polyphenol oxidase activity in tomato plants increases after jasmonic acid application and declines after a few days (Thaler *et al.* 2001). In greenhouse experiments without insect feeding, tomato plants treated with jasmonic acid have elevated levels of polyphenol oxidase activity that also declines over time, but may stay higher than controls for a few weeks (Redman *et al.* 2001). In tomato plants, there is evidence of negative cross-talk between jasmonic acid- and salicylic acid-mediated responses (Thaler *et al.* 2002b), and plants in the field could be exposed to pathogens or insects that activate salicylic acid-induced pathways.

Phenol levels can increase in plants treated with PGPR followed by pathogen inoculation (Nakkeeran *et al.* 2006), so it was expected that levels would be elevated in the PA23+pathogen treatments, but this was not seen in the results. There was however an increase in phenolics with the addition of insect feeding at 24 h, followed by a decline at 48 h. An increase was expected in all treatments with the addition of insect feeding because they can be stress-induced (Vermerris and Nicholson 2006), but no changes were seen in the control with the addition of insect feeding.

Increased glucosinolate levels in *B. napus* do not deter feeding by other *Brassica* specialist insects, including the flea beetles *Phyllotreta cruciferae* (Bodnaryk and Palaniswamy 1990) and *Psylliodes chrysocephala* (L.) or the lepidopteran *P. rapae* (Giamoustaris and Mithen 1995), but changes in glucosinolate levels can still affect specialist insects. Increasing glucosinolate levels negatively affects the relative growth rate of *P. xylostella* (Li *et al.* 2000), but may have no effects (Li *et al.* 2000) or a curvilinear relationship with the amount of leaf area consumed (Siemens and Mitchell-Olds 1996).

Recent work has suggested that glucosinolate levels do not play an important role in plant defence against *P. xylostella*. Analysis of *P. xylostella* faeces by HPLC found that desulpho-glucosinolates were present, suggesting that a sulphatase enzyme is used by these insects (Ratzka *et al.* 2002). Gut extracts from fourth instar larvae were used for gene sequencing, and revealed genes similar to sulphatase that can compete with myrosinase, the enzyme responsible for hydrolyzing glucosinolates into toxic breakdown products (Chew 1988), producing non-toxic desulpho-glucosinolates (Ratzka *et al.* 2002). Increased levels of myrosinase activity negatively affects feeding time and amount of leaf

area damaged *P. xylostella* larvae on *Brassica juncea* (L.) (Li *et al.* 2000), indicating that the effectiveness of *P. xylostella* sulphatase depends on competition with myrosinase.

In my research, larval relative growth rate was reduced, and was attributed to the reduced larval feeding, and therefore the effect of treatment appears to be deterrent rather than antibiotic. Increased peroxidase activity can increase lignin deposition, and work done using other elicitors of induced resistance has indicated that this may reduce feeding by *P. xylostella* (Hodge *et al.* 2006). Phenolic compounds are involved in host plant acceptance (Simmonds 2001), and can negatively affect larval development (McCloud and Berenbaum 1994), so changes in phenolic concentration may partially explain the effects on *P. xylostella*. Increased glucosinolate levels do not affect the time spent feeding or leaf area damaged by *P. xylostella* larvae, but do have a negative effect on relative growth rate of larvae (Li *et al.* 2000). In my research there were no significant treatment effects on the efficiency of biomass conversion, but the effects on *P. xylostella* may have included some antibiotic effects of increased glucosinolate levels on larval relative growth rate.

The intrinsic rate of natural increase (r_m) for insects was first described by Birch (1948) as the growth of a population with a stable age distribution under unlimited environmental conditions. This measure is very useful in ecological studies, but can be difficult to estimate because of the need for life history data for the insect (Birch 1948, Wyatt and White 1977). Modifications of r_m have been used to provide simplified measures of intrinsic rate of increase, but many of their assumptions are not consistent with aphid ecology in which the reproductive period is short and most young are produced in the first few days (Wyatt and White 1977). Wyatt and White's (1977)

estimator of r_m, which I used, has been used to investigate host plant influences on performance of aphids for numerous species (Castle and Berger 1993, Cole 1997, Sylwia *et al.* 2006, Ranger *et al.* 2007), including *L. erysimi* (Amjad and Peters 1992).

Amjad and Peters (1992) investigated the effects of different varieties of oilseed *Brassica* on *L. erysimi* survival, development and reproduction using *B. campestris* (L.) cv. Toria-A as a standard control check for susceptibility. The r_m values calculated in the present study were larger than those found by Amjad and Peters (1992), except for those in the jasmonic acid treatment which were similar to their controls. Earlier work done by Landin and Wennergren (1987) on the effects of temperature on *L. erysimi*, showed similar r_m values between 20–25˚C to those found in the present study. Although their calculation used the Birch (1948) equation, the r_m equation used in the present study is predicted to produce reasonably comparable results (Wyatt and White 1977).

The jasmonic acid treatments reduced reproduction and increased development time resulting in significantly lower r_m values than the control. The aphid experiment was designed so that aphids would undergo two reproductive generations to allow the second generation to be exposed to treatments while developing as embryos within the mother, and later when feeding directly on the treated plants. The measure of days to second young or total development was predicted to be a more important measure than days to first young, because of the longer exposure to treatments. Although there were no significant effects on days to first young, there were significant effects of treatment on the days to second young and total development time, and a Dunnett's test showed that jasmonic acid was significantly different from the controls for all significant means. These results indicate that jasmonic acid treatments were acting on the insects only a few

days after application and persisted for long enough to affect the development rates of the next generation.

Although effects of jasmonic acid on *P. xylostella* larvae appear to be a response to plant chemistry rather than to residues on the leaf surface, this does not necessarily mean that aphid population is also responding to plant chemistry. The aphid response could be to surface residues of jasmonic acid on the leaf. However, as with *P. xylostella*, previous studies indicate that jasmonic acid does not directly affect aphid development and reproduction. Studies on the potato aphid (*Macrosiphum euphorbiae* Thomas) have shown that there are no effects on survivorship or production of young when aphids are sprayed with low and high doses of jasmonic acid (0.5mM and 1.5 mM) relative to control treatments (Thaler *et al.* 2001).

The greater levels of phenolics and glucosinolates in the jasmonic acid treatments compared with controls could be involved in the increased development time and reduced reproduction seen in the aphid study. Aphid feeding, wounding or jasmonic acid treatment can induce greater levels of enzymes involved in phenolic synthesis (McConn *et al.* 1997, Moran and Thompson 2001) and phenolics can aid in plant defence against aphids by negatively effecting aphid reproduction (Chaman *et al.* 2003). The reduction in reproduction and r_m may partially be attributed to the increase in phenolic concentration in the jasmonic acid treated plants. Glucosinolates are also believed to effect aphid development (Thompson and Goggin 2006) as they are transported through phloem (Chen *et al.* 2001). Glucosinolates can have a negative or positive effect on the intrinsic rate of increase of aphids, depending on the plant and aphid species (Cole 1997, Levy *et al.* 2005). The increased levels of glucosinolates found in my research may have

contributed to the reduction in r_m, as high levels of total glucosinolates in other *Brassica* spp. have been shown to negatively affect the fecundity of *L. erysimi* (Malik *et al.* 1983). The increased levels of glucosinolates in jasmonic acid treated plants may play a role in defence against *L. erysimi*, but more work is still required to fully understand the response of these insects.

It was expected that jasmonic acid treatments would induce the highest levels of defence compounds, because induction of defence genes is generally greater for plants treated with signalling molecules than other elicitors, such as wounding (Bodnaryk and Rymerson 1994) or pathogen inoculation (Schenk *et al.* 2000). Based on previous studies it was expected that there would also be induction of plant defences in treatments with PGPR, and that these would affect the insect herbivores. Neither *P. xylostella* nor *L. erysimi* responded significantly to the bacterial treatments.

