Plant-mediated interactions between the potato cyst nematode, *Globodera pallida* and the peach potato aphid, *Myzus persicae*

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Abstract

This study elucidated the relationship between the potato cyst nematode, *Globodera pallida* and the peach-potato aphid, *Myzus persicae*, two pests that can infect the potato plant and often so simultaneously.

The biological, biochemical and molecular responses of potato plants suffering nematode and aphid stress were characterised both singly and in combination. It was established that the reproductive success of aphids was significantly greater on potato plants pre-infected with nematodes compared to non-infected control plants. Endogenous levels of salicylic acid (SA) increased systemically in the leaves of potato plants following nematode and aphid infection singly with a corresponding increase in expression of the SA-mediated marker gene *PR*-5. Measurements of endogenous levels of jasmonic acid (JA) as well as the expression of JA-mediated defence genes increased in plants infected with aphids but were suppressed when plants were co-infected with both nematodes and aphids. This suppression of JA in co-infected plants shows a different and unique response to that found when the plant was infected with either pest in isolation. These results may indicate antagonistic crosstalk between molecular pathways in the plant following infection of the plant with *G.* pallida and *M. persicae*.

M. persicae had a negative amensalism effect on *G. pallida* below-ground whereby pre-infestation of potato plants with *M. persicae* stimulated a significantly lower cumulative hatch of second-stage juveniles (J2s) when cysts of *G. pallida* were incubated in root diffusates from aphid-infested plants. A dose-dependent response was responsible for this with the presence of 50 aphids on the foliar parts of the plant causing a significant reduction in the emergence of J2s from cysts. Sequence of arrival experiments showed that pre-infestation of *M. persicae* significantly reduced the infection rate of *G. pallida*.

This work comprises the first study into dual nematode and aphid attack in the potato crop and the study highlights how multiple stresses elicit a unique molecular response to attack compared to singly stressed plants.