From the analysis of changes in plant chemistry there were no changes in the PA23 or PA23+pathogen treatments alone, but with the addition of insect feeding there were increases in peroxidase in the PA23 treatment with insect feeding at 24 h and phenol at 24 h in the PA23 and PA23+pathogen treatments with insect feeding, followed by declines at 48 h. It is possible that the bacterial treatments induce defence compounds that were not investigated and do not affect the insects examined, or the bacterial treatments may not be inducing chemical changes in the plants.

In laboratory studies performed by Fernando *et al*. (2007), canola inoculated with PA23 and followed 24 h later by *S. sclerotiorum* inoculation showed significant increases in enzymes involved in defence against fungal pathogens. Corresponding increases were not seen in control plants or plants inoculated with PA23 or *S. sclerotiorum* alone. In my

research, canola inoculated with PA23 or PA23 followed 24 h later by inoculation with the blackleg pathogen did not produce significant increases in any of the defence-related compounds investigated. There are several reasons why this may have occurred.

The activation of induced systemic resistance by PGPR is usually accomplished through the application of bacteria to plant seeds, roots or surrounding soil (Kloepper 1996, van Loon *et al.* 1998), as this is the location of PGPR symbiosis with the plant (Molla *et al.* 2001), and spatial separation of bacteria and pathogen inoculation allows researchers to confirm that resistance is due to a systemic induction not direct effects of the bacteria (Hoffland *et al.* 1995). Bacteria can also be applied to the above ground plant parts through pressure infiltration (Pieterse *et al.* 1996), indicating that the same systemic signalling pathways can be activated when bacteria are applied to roots or leaves, but plant wounding is required for pressure infiltration (Swanson *et al.* 1988). Foliar sprays of PGPR may activate induced systemic resistance (Fernando *et al.* 2007), but there are few examples of this application method in the literature, and this method may not induce resistance, as compared with root inoculation (Kilic-Ekici and Yuen 2004). My research plants were kept in a growth chamber where they were subjected to wind that caused some wounding; however, pressure infiltration of PGPR forces bacteria into plant wounds (Swanson *et al.* 1988, Pieterse *et al.* 1996), and therefore may be more effective at activating signalling pathways than a foliar spray.

Petals infected with *S. sclerotiorum* can be an important source of infection in canola (Gugel and Morrall 1986), so it is desirable to apply these bacterial antagonists to above ground plant parts to control this pathogen on plant surfaces. When foliar sprays are used, PA23 and BS6 are able to colonize the surface and suppress *S. sclerotiorum*

(Savchuck and Fernando 2004, Zhang 2004). This demonstrates that this application method can be successful in pathogen control, but it is possible that the application of these bacterial strains alone may not activate induced systemic resistance.

In *Arabidopsis thaliana* (L.), the exogenous application of salicylic acid or methyl jasmonate triggers the expression of defence-related genes and can induce pathogen resistance (van Wees *et al.* 1999). Treatment with the bacterial strain *Pseudomonas fluorescens* WCS417r can also defend *A. thaliana* against bacterial and fungal pathogens (Pieterse *et al.* 1996), but there is no induction of defence-related genes following treatment with the bacteria (van Wees *et al.* 1999). Only when *A. thaliana* is treated with *P. fluorescens* WCS417r followed by inoculation with a pathogen are defence-related genes expressed, indicating that these plants only activate defence-related genes following pathogen challenge (van Wees *et al.* 1999). This enhanced ability of plants to induce defences in response to pathogens or other elicitors is referred to as priming or potentiation (Conrath *et al.* 2002). It has been suggested that pathogen-induced salicylic acid is required for expression of induced systemic resistance in PGPR-treated plants (van Wees *et al.* 1999), and priming is commonly associated with induced systemic resistance by PGPR (Pozo *et al.* 2005).

To investigate whether priming was needed to produce effects on insects, a PA23+pathogen treatment was introduced. However, this treatment did not affect *P. xylostella* larval growth, aphid reproduction or development or induce defence compounds for which I tested. These results do not rule out priming, as the laboratory studies may not have allowed for enough time for plants to be primed. Bacterial treatments were applied 24 h prior to the start of the experiments (or 24 h prior to

pathogen inoculation, 48 h prior to the start in later studies) because this is the ideal application timing for *S. sclerotiorum* control by PGPR in the laboratory (Savchuck and Fernando 2004). Other studies on induced systemic resistance by PGPR involve longer time periods between bacterial treatments and challenge inoculation with pathogens (Liu *et al.* 1995a, Pieterse *et al.* 1996). Studies using jasmonic acid treatments generally involve shorter periods between treatment application and insect feeding or leaf analysis (Bodnaryk and Rymerson 1994, Thaler *et al.* 1996) and this may explain why only jasmonic acid treatments significantly induced defence related compounds in this study.

4.7.1 Implications of insect responses for use of PGPR

Analysis at the genomic level can provide a greater understanding of induced plant responses by elucidating which genes are involved. Transcriptional profiling has been done for the cultivar used in these experiments (*B. napus* cv. Westar) by Sarosh and Meijer (2007). Their work revealed that induced responses are similar for jasmonate treatment, wounding or larval feeding, but differences exist in the levels of gene induction with each treatment. Although the methods used may not be able to detect all changes occurring in the plants, there were significant effects found for jasmonic acid treatments. This research showed that the jasmonic acid treatments are not acting directly on *P. xylostella* larvae, but are affecting development and reproduction by acting systemically on the plant. Based on the analysis of peroxidase and phenol activity and glucosinolate levels, the jasmonic acid treatments are believed to be affecting *P. xylostella* and *L. erysimi* through the activation of these and potentially other plant defences.

Induced systemic resistance can be used to enhance plant defences and might be useful in the control of insect herbivores of canola. Jasmonic acid is one method of inducing systemic resistance, but may not be practical due to cost, efficacy of treatment and the complexity of plant responses to treatment. Research has shown that PGPR have the potential to be useful in the control of plant pathogens, but there are numerous problems with widespread adoption including application methods, costs and reliability (Cook *et al.* 1996). These elicitors of induced plant resistance may be important methods of control for insects and diseases, making this a worthwhile area of research.

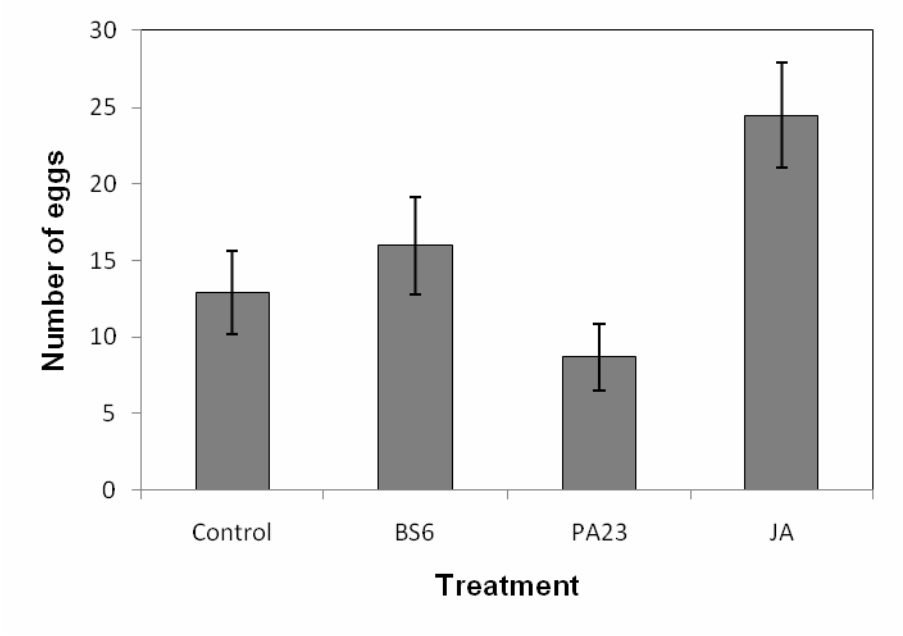

Figure 4.1. Mean (±SEM) number of eggs laid per plant for each treatment (N=29).