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List of Abbreviations

ABA = Abscisic Acid

AOC = Allene Oxide Cyclase

AOS = Allene Oxide Synthase

Avr-genes = Avirulence genes

CHIB = Basic Chitinase

COI1 = Coronatine Insensitive 1

DPI = Days Post Inoculation

dsRNA = Double-Stranded RNA

EIN1 = Ethylene Insensitive Mutant

ESI = Electrospray Ionisation

ET = Ethylene

ETI = Effector Triggered Immunity

ETS = Effector Triggered Susceptibility

FDR = False Discovery Rate

GA = Gibberellic Acid

HCl = Hydrochloric Acid

HEL = Hevein Like Protein

HR = Hypersensitive response

ICS = Isochorismate Synthase

ISC = Initial Syncytial Cell

J2 = Second-Stage Juvenile

J3 = Third Stage Juvenile

J4 = Fourth Stage Juvenile

JA = Jasmonic Acid

JA-Ile = Jasmoynyl-Isoleucine

JAR1 = Jasmonic Acid Resistant 1

JAZ = Jasmonate Zim Domain

LC-MS = Liquid Chromatography coupled with Mass Spectrometry

LC-HRMS = Liquid Chromatography coupled with High Resolution Mass Spectrometry

LOX = Lipoxygenase

MAMPs = Microbial Associated Molecular Patterns

MeJA = Methyl Jasmonic Acid

MeSA = Methyl Salicylate

MS = Mass Spectrometry

MTI = Microbial Triggered Immunity

NaOCl = Sodium Hypochlorite

NB-LRR = Nucleotide Binding Leucine Rich Repeat

NMR = Nuclear Magnetic Resonance

NPR1 = Non Expressor of PR-1

OPDA = 12-oxo-phytodienoic acid

PAL = Phenylalanine Ammonia Lyase

PAMPs = Pathogen Associated Molecular Patterns

PCA = Principal Components Analysis

PCN = Potato Cyst Nematode

PDF1.2 = Plant Defensin

PI = Proteinase Inhibitor

PLRV = Potato Leaf Roll Virus

PPN = Plant Parasitic Nematodes

PR = Pathogenesis-Related

PRD = Potato Root Diffusate

PRRs = Pattern Recognition Receptors

QC = Quality Control

R-genes = Resistant genes

RNAi = RNA Interference

RP-HPLC = Reverse Phase-High Pressure Liquid Chromatography

RKN = Root Knot Nematodes

ROS = Reactive Oxygen Species

SA = Salicylic Acid

SAR = Systemic Acquired Resistance

SAG = Salicylic Acid *O*-*β*-glucoside

SAGT = Salicylic Acid Glucosyltransferase

SCF^{COI1} = Skp/Cullin/F-box Complex

TCA = Tricarboxylic Acid

TMV = Turnip Mosaic Virus

TRD = Tomato Root Diffusate

UPLC = Ultra Performance Liquid Chromatography

UV = Ultra Violet

VSP2 = Vegetative Storage Protein

VOC = Volatile Organic Compound

List of Suppliers

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Chapter 1
Introduction

1.1. Biotic stress

As plants are sessile they are a constant target for a range of different pests and pathogens including fungi, bacteria, nematodes and insects. All of these pathogens utilise the photosynthate produced by plants for their own benefit, therefore the study of the biotic stress responses is vital in order to control the loss of economically important crops (Hammond-Kosack and Jones, 2000). Plants however have evolved to develop an array of sophisticated mechanisms to defend themselves to deal with such attacks (Dangl and Jones, 2001). These include a range of preformed, constitutive defences as well as induced defences that are deployed by the challenged plant (Walling, 2009). The first line of defence a plant possesses is an array of barriers such as rigid cell walls, trichomes and thick waxy cuticles. However, the pathogens that overcome this pre-invasive layer of defence activate two over-lapping yet different forms of post-invasive defence that enables the plant to recognise its attacker and turn this perception into an effective immune response (Jones and Dangl, 2006). Plants can elicit both non-specific basal defence responses, as well as specific responses that recognise specific types of pathogen (Figure 1.1). Both of these responses activate downstream defence responses but one key difference between the two is the timely recognition of the invading pathogen by specific defence responses and the effective activation of the resulting defence mechanisms (Yang *et al.*, 1997).

1.1.1 The basal defence response

The non-specific basal defence response of a plant is triggered by the recognition of general elicitors termed microbial (or pathogen)-associated molecular patterns (MAMPs/PAMPs), for example flagellin (Hammond-Kosack and Kanyuka, 2007). The term MAMPs replaced the term PAMPs as this classification did not distinguish

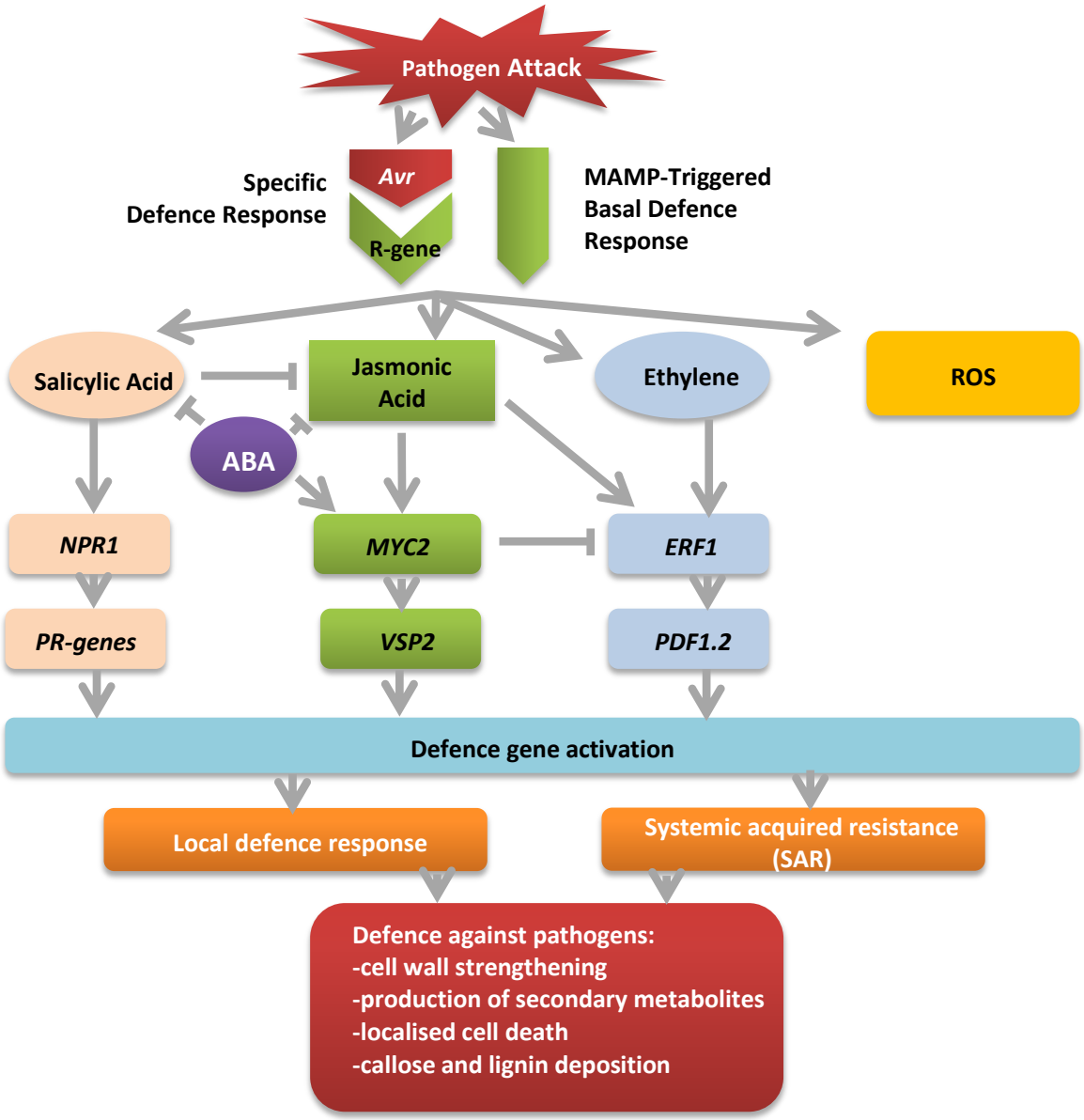

Figure 1.1 Plant defence response upon pathogen attack (Atkinson, 2011).