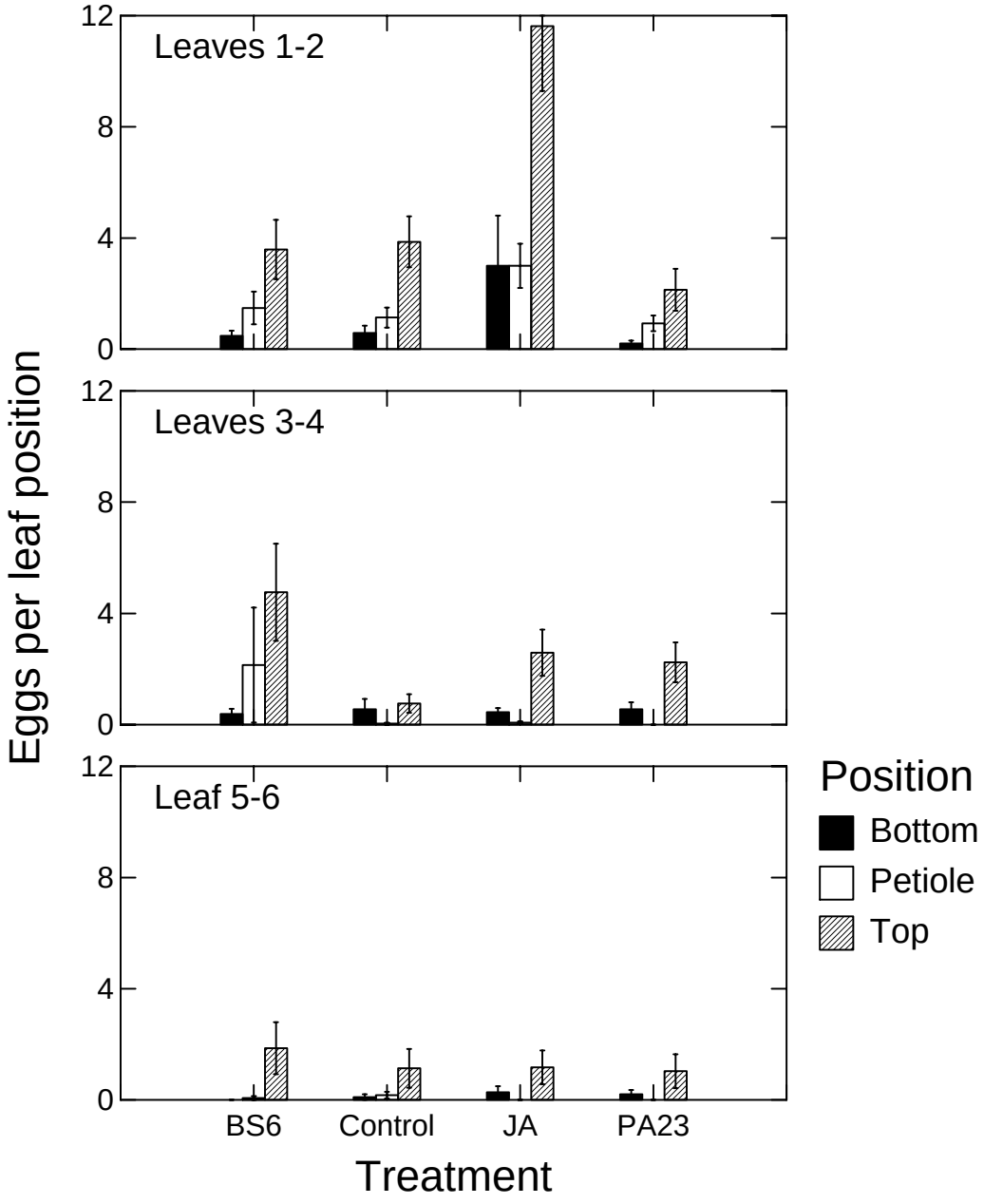

Figure 4.2. Mean (±SEM) number of eggs laid on bottom, petiole or top of leaves 1–2, 3–4 and 5–6 for each treatment (N=29).

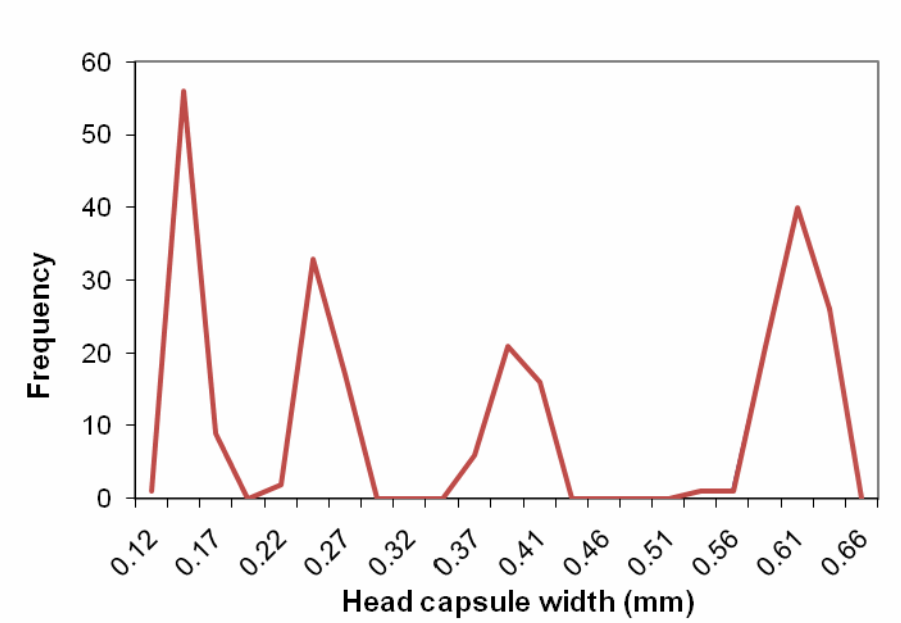

Figure 4.3. Frequency distribution of *Plutella xylostella* head capsule widths.

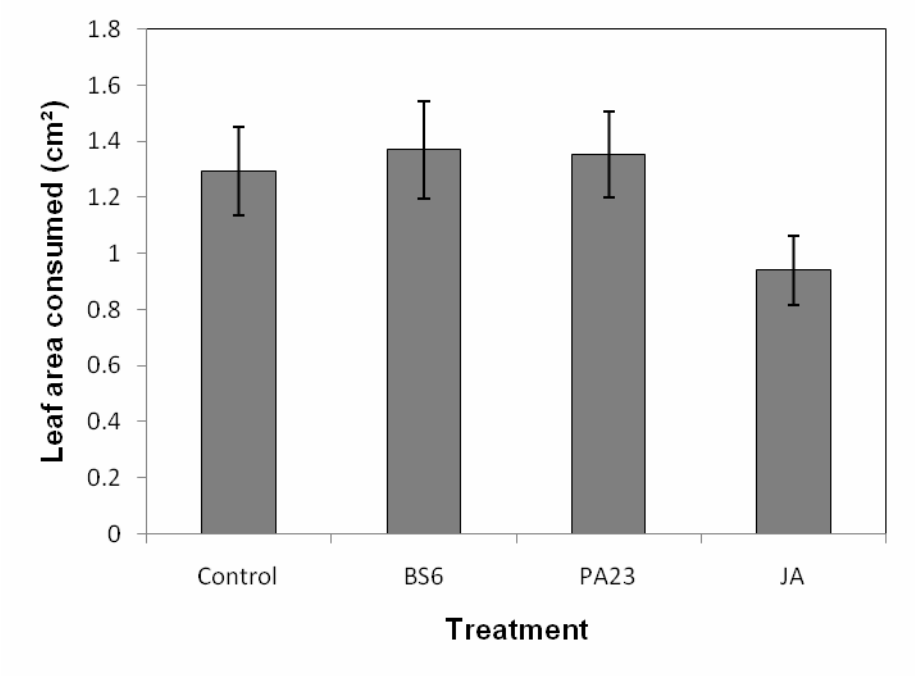

Figure 4.4. Mean (±SEM) leaf area consumed by larvae in each treatment (N=31).

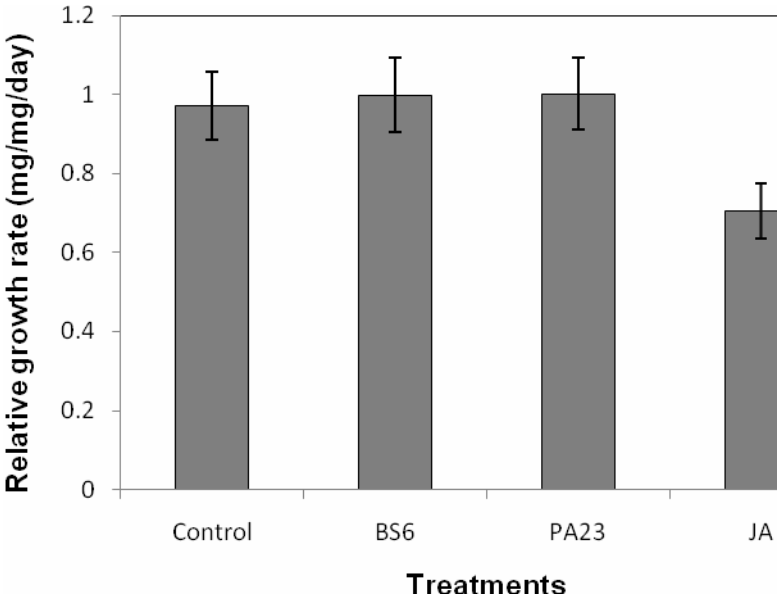

Figure 4.5. Mean (±SEM) relative growth rate of larvae in each treatment (N=29).

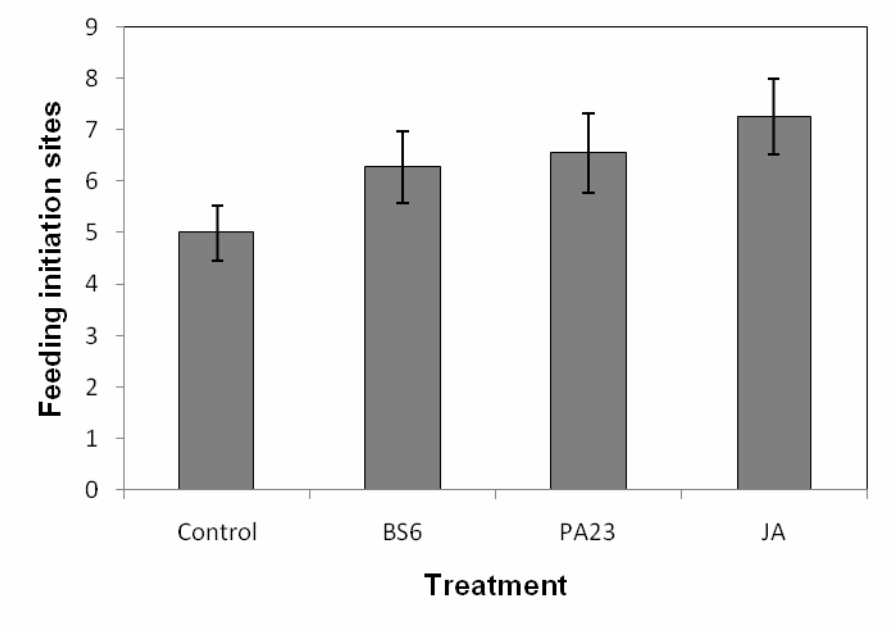

Figure 4.6. Mean (±SEM) number of larval feeding initiation sites per leaf in each treatment (N=31).

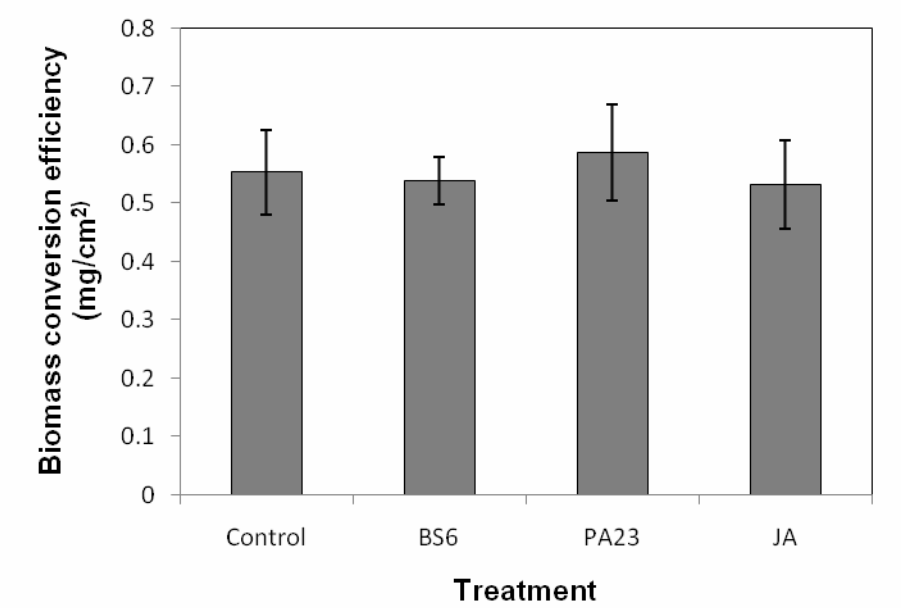

Figure 4.7. Mean (±SEM) biomass conversion efficiency (mg/cm^2) for larva in each treatment (N=31).

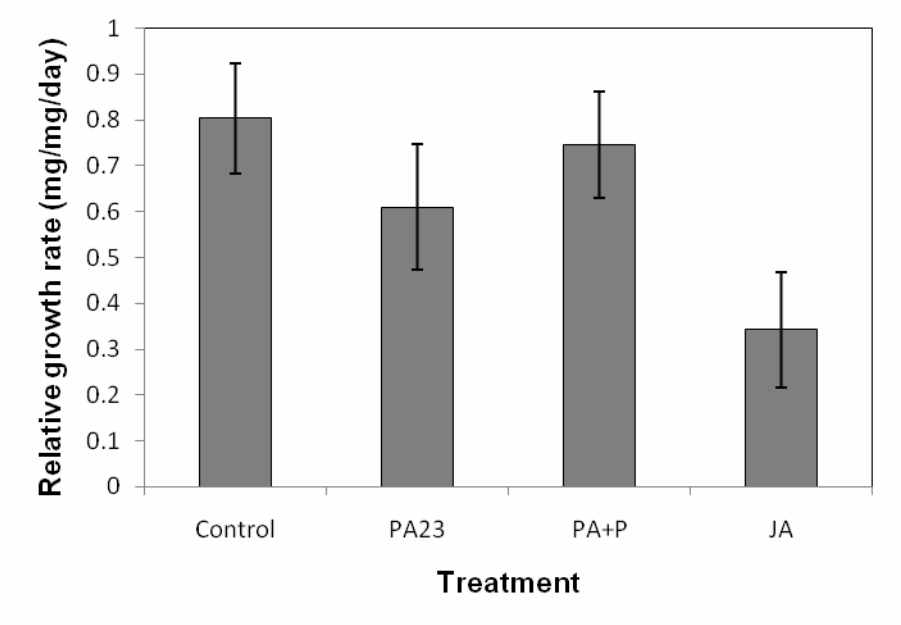

Figure 4.8. Mean (±SEM) relative growth rate of larvae in each treatment in the plant chemistry experiment (N=6).