The basal defence response of a plant is triggered by the recognition of microbial associated molecular patterns (MAMPs). The specific defence response is mediated by resistance (*R*)-genes and the response is triggered by specific recognition of the attacker by these plant *R* genes. Both these pathways lead to the elicitation of phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). These hormones lead to the activation of transcription factors, which then leads to the expression of defence genes locally. Defence compounds can also accumulate systemically. This is termed systemic acquired resistance (SAR). Defence against pathogens include cell wall strengthening, production of secondary metabolites, localised cell death and callose and lignin deposition.

between mutualistic and parasitic symbioses (Staal and Dixelius, 2007). The plant recognises it is being attacked when a class of plasma-membrane bound extracellular receptors called pattern recognition receptors (PRRs) (Beck *et al.*, 2012), recognise microbial elicitors or MAMPs and it is these PRRs that result in an active defence response (Hammond-Kosack and Jones, 1996), and which ultimately will stop the spread of the pathogen throughout the plant. The heterodimerisation of MAMPs and PRRs is the earliest event in activating the microbial triggered immunity (MTI) signalling downstream (Muthamilarasan and Prasad, 2013). Downstream of this early signalling response plants activate an array of molecular and biochemical defence responses to halt pathogen attack including oxidative bursts.

Oxidative bursts can be defined as a rapid and transient production of reactive oxygen species (ROS) following an attempted invasion from a pathogen. ROS are by-products of a cell, which are formed as a result of successive one-electron reduction of molecular oxygen (O_2). During normal metabolic processes plant cells produce a range of ROS: The superoxide radical ($\cdot O_2$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\cdot OH$) and nitric oxide (NO) and these are maintained at their lowest levels by relevant protective mechanisms (Daudi *et al.*, 2012). However, during times of stress or when levels get too high protective mechanisms are overridden and an oxidative burst occurs (Bolwell and Wojtaszek, 1997). ROS have been reported to be involved in many defence mechanisms including antimicrobial roles (Peng and Kuc, 1992), the hypersensitive response (HR) (Lamb and Dixon, 1997), cell wall protein cross-linking (Brown *et al.*, 1998), phytoalexin production (Apostol *et al.*, 1989, Daudi *et al.*, 2012), induction of defence gene expression, for example gluthathione S-transferase, a family of enzymes protective against oxidative stress, and phenylalanine ammonia-lyase (PAL) involved in the phenylpropanoid biosynthetic pathway (Desikan *et al.*, 2000) and systemic acquired resistance (SAR) (Alvarez *et al.*, 1998, Lamb and Dixon, 1997). ROS are chemically reactive molecules and so can react with proteins, DNA and membrane lipids to reduce photosynthesis, increase electrolyte leakage, and accelerate senescence and cell death (Sharma and Davis, 1997).

1.1.2. The specific defence response

During the co-evolutionary arms race between pathogens and their host plants, pathogens acquired effector molecules that can interfere with MTI, suppress its effects (effector triggered susceptibility; ETS) and thus promote pathogen virulence (Jones and Dangl, 2006). In turn, plants acquired resistance (R) proteins that recognise these effectors and trigger a secondary immune response termed effector-triggered immunity (ETI). The specific defence response is mediated by resistance *R*-genes and the response is triggered by specific recognition of the pathogen by these plant *R* genes (Flor, 1971). Upon recognition of the invading pathogen, plant defences, which overlap with the plant's basal defence response, are initiated to localise the invasion (Figure 1.1) (Kaloshian, 2004). The specific recognition of an avirulence (*avr*) gene product by an *R* gene product results in a signal transduction cascade that activates plant defences (Keen, 1990). Plant resistance will only occur when a plant possesses a dominant *R* gene and the pathogen possesses the complementary *avr* gene, thus an incompatible interaction in a plant is determined by complimentary pairs of dominant genes. If there is a change in either the *R* gene or the *avr* gene or if either of the genes are absent then a compatible interaction ensues and disease occurs (Hammond-Kosack and Kanyuka, 2007). The majority of *R* genes belong to the intracellular nucleotide-binding leucine-rich repeat (NB-LRR) family (Jones and Dangl, 2006) with a second major class of R proteins belonging to the extracellular LRR (eLRR) proteins group (Nishimura *et al.*, 2010). Effectors that are released into the plant are recognised by one of two modes: there can be a direct interaction between the effector and the NB-LRR receptor or there can be an indirect interaction mediated by accessory-proteins that the immune receptor associates with effector-triggered modifications (Dodds and Rathjen, 2010). The specific innate defence response is an addition onto the basal defence response and a function of *R*-mediated signalling is to more rapidly and effectively activate downstream mechanisms than the basal response (Yang *et al.*, 1997).