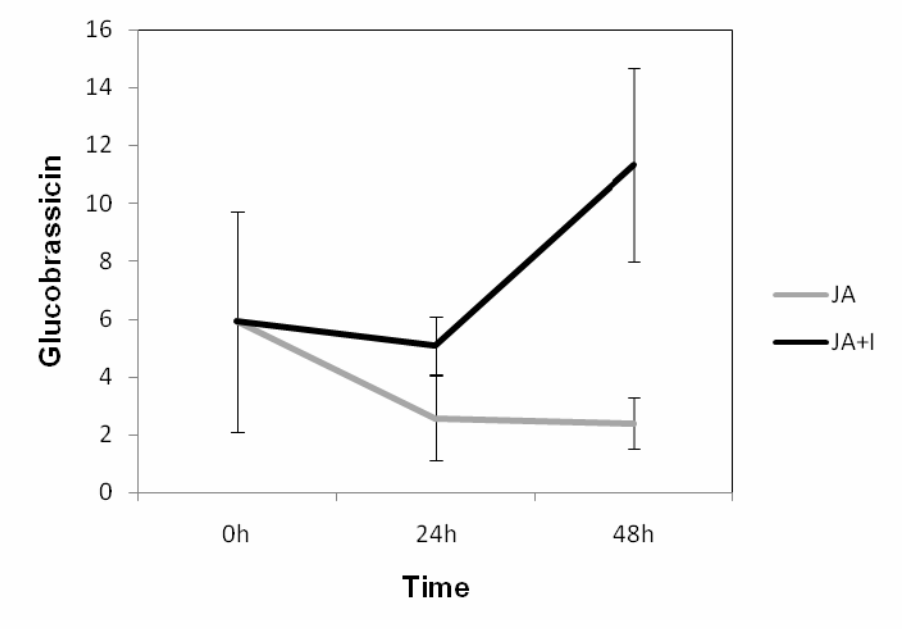

Figure 4.9. Ratio of peak area (mean ±SEM) of glucobrassicin to the area of the peak for the standard (sinigrin) for jasmonic acid treatments with and without insect feeding (N=4).

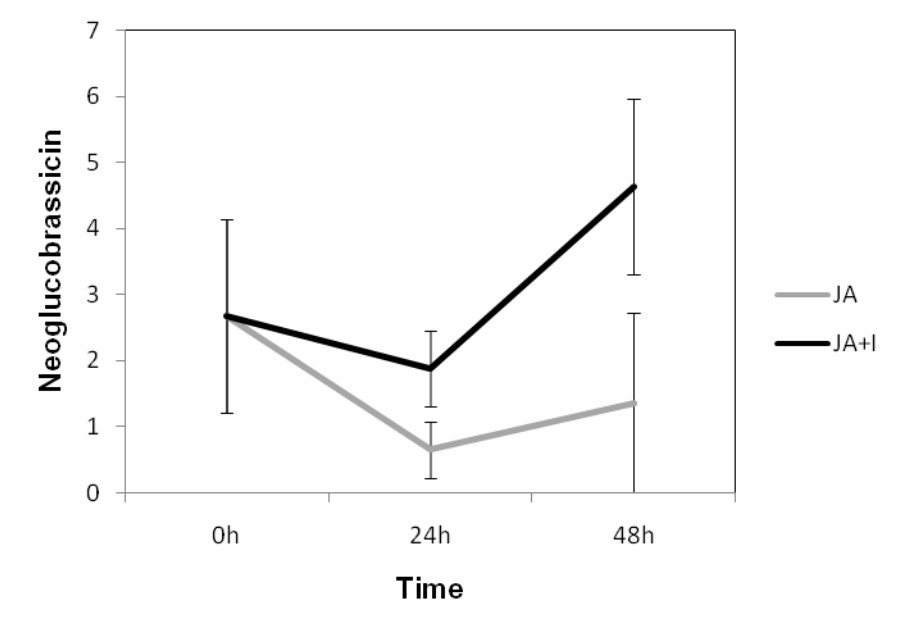

Figure 4.10. Ratio of peak area (mean ±SEM) of neoglucobrassicin to the area of the peak for the standard (sinigrin) for jasmonic acid treatments with and without insect feeding (N=4).

Table 4.1. Levels of peroxidase in relation to treatments and the presence or absence of insects.

Treatment	Time since insect introduction (h)	Peroxidase ΔOD $min^{-1}mg^{-1}$*	
		Without insects	With insects
Control	0	0.22±0.05	
	24	0.20±0.14	0.21±0.04
	48	0.28±0.05	0.21±0.02
Jasmonic Acid	0	0.47±0.15	
	24	0.24±0.05	0.54±0.16
	48	0.68±0.07	0.55±0.17
PA23	0	0.19±0.00	
	24	0.27±0.03	0.46±0.08
	48	0.26±0.07	0.34±0.11
PA23 + pathogen	0	0.22±0.04	
	24	0.20±0.04	0.28±0.11
	48	0.27±0.10	0.27±0.05

*Rate of change in optical density at 420 nm.

Table 4.2. Analysis of variance for levels of peroxidase.

Source	df	MS	F ratio	P
Overall Model	19	0.08	2.54	0.003
Error for overall model	58	0.03		
Treatment effect (pooled over time and insect)	3	0.30	9.64	<0.001
Time effect (pooled over treatment and insect)	2	0.04	1.39	0.258
Insect effect (pooled over treatment and times 24 and 48 h)	1	0.04	1.37	0.246
Treatment x time (pooled over insect)	6	0.03	1.07	0.389
Treatment x insect (pooled over 24 and 48 h)	3	0.02	0.57	0.638
Insect x time (24 and 48 h) (pooled over treatment)	1	0.12	3.73	0.584

Table 4.3. Phenol activity in relation to treatments and the presence or absence of insects.

Treatment	Time since insect introduction (h)	Phenol activity (mg catechol equivalents/g)	
		Without insects	With insects
Control	0	69.03±1.89	
	24	68.51±6.19	68.68±5.77
	48	60.25±4.54	57.72±2.66
Jasmonic Acid	0	94.77±12.29	
	24	143.03±11.95	87.02±7.30
	48	71.41±2.48	95.07±14.61
PA23	0	73.61±4.44	
	24	64.81±5.65	90.40±11.44
	48	61.94±3.95	64.11±2.62
PA23 + pathogen	0	68.27±1.88	
	24	60.53±6.57	110.06±8.39
	48	66.63±2.12	60.12±3.93

Table 4.4. Analysis of variance for phenol activity.

Source	df	MS	F ratio	P
Overall Model	19	1599.05	8.74	<0.001
Error for overall model	64	182.85		
Treatment effect (pooled over time and insect)	3	4312.48	23.58	<0.001
Time effect (pooled over treatment and insect)	2	3152.47	17.24	<0.001
Insect effect (pooled over treatment and times 24 and 48 h)	1	337.78	1.85	0.179
Treatment x time (pooled over insect)	6	231.54	1.27	0.286
Treatment x insect (pooled over 24 and 48 h)	3	1139.16	6.23	<0.001
Insect x time (24 and 48 h) (pooled over treatment)	1	1.62	0.01	0.925

Table 4.5. Ratio of peak area (Mean ±SEM) of the glucosinolate glucobrassicin relative to the area of the known standard (sinigrin) in relation to treatments and the presence or absence of insects (N=4).

Treatment	Time since insect introduction (h)	Ratio of glucobrassicin to standard	
		Without insects	With insects
Control	0	0.05±0.04	
	24	0±0.0	0.01±0.01
	48	0±0.0	0.03±0.02
Jasmonic Acid	0	5.91±3.81	
	24	2.56±1.46	5.08±1.02
	48	2.39±0.89	11.32±3.34
PA23	0	0.03±0.03	
	24	0.27±0.13	0±0.0
	48	0±0.0	0.01±0.01
PA23 + pathogen	0	0.19±0.13	
	24	0.13±0.05	0±0.0
	48	0±0.0	0.02±0.01

Table 4.6. Analysis of variance for glucobrassicin.

Source	df	MS	F ratio	P
Overall Model	19	0.40	18.58	<0.001
Error for overall model	65	0.02		
Treatment effect (pooled over time and insect)	3	2.22	102.22	<0.001
Time effect (pooled over treatment and insect)	2	0.00	0.13	0.880
Insect effect (pooled over treatment and times 24 and 48 h)	1	0.18	8.47	0.005
Treatment x time (pooled over insect)	6	0.01	0.52	0.788
Treatment x insect (pooled over 24 and 48 h)	3	0.26	12.08	<0.001
Insect x time (24 and 48 h) (pooled over treatment)	1	0.06	2.97	0.090

Table 4.7. Ratio of peak area (Mean ±SEM) of the glucosinolate neoglucobrassicin relative to the area of the known standard (sinigrin) in relation to treatments and the presence or absence of insects (N=4).

Treatment	Time since insect introduction (h)	Ratio of neoglucobrassicin to standard	
		Without insects	With insects
Control	0	0±0.0	
	24	0±0.0	0±0.0
	48	0±0.0	0±0.0
Jasmonic Acid	0	2.68±1.46	
	24	0.65±0.42	1.88±0.57
	48	1.36±0.38	4.63±1.33
PA23	0	0±0.0	
	24	0.09±0.05	0±0.0
	48	0±0.0	0±0.0
PA23 + pathogen	0	0.01±0.01	
	24	0.05±0.03	0±0.0
	48	0±0.0	0±0.0

Table 4.8. Analysis of variance for neoglucobrassicin.

Source	df	MS	F ratio	P
Overall Model	19	0.18	15.59	<0.001
Error for overall model	65	0.01		
Treatment effect (pooled over time and insect)	3	0.92	80.64	<0.001
Time effect (pooled over treatment and insect)	2	0.02	1.63	0.203
Insect effect (pooled over treatment and times 24 and 48 h)	1	0.10	8.37	0.005
Treatment x time (pooled over insect)	6	0.03	2.36	0.040
Treatment x insect (pooled over 24 and 48 h)	3	0.12	10.47	<0.001
Insect x time (24 and 48 h) (pooled over treatment)	1	0.01	1.06	0.306

Table 4.9. Effects of treatment on aphid development time, reproduction and intrinsic rate of increase (N=23).

	Treatment					
	Control	PA23	PA23+P	JA	F	*P*
Days to first young	7.80±0.14	7.70±0.10	7.70±0.11	8.07±0.12	2.62	0.056
Days to second young	7.30±0.09	7.30±0.10	7.38±0.11	7.68±0.08*	5.64	0.001
Total days	15.11±0.14	15.00±0.15	15.08±0.14	15.68±0.16*	8.54	<0.001
Total offspring	60.78±1.90	60.88±1.98	61.33±1.50	58.14±2.04*	3.93	0.011
Intrinsic rate of increase r_m	0.415±0.006	0.416±0.007	0.413±0.007	0.390±0.004*	12.80	<0.001

* Indicates that a mean differs from the control (two-tailed Dunnett's test α=0.05)

Chapter 5

GENERAL DISCUSSION

The objective of this research was to investigate the effects of two types of elicitors, the plant growth-promoting rhizobacteria (PGPR) *Pseudomonas chlororaphis* strain PA23 and *Bacillus amyloliquefaciens* strain BS6 and jasmonic acid, on insect pests of canola (*Brassica napus* L.) through field and laboratory studies.

In the laboratory, choice experiments were conducted using PA23, BS6, jasmonic acid and control treatments to investigate oviposition preference of the diamondback moth (*Plutella xylostella* L.). The results showed that jasmonic acid affects diamondback moth oviposition preference, with greater numbers of eggs laid on the jasmonic acid treated plants. Laboratory experiments conducted on *P. xylostella* showed that larvae feeding on jasmonic acid treated plants consumed less plant material and had lower growth rates. Despite these significant laboratory results, there was no evidence of any effects of jasmonic acid on *P. xylostella* in the field. The field experiment would have been more likely to detect treatment effects on oviposition preference than on feeding rate, because beat cloth and sweep net samples indicate numbers of insects, rather than the amount of larval feeding. The treatment application may not have been timed appropriately to test for this effect.

The second application of treatments was made on 19 July 2006 at the 30–50% bloom stage, as this stage was used in earlier studies with the bacterial treatments to control *Sclerotinia sclerotiorum* (Lib.) de Bary (Fernando *et al*. 2007). This application date appears to have been after eggs had been laid on the canola, as there were many

large larvae present during the first sampling date (26 July), one week after the application of treatment. On the second sampling date, (9 August) there were few very few larvae present, indicating that the majority of oviposition had occurred prior to the 19 July application of treatments, and therefore could not influence *P.* xylostella oviposition. Although diamondback moths are blown north in the spring and are not believed to overwinter in Canada (Talekar and Shelton 1993), they still undergo several generations per season in Canada (Harcourt 1957). Had the first and second treatment applications both been made to the same plants (5 June field), as had originally been planned, the first treatment may have had an effect on oviposition preference; however, problems with the weather conditions and the type of seeder used for planting prevented this.

In canola, laboratory studies have also shown that jasmonic acid treatments can induce defence compounds and reduce feeding by insect herbivores (Bodnaryk and Rymerson 1994). Similarly, laboratory and field studies on tomato have found that jasmonic acid treatments can also induce defence compounds and negatively affect insect herbivore populations (Thaler *et al.* 1996, 2001). Based on these examples of insect control, jasmonic acid treatments were predicted to induce defence compounds in *B. napus* and negatively affect insect herbivores of canola. Although there were no significant effects on flea beetle injury, there was a significant effect on flea beetles in the beat cloth samples from July 26. The jasmonic acid treatments may have increased the levels of glucosinolates in the plants resulting in increased attraction of flea beetles, although none of the treatments differed significantly from the control. There were also no effects of treatment on any of the other insects sampled, seed yield or quality, although the data for seed yield and glucosinolate levels was nearly significant, with the

greatest levels in the jasmonic acid treated plants. Contrasts between control and jasmonic acid were performed, but the effect of treatment was still not quite significant in either case.

In the laboratory studies, the jasmonic acid treatments also had greater levels of glucosinolates, and the test of systemic influences and the experiments on plant chemistry demonstrated a systemic effect of treatment. There are numerous glucosinolates found at varying concentrations in all parts of *B. napus* (Mithen 1992). The levels of glucosinolates in leaves are not related to the levels in the seed (Mithen 1992), but are determined by environmental factors (Inglis *et al.* 1992). The jasmonic acid treatments may have an effect on plant chemistry in the field as well as the laboratory, but further examination of plant chemistry, including analysis of leaf tissue is needed to more fully understand the effects.