1.1.3. Phytohormones involved in pathogen signalling

Plants produce a range of phytohormones including auxins, gibberellins (GA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and cytokinins (CK) (Bari and Jones, 2009) and each of these play a role in the growth, development and reproduction of the plant as well as a major role in defence signalling upon pathogen attack (Verhage *et al.*, 2010). It has been well established that SA, JA and ET are the primary signals involved in plant defence (Pieterse *et al.*, 2009). SA is a more effective defence mechanism against pathogens that require a living host (biotrophs) whereas pathogens that obtain their nutrients from dead or dying tissues (necrotrophs) are more sensitive to the JA/ET defence pathway (Glazebrook, 2005). The SA, JA and ET pathways interact with each other.

The SA and JA signalling pathways are mutually antagonistic and the expression of SA-mediated genes may result in the repression of JA-mediated genes and *vice versa* (Glazebrook, 2005). However, some synergistic interactions between these pathways have been reported (Beckers and Spoel, 2006). The induction of some genes may require both JA and ET; however other genes may require just one of these signals. In addition, there have been reports of a negative interaction between the ET and JA pathways (Glazebrook, 2005). These interactions have been discovered through the analysis of a variety of hormone signalling mutants (Jalali *et al.*, 2006).

Salicylic acid, 2-hydroxy benzoic acid, is a phenolic compound and has been well established as a key player in plant defence signalling. There are two pathways from which SA can be synthesised, the isochorismate pathway (Wildermuth *et al.*, 2001) or the phenylpropanoid pathway (Ribnicky *et al.*, 1998) both of which require the primary metabolite chorismate (Vlot *et al.,* 2009). The bulk of pathogen-induced SA in *Arabidopsis, Nicotiana benthamiana* and tomato involves the conversion of chorismate through the isochorismate pathway and isochorismate synthase and isochorismate pyruvate lyase. *Arabidopsis* encodes two ICS enzymes and mutants lacking functional ICS1, which accounts for approximately 90% of SA production induced by pathogens, are severely compromised in SA production as well as pathogen resistance. However, in an *ics1/ics2* double mutant, residual SA was

detected suggesting that the ICS pathway is not the only source of SA (Garcion *et al.*, 2008). The phenylpropanoid pathway converts chorismate derived L-phenylalanine into SA via benzoate intermediates or coumaric acid through a series of enzymatic reactions that are initially catalysed by phenylalanine ammonia lyase (PAL) (Vlot *et al.*, 2009). The majority of SA produced by a plant is converted into SA *O-β*-glucoside (SAG) by an SA glucosyltransferase (SAGT) enzyme, which is inducible by pathogens (Dean and Mills, 2004). SA is synthesised in the chloroplasts of *Arabidopsis* (Garcion *et al.*, 2008) whereas in tobacco SAGT localises in the cytosol (Dean *et al.*, 2005). SAG is moved from the cytosol to the vacuole where it is stored in an inactive form, which can be converted back to SA when required by the plant (Dean and Mills, 2004).

Upon pathogen attack, SA accumulates locally and activates a range of downstream defence responses. Immediately following pathogen invasion and recognition by plant R-genes, the expression of the signalling molecules *SID1, EDS5* and *PAD4* is induced and production of SA occurs. SA then leads to the expression of the transcriptional activator *NPR1* encoded by the *NON EXPRESSOR OF PR1* gene, which in turn activates downstream defence genes such as pathogenesis-related (*PR*) genes (Kunkel and Brooks, 2002). Following pathogen attack, plants can also prime themselves for further attack by accumulating SA in distal uninfected tissues. This is termed systemic acquired resistance (SAR) and *PR*-genes in these tissues are widely used as molecular markers for the activation of SAR (Cao *et al.*, 2011, Van Loon *et al.*, 2006). In addition, salicylic acid is required for the hypersensitive response, the rapid and localised plant cell death induced during resistance to certain plant pathogens (Alvarez, 2000). When transgenic *Arabidopsis* and tobacco plants expressing the bacterial *NahG* gene, which encodes the SA-metabolising enzyme salicylate hydroxylase, were challenged with virulent pathogens, they were found to be more susceptible to attack. This is postulated to be because these plants were unable to accumulate high levels of SA and therefore failed to mount resistance in uninfected parts of the plant or express *PR*-genes (Gaffney *et al.*, 1993, Delaney *et al.*, 1994). There has been a lot of research on the mobile signal responsible for the activation of SAR. Initially, SA was thought to be as the phloem mobile signal

associated with SAR but grafting experiments using tobacco expressing the bacterial *nahG* gene dismissed its involvement (Vernooij *et al.*, 1994). Methyl salicylate (MeSA) has also been reported to be the mobile signal involved in SAR. Levels of MeSA found in primary leaves and petiole exudates of tobacco infected with tobacco mosaic virus paralleled similar increases in MeSA in systemic leaves. This was further confirmed by grafting experiments using *NtSAM1*-silenced plants and wild-type tobacco plants. *NtSAM1* encodes a MeSA synthesising SA-methyl transferase and the grafting experiments showed that its function in SAR was required in the primary infected leaves but not in the systemic leaves suggesting that MeSA synthesised in primary infected leaves was required for activation of SAR in the systemic tissue (Park *et al.*, 2007). However, in a separate study accumulation of SAR was intact when a SA methyltransferase (*bsmt1*) in tobacco was silenced (Attaran *et al.*, 2009). The mobile signal involved in SAR is now thought to be azelaic acid, a nine-carbon dicarboxylic acid metabolite, which is found naturally in a number of plants and primes systemic defences in *Arabidopsis* (Shah, 2009). Elevated levels of azelaic acid were found in petiole exudates of Arabidopsis when challenged with a SAR-eliciting strain of *Pseudomonas syringae* compared to control leaves. Radiolabelled azelaic acid was found in petiole exudates as well as in the distal leaves, suggesting systemic movement of the metabolite. In addition, exogenous application of azelaic acid induced disease resistance in distal tissues (Jung *et al.*, 2009).

Jasmonic acid (JA) is an oxylipin, which was first associated with defence signalling following the discovery that it was a potent activator for the expression of proteinase inhibitors (PI) in tomato (Farmer and Ryan, 1990). JA signalling plays a large role in promoting plant defence against herbivore attack both directly, by providing resistance against necrotrophs and insect pests and indirectly by producing volatile emissions (Halitschke and Baldwin, 2004, War *et al.*, 2012). JA has been shown to increase locally upon pathogen infection or upon mechanical wounding of the tissue and when exogenously applied induces the expression of defence-related genes (Lorenzo and Solano, 2005, Wasternack, 2007). Studies have shown that JA is up-regulated in response to attack by caterpillars in tobacco (Kahl

et al., 2000), spider mite in tomato (Li *et al.*, 2002) and *Pieris rapae* caterpillars in *Arabidopsis* (Reymond *et al.*, 2004). JA is synthesised when phospholipases are activated to release α-linolenic acid from membrane lipids. Linolenic acid is oxygenated by lipoxygenase (LOX) to form 13(S)-hydroxy linolenic acid (13-HPOT), which is then converted to 12-oxo-phytodienoic acid (OPDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC). JA is then synthesised from OPDA through reduction and three steps of β-oxidation after which it is catabolised further by JA carboxyl methyltransferase (JMT) to form the volatile compound methyl jasmonate (MeJA) (Seo *et al.*, 2001, Dar *et al.*, 2015). The three main signalling components involved in the jasmonic acid signalling pathway include coronatine insensitive 1 (COI1), jasmonate resistant 1 (JAR1) and jasmonate insensitive 1/MYC2 (JIN1/MYC2) (Bari and Jones, 2009) and mutants in these components are defective in biosynthesis or perception of JA signalling (Bari and Jones, 2009). The JA signalling pathway has been mostly unravelled from studies using the *Arabidopsis* COI1 mutant, which encodes an F-box protein (Xie *et al.*, 1998) and is deficient in JA biosynthesis (Mandaokar *et al.*, 2006). From these studies it was found that the Skp/Cullin/F-box complex (SCF^{COI1}), a type of E_3 ubiquitin ligase protein (Deshaies, 1999) controls the perception and signalling of jasmonates (Dar *et al.*, 2015). Following the synthesis of jasmonates from the oxylipin biosynthetic pathway, jasmonates conjugate with isoleucine in the presence of the enzyme JA-conjugate synthase (JAR1) (Staswick and Tiryaki, 2004) to form the active form of jasmonates jasmonyl-isoleucine (JA-Ile) (Dar *et al.*, 2015). The JA-Ile binds to its receptor COI1, it causes degradation of Jasmonate-Zim Domain (JAZ) proteins through the 26S proteasome (Chini *et al.*, 2007, Thines *et al.*, 2007). When JAZ proteins interact with transcriptional activators, jasmonate signalling is repressed, however when cells are stimulated by JA, they cannot interact with JAZ proteins and so JA-responsive genes are activated (Chini *et al.*, 2007, Memelink, 2009).