In the laboratory studies, I found that jasmonic acid treatments increased glucosinolates, important defence compounds found in canola and other Brassicaceae (Halkier and Gershenzon 2006). When plants are damaged, glucosinolate hydrolysis occurs in the presence of the enzyme myrosinase, producing several toxic compounds that can have negative effects on insects and pathogens (Halkier and Gershenzon 2006). Although glucosinolates are believed to have evolved as a method of plant defence (Rosenthal and Janzen 1979), specialist pathogens (Giamoustaris and Mithen 1997) and insects (Giamoustaris and Mithen 1995) are not deterred by these defences and many insect specialists have adapted to use them as cues in host plant identification (Fraenkel 1959).

Insect pests of Brassicaceae have developed various methods of dealing with toxins found in their host plants. *Plutella xylostella* is able to avoid the potentially toxic hydrolysis products of glucosinolates through the production of glucosinolate sulfatase (Ratzka *et al.* 2002), which can interfere with myrosinase and reduce its availability (Shikita *et al.* 1999). The turnip aphid *L. erysimi* releases alarm pheromones that contain isothiocyanates (Dawson *et al.* 1987), a toxic product released from the hydrolysis of glucosinolates by the enzyme myrosinase (Halkier and Gershenzon 2006), indicating that these insects use myrosinase to produce isothiocyanates. Myrosinase similar to those found in plants has also been identified in the aphid *Brevicoryne brassicae* (L.) (Pontoppidan *et al.* 2001). *Brevicoryne brassicae* sequesters myrosinase and glucosinolates in the body as a defence against predators (Kazana *et al.* 2007).

These examples highlight how some insect specialists have overcome evolutionary defences in plants. Although glucosinolates may be less effective against these insects, there is potential for related compounds to function in defence against insect specialists. For example, plants with increased levels of myrosinase may counteract defences in *P. xylostella* through competition with sulphatase (Li *et al.* 2000). Increased levels of myrosinase in plants could increase protection against this insect, but the potential effects of modifying the myrosinase levels in plants on aphids and other insects are unknown, so further research is needed.

Secondary plant compounds have been implicated in plant defence against insects and pathogens (Wittstock and Gershenzon 2002), but the role of many of these compounds in plant defence is not well understood (Bennett and Wallsgrove 1994, Hammerschmidt 1999). In laboratory experiments, this research looked at peroxidase

activity and phenol concentration because these compounds can be induced by PGPR (Nakkeeran *et al.* 2006) and jasmonic acid (Gundlach *et al.* 1992, Redman *et al.* 2001), and can be important in plant defence against both pathogens and insects.

Studies using transgenic plants with suppressed production of suspected defence compounds have shown that phenolics are important in plant defence against pathogens. Transgenic tobacco plants with suppressed phenylalanine ammonia-lysase activity, the enzyme responsible for most phenolic synthesis (Bate *et al.* 1994), are more susceptible to pathogens than wild-type plants (Maher *et al.* 1994), indicating that phenolic compounds are required for plant defence.

Peroxidases have also been implicated in plant defence against pathogens, as levels of these enzymes increase significantly more in pathogen-resistant than susceptible plants (Gay and Tuzun 2000). Peroxidases catalyze cell wall lignification (Mäder and Füssl 1982) which can help prevent pathogen infection (Passardi *et al.* 2004). Following pathogen attack on plants, peroxidases can form hydrogen peroxide which limits the spread of pathogens through antimicrobial activity (Peng and Kuc 1992).

Phenolic compounds and peroxidase can also play a role in plant defence against insect herbivores, although there is significant controversy as to their importance in the control of insects (Bernays *et al.* 1989, Appel 1993, Duffey and Stout 1996). Feeding by insect herbivores increases peroxidase activity and can negatively affect insect growth rates (Bi and Felton 1995, Moore *et al.* 2003a), but many insects are able to remove some hydrogen peroxide with midgut enzymes, most importantly catalases (Felton and Duffey 1991). Phenolic compounds can also negatively affect insect development, through the formation of potentially toxic quinones that bind with other molecules, including

peroxidases, to produce free radicals that can destroy insect proteins (Summers and Felton 1994, Duffey and Stout 1996). Although phenolics appear to be important in defence in some plant species, studies on tobacco have found that increased levels of plant phenolics do not have corresponding negative effects on insect herbivore development and survival (Bi *et al.* 1997b) and in some instances may be beneficial to insects (Johnson and Felton 2001). This work demonstrates that in some insect-plant interactions phenolics do not function in plant defence.

In this research, laboratory studies found that there were significant effects of jasmonic acid on *P. xylotsella* oviposition preference and larval feeding and growth rate. There were also effects of jasmonic acid on *L. erysimi* development and reproduction. From the studies on *P. xylotsella* larval feeding and growth rate and the analysis of plant chemistry, the effects of jasmonic acid were attributed to systemic responses induced in the plants. There were no effects of either bacterial treatment or of the PA23+pathogen treatment on either of the insects examined.

Mechanical wounding and other stresses can activate defence compounds in plants, (Gatehouse 2002) so it was predicted that there would be increases in defence compounds in all plants with the addition of insect feeding. Although there were no increases in any of the compounds investigated in the control plants with or without insect feeding, there were changes in the other treatments. The level of peroxidase activity increased in the PA23 treatment with insect feeding and phenol concentration increased in the PA23 and PA23+pathogen treatments with insect feeding. These results indicate that the bacterial treatments may be priming the plants to activate enhanced defences after the challenge of insect feeding.

Based on previous studies by Fernando et al (2007), defence compounds were activated in PA23 treated plants only after inoculation with the pathogen *S. sclerotiorum*, indicating that the PA23 treatment primed the plants to later induce defences (Conrath *et al*. 2002). In this research, the blackleg pathogen was added as a priming agent, by providing a stimulus to activate plant defences. Although there was evidence of disease at the pathogen inoculation site, it is possible that the pathogen used did not adequately stimulate the plant to induce defences. The addition of insect feeding led to increases in peroxidase and phenolic compounds in the bacterial treatments, but no changes in the control treatments, indicating that the PA23-treated plants may have been primed but required the addition of insect feeding to activate defences.

Numerous studies have found that jasmonic acid treatments can negatively affect insect herbivores and may be important in insect control (Avdiushko *et al.* 1997, Thaler *et al.* 2001, Black *et al.* 2003), but there are relatively few studies that have demonstrated potential to control insect pests through the use of bacterial biocontrol agents. The only significant work in this area has been done by Zehnder *et al*. (1997a), who found that PGPR used to activate induced systemic resistance against diseases in cucumber could also be used to control insect herbivores. Cucumber plants treated with PGPR are fed upon significantly less by cucumber beetles and the plants have significantly reduced levels of cucurbitacin, feeding stimulants for these insects (Zehnder *et al.* 1997a). This work demonstrates that PGPR treatments that alter plant chemistry, such as by reducing feeding stimuli, have the potential to increase resistance to insect herbivores. Although my research did not yield any consistent effects of the bacterial treatment on insect herbivores, there was evidence that the bacterial treatments primed the plants to induce

defences following insect feeding, and this warrants further research into the effects of PGPR on canola and other crop species.