JAs interact with ethylene responses in plant pathogen interactions as both hormones are synthesised when the plant is wounded or attacked by a pathogen (Creelman *et al.*, 1992, Kuć, 1997). JA and ethylene interact synergistically through

a number of defence-related genes including *PR*-5 and a plant defensin (*PDF*1.2) (Penninckx *et al.*, 1998, Xu *et al.*, 1994). In addition, when both phytohormones are exogenously applied to *A. thaliana* there is a synergistic induction of *PDF1.2*, a hevein-like protein (*HEL*) and a basic chitinase (*CHIB*) (Norman-Setterblad *et al.*, 2000, Penninckx *et al.*, 1998) and also *osmotin* and *PR1-b* in tobacco (Xu *et al.*, 1994). Furthermore, the induction of such defence-related proteins is blocked in the JA-insensitive mutant *coi1* and ethylene-insensitive mutant *ein1* suggesting that there is a requirement for both of these signalling pathways for their induction (Lorenzo *et al.*, 2003). There are few reports to suggest that ethylene and JA act antagonistically. In ethylene-insensitive mutants, the JA-responsive vegetative-storage protein, *VSP*2 was up-regulated suggesting that ethylene represses the induction of *VSP*2 (Rojo *et al.*, 1999). In addition, exogenous application of ethylene has a negative effective on JA-induced genes in *Arabidopsis* (Matsushima *et al.*, 2002). Although, there have been reports of a negative interaction between JA and ethylene, this negative effect on the induction of defences genes still influences the resistance of plants to herbivory (Rojo *et al.*, 2003). In *Arabidopsis*, resistance to the cotton leaf worm, *Spodoptera littoralis* is mediated by JA (Stotz *et al.*, 2002), however it is enhanced in ethylene-insensitive mutants but following the application of etephon, resistance is decreased. Etephon is an ethylene releasing compound and when this is applied exogenously JA-mediated defences are down-regulated (Rojo *et al.*, 2003). The JA/ethylene negative interaction has not only been reported in *Arabidopsis* but also in *Nicotiana attenuata.* Ethylene defences were induced following feeding by *Manduca sexta* and this had an antagonistic effect on wound and JA-induced nicotine production (Winz and Baldwin, 2001). This burst of ethylene may be an indirect defence mechanism against *Manduca sexta*. Tobacco with a lower level of nicotine resulted in a lower nicotine levels in *M. sexta* thus making it more susceptible to parasitoid wasps (Winz and Baldwin, 2001).

1.2. The biology of plant-parasitic nematodes

Plant parasitic nematodes (PPN), are highly evolved pathogens of economically important crops worldwide (Davis *et al.*, 2008) that have developed a wide range of parasitic strategies (Sijmons *et al.*, 1994). PPN are obligate parasites and they

survive by feeding on the cytoplasm of living plant cells (Williamson and Gleason, 2003). Although the majority of the vascular plant can be infected by at least one species of PPN (Jagdale and Grewal, 2006); the species that are responsible for major, global economic loss to crops are those that have the ability to parasitise the root (Fuller *et al.*, 2008). PPN can be categorised into three main groups based on their biology, migratory ectoparasites, migratory endoparasites and sedentary endoparasites (Trudgill, 1991). Migratory ectoparasites never enter the host but migrate through the soil but use roots as a short-term food source as they come into contact with them. Migratory endoparasites enter the host and cause extensive tissue damage as they migrate through the root (Mantelin *et al.*, 2015). Sedentary endoparasites are the most highly evolved and specialised PPN and are the most economically important. They attack the host plant and migrate through the root before forming specialised feeding sites called giant cells or syncytia. The most widely studied nematodes are the root-knot nematodes and cyst nematodes due to their global economic importance (Hewezi *et al.*, 2010).

1.2.1. Root knot nematodes

Root-knot nematodes (RKN), belonging to the genus *Meloidogyne*, are a polyphagous group of obligate plant parasitic nematodes of economic importance due to their ability to infect the majority of higher plants (Moens *et al.*, 2009) and their ability to reproduce by parthenogenesis (Castagnone-Sereno, 2006). The level of damage that RKN cause to a host plant is influenced by a variety of factors including the host (species-specificity), crop rotation, season and soil type (Greco *et al.*, 1992). Female RKNs lay eggs into gelatinous masses, which safeguard them from external conditions and predation. A second-stage infective juvenile (J2) forms within the egg and in response to a range of factors that include temperature, soil moisture and root diffusate, the J2 hatches and invades a suitable host. Following root invasion the J2 begins to migrate through the root in order to form a permanent feeding site made up of giant cells. Giant cells are formed as a result of secretions produced in the RKN esophageal glands that cause cell enlargement in the vasculature cylinder, increased cell division in the pericycle, partial dissolution of cell walls and expansion of nuclei forming a multinucleate feeding site (Moens *et*

al., 2009). Following the formation of the giant cells, the RKN becomes sedentary and swells, and if the conditions are favourable moult into the third-stage juvenile (J3), after approximately two weeks, then to the fourth-stage juvenile (J4) and finally to the adult stage (Greco *et al.*, 1992). Vermiform males are sometimes present; however, there have been no reports to suggest that they feed (Moens *et al.*, 2009). Furthermore, males can be produced in parthenogenetic species if conditions become unfavourable i.e. when populations become too high or conditions are not suitable for female development (Greco *et al.*, 1992).