Although these elicitors may be beneficial for insect and pathogen control, there are also several potential problems associated with their use, including unknown effects on humans, other animals and microbial communities (Cook *et al.* 1996). There is always a concern over the relative safety to humans of new products introduced for use in agricultural production. PGPR and elicitors that mimic plant hormones such as jasmonic acid are generally considered to be safe for use in agriculture, as these elicitors are not directly toxic, but act on the plant through induced systemic resistance (Stout *et al.* 2002, Black *et al.* 2003). Concerns have nevertheless been raised about the potential negative effects on humans of compounds involved in defence against insects and pathogens (Lyon *et al.* 1995). Induced compounds such as phytoalexins can function as antioxidants (Sakihama *et al.* 2002) or may be involved in cancer prevention (Birt *et al.* 2001), but the effects of some induced compounds on animal health are complicated and may be variable. For example, glucosinolates found in canola and other Brassicaceae that can adversely affect thyroid functions in livestock (Heaney and Fenwick 1995), but there is also evidence that they may provide nutritional benefits in humans (Mithen *et al.* 2000). There is an obvious need to ensure that products are well tested in conjunction with specific crop species prior to agricultural use.

The application of PGPR to agricultural areas introduces bacteria over large areas in high concentrations, and has the potential to affect microbial populations in the soil; however, it is difficult to predict the effects of introduction (Castro-Sowinski *et al.* 2007). Research has demonstrated that inoculation with PGPR can have negative effects (Walsh

et al. 2003) or no effects (Herschkovitz *et al.* 2005) on naturally occurring rhizobacteria communities. To prevent negative impacts on the native microflora, management strategies such as those suggested by Cook *et al.* (1996) are advisable. The authors outline several pertinent safety issues to be considered in terms of target and non-target effects of microorganisms used for biological control. These issues include the potential for displacement of, and pathogenic or toxic effects on, non-target microorganisms, as well as pathogenic, allergenic or toxic effects on humans and other animal. Management practices that require a high degree of knowledge about the biocontrol agents prior to use and strategies for monitoring the environment before and after application are recommended.

Induced plant defences may be useful in pest management, but costs incurred by plants must be considered (Cipollini *et al.* 2003, Vallad and Goodman 2004). Induced plant defences are believed to have evolved as a way to reduce the fitness costs associated with defence since they are only activated as needed (Coley *et al.* 1985, Simms and Fritz 1990); however, it is difficult to measure the differences between costs associated with induced defences and benefits received through increased protection from herbivores and disease. Numerous studies have attempted to compare the benefits received by the plant with the costs of induced defences, but as suggested by Mole (1994) it is very difficult to quantify physiological costs and benefits without some "common physiological currency". For example, seed production in *Nicotiana attenuata* (Torr.) is reduced by about 50% when treated with methyl jasmonate, but these plants are better defended against herbivore attack, and in the presence of herbivory plants mature about 11% more viable seeds than control plants (Baldwin 1998). Under these circumstances

the induced responses confer a fitness advantage, but it is difficult to predict the effects of induced responses in the field. Induced defences may be most beneficial to plants when they are variable, because variability may reduce herbivore performance by preventing herbivores from learning which plants are less nutritious (Karban *et al.* 1997). Unfortunately, variability is not a desirable trait to growers and it is important consider whether uniformity of agronomic and yield characters can be maintained while herbivore defences vary.

In addition to these potential costs, induced plant defences can be further complicated by the potential for cross-talk – interactions between signalling pathways involved in pathogen and herbivore defence (Bostock 2005). These interactions are highly variable even within the same plant species depending on experimental conditions, so information obtained about one plant species may not provide useful information about other plant species (Bostock *et al.* 2001). In canola, both pathogen infection (Yang *et al.* 2007) and insect feeding (Sarosh and Meijer 2007) can activate genes involved in both the jasmonic acid and salicylic acid defences. This indicates that there is a significant amount of crosstalk occurring, but the effects of cross-talk on plant defences against different pests have not been studied, and more research is required in this area.

In field and laboratory studies done for my research, there was no evidence of any costs to the plant through the application of treatments, with the exception of occasional yellow-brown spots produced on some leaves of jasmonic acid treated plants in the laboratory. This browning is attributed to the role played by jasmonic acid in leaf senescence (Creelman and Mullet 1997) and can occur due to high concentrations of jasmonic acid (Thaler *et al.* 1996) that may have resulted from treatments pooling and

drying on one area of the leaf. Although laboratory studies were too short to see any long-term effects of treatment, in the field, seed yield and quality were compared and there were nearly significant effects of treatment on yield and glucosinolates, with the lowest yield and greatest glucosinolate levels in the jasmonic acid-treated plots. These results were still not significant when a contrast was performed between jasmonic acid and control.

Jasmonic acid-induced defences have been found to have fitness costs in other Brassicaceae. Wild radish (*Raphanus raphanistrum* L.) treated with jasmonic acid are more resistant to the generalist caterpillar *Spodoptera exigua* (Hübner) (Agrawal 1999), but in the absence of herbivory there is a reduction in the number of pollen grains produced and a delay in time to first flower (Agrawal ***et al.*** 1999). Increases in glucosinolate levels were used by Agrawal *et al*. (1999) to indicate that jasmonic acid treatments had induced responses in the plants. In my research it is possible that the lower seed yield in the field samples may be related to the higher levels of glucosinolates, and it would be of interest to examine other measures of fitness to gain a better understanding of the effects of jasmonic acid in the field.

Despite potential problems, elicitors of plant defences are desirable for use in pest management as they can be effective against numerous pests (van Loon ***et al.*** 1998, Thaler ***et al.*** 2001), are generally considered to have less of a negative impact on the environment than pesticides (Lyon and Newton 1997, Stout ***et al.*** 2002), and the use of elicitors is considered less likely to lead to the development of pest resistance because they can activate a wide range of plant defences and are not likely to exert high enough

selection pressures on pests to lead to the development of resistance (Van der Plank 1968, Lyon and Newton 1997).

Current agricultural practices depend heavily on chemical pesticides to control insects and diseases, and there is a desire to move away from these practices. Integrated Pest Management that incorporates numerous strategies in the control of all types of pests is needed to provide alternative control measures and reduce the dependence on pesticides. The effectiveness of many practices that are currently used to control insects and pathogens may be compromised through increased agricultural production. Over the next few years canola production is predicted to increase from approximately 9 million tonnes annually to 15 million (Canola Council of Canada 2006), and this could lead to significant increases in pest related problems.

Breeding practices are one extremely important method of producing disease resistant crops, but in some crop species the duration of effective resistance is declining as agricultural production increases (Tilman *et al.* 2002). Pest control by natural enemies is an important aspect of controlling insect outbreaks, but the reduction in landscape complexity through increased agricultural production can lead to a loss of natural insect pest control through reduced levels of parasitism (Thies and Tscharntke 1999). The increased risk of insect herbivore and disease outbreaks through increased production indicates that the development of alternative control measures is desirable.

Plant-growth promoting rhizobacteria are potentially important biocontrol agents because they can protect plants from pathogens through the production of various bacterial metabolites and the activation of induced systemic resistance (van Loon *et al.* 1998). Although the effects of these bacterial species on naturally occurring bacterial

communities can be difficult to predict (Castro-Sowinski *et al.* 2007), research has shown that negative effects can be comparable to the effects of chemical fungicides (Walsh *et al.* 2003). Although my research did not find any significant effects of the PGPR on the insect herbivores investigated, I also did not find any significant effects on beneficial insect species. This provides additional safety information for the use of PGPR and may be important if products are registered for use in agriculture.

Through the research presented in this thesis and work done by others, jasmonic acid treatments have been shown to control numerous insect herbivores in a number of plant species. Research on transgenic plants (Berger 2002, Devoto and Turner 2005), and the analysis of genes activated by different elicitors (Sarosh and Meijer 2007) has given us a greater understanding of induced plant defences, and in the future there may be potential to exploit these defences in an agricultural setting (Hedden and Phillips 2000). Although these treatments may not provide complete control of any insect pest or pathogen, this research makes an important contribution to the development of alternative approaches to sustainable agriculture by enhancing our understanding of the interaction between elicitors and plants.

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