1.2.2. The biology of cyst nematodes

Cyst nematodes consist of approximately seventeen genera (Sasser and Freckman, 1987), form intricate relationships with their hosts and are one of the most successful biotrophs in global agriculture. The most studied cyst nematodes are those belonging to the genera *Globodera* and *Heterodera* (Lilley *et al.*, 2005). Similar to RKN, when favourable conditions are present or following the detection of root diffusate from a suitable host, infective stage-two juveniles (J2s) enter the plant near the root tip within the area of cell elongation and cell differentiation (Dropkin, 1955). Cyst nematodes use their hollow, protrusible stylets, along with secretions containing cell-wall degrading enzymes to make perforations along the cell wall, enabling them to make a slit into which they can invade the root (Turner *et al.*, 2006). As the nematodes migrate intracellularly through the root, protoplasts breakdown and its exploratory path is lined with damaged cells (Figure 1.2) (Wyss and Zunke, 1986). Once the J2 reaches the vasculature cylinder the nematode selects an initial cell in order to create a permanent feeding site made up of fused cells (Wyss, 2002). The nematode probes an initial cell with its stylet and if the protoplast of the probed cell does not breakdown and the cell is recognised as functional, then the stylet is inserted for a feeding-preparation period (Wyss, 2002). Following the feeding-preparation period the nematode pumps secretions, produced in the oesophageal glands, into the cell causing re-differentiation of the initial cell (Jones, 1981). The initial cell increases in size by incorporating up to two hundred cells by partial dissolution of the cell wall and protoplast fusion leads to a large feeding site called a syncytium. This feeding site acts as a nutrient reservoir

for all stages of sedentary cyst nematodes and a unique feeding tube is formed at the apex of the stylet every time the nematode enters for a feeding cycle (Lilley *et al.*, 2005). Feeding tubes are blind ended structures that are formed at the stylet orifice but within the cytoplasm and from which host cell assimilates are withdrawn through the walls of the tube. Blocking of the stylet would be fatal to the nematode but as a new tube is formed before each feed, blockages can be removed (Eves-van den Akker *et al.*, 2014).

The life cycle of cyst nematodes varies from species to species but largely reflects temperature. As the temperature increases the time it takes for a species to complete its life cycle decreases. For example, *Heterodera oryzicola* completes a life cycle in 23 days at 27°C and *H. glycines* in 21 days at 25°C whereas temperate species such as *H. trifolii* requires 31 days at 20°C but 45 days at 15.5°C (Turner *et al.*, 2013). The pathogen undergoes three moults each lasting approximately 3-4 days before becoming an adult (von Mende *et al.*, 1998). Males and females develop at the same rate and both emerge from the root at the fourth moult. Males are non-feeding, free-living nematodes and only survive in the soil for a short period of time. During this time they are attracted to pheromones produced by the female and fertilisation occurs (Lilley *et al.*, 2005). Following fertilisation, the female remains in the root inactive and swells as embryos develop within the egg as far as the formation of the second stage juvenile (J2), while still contained inside the females body wall (Turner *et al.*, 2013). The female then dies and the body becomes tanned by a polyphenol oxidase and forms a resilient saccate shaped cyst, which can enclose up to 500 hundred eggs, however this is dependent on the species and environmental conditions (Brodie *et al.*, 1993). As the host plant dies the cysts eventually detach from the roots and then remain dormant in the soil with eggs inside remaining viable until the next suitable host grows nearby (Turner and Evans, 1998).

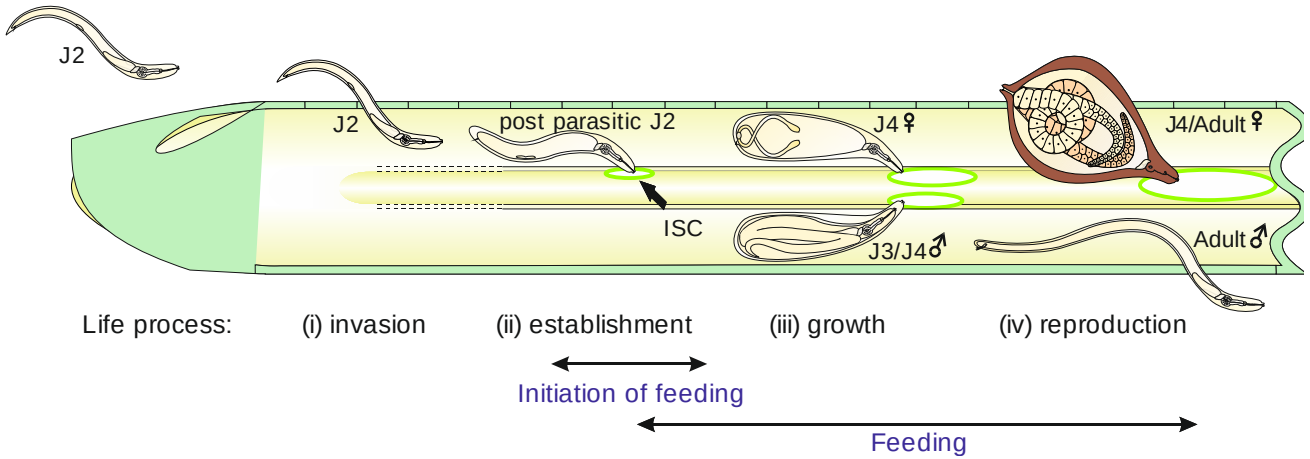

Figure 1.2 Life cycle of a cyst nematode (Lilley, *et al*, 2005).

Second-stage juveniles (J2s) contained in an encapsulating cyst in the soil migrate towards the tip of the root and enter at the zone of elongation, migrate intracellularly and cross the cortical tissue towards the vascular cylinder where using its retractable stylet selects an initial syncytial cell (ISC). Effector proteins from the nematodes oesophageal gland are secreted into the ISC and the formation of a permanent feeding site, the syncytium is initiated. Following syncytium induction the nematode undergoes three more moults to reach adulthood. At the end of J3 stage the male stops feeding, becomes motile and leaves the root in order to fertilise the female. The female remains sedentary and continues to feed from the syncytium and undergoes a final moult resulting in the formation of a spherical, lemon-shaped adult nematode. Following successful fertilisation the female lays eggs inside the cyst and dies. The body wall of the dead female tans and hardening enclosing the eggs inside.

1.2.3. Effects of cyst nematodes on their hosts

Cyst nematodes can affect host plants in a number of ways: (a) direct parasitic strategies such as extracting nutrients from the plant; (b) physiological damage that can impact on the development and function of roots; and (c) systemic effects that can alter the growth, development or physiology of foliar parts of the host plant (Trudgill, 1991).

Root damage can be caused directly by the invasion of the cyst nematode and this can reduce root extension rates (Evans and Stone, 1977), in addition to decreasing the rates at which aerial tissues can take up macro-nutrients (Trudgill, 1980). However, quantification of the damage that cyst nematodes cause is hard to estimate due to the lack of clear symptoms that often leads farmers to overlook infestation until extensive damage is done, thus causing significant losses to valuable crops (Gurr *et al.*, 1991). The main indicator of cyst nematode infection is the visible presence of the female nematode, attached to the root following weeks of parasitism (Lilley *et al.*, 2005). Reduced growth and development is also an indication of cyst nematode infestation with infested plants weighing less than plants without nematodes. The most damaging cyst nematodes include the soybean cyst nematodes (*Heterodera glycines*), potato cyst nematodes (*Globodera pallida* and *G. rostochiensis*) and cereal cyst nematodes (*Heterodera avenae* and *H. filipjevi*) (Mantelin *et al.*, 2015). In the USA, soybean cyst nematodes have been estimated to cause a loss of $US1.5 billion annually (Chen *et al.*, 2001), while cereal cyst nematodes can cause an estimated loss of 90% in some environments, however this is dependent on environmental conditions (Nicol *et al.*, 2011). *G. pallida* cause approximately £50 million of yield losses each year in the UK and cause an estimated £900 million globally to the potato industry (Haydock and Evans, 1998). Furthermore, these cyst nematodes are of particular concern in the UK due to their presence in approximately 64% of fields in England and Wales (Minnis *et al.*, 2002). The infestation of cysts nematodes and their effects on economically important crops are increased due to the fact these particular pathogens can survive in the soil for several years without needing the presence of a host (Turner *et al.*, 2006).

1.3. The biology and behaviour of aphids

Aphids are phytophagous, phloem-feeding insects belonging to the superfamily Aphidoidea, within the order Hemiptera. Aphids attack all parts of their host plant, both above and below the ground and they have an adverse effect on their hosts in part due to their ability to reproduce extremely quickly (Goggin, 2007). Unlike many other sap feeders, aphids can reproduce by parthenogenesis and bear live young called first instar nymphs (Figure 1.3) (Powell *et al.*, 2006). In addition, an aphid's generation time is extremely short, with a nymph of some species such as the cotton aphid, *Aphis gossypii*, reaching maturity in as little as five days (Dixon, 1987). Furthermore, aphids of the same clone can produce wingless (apterous) adults that reproduce, or winged (alate) aphids, which depending on the poor quality of food or high numbers of aphids can colonise new host plants (Powell *et al.*, 2006) (Figure 1.3). Aphids invade plants in order to extract their phloem sap that is high in sugar, by inserting long stylets into the sieve elements, and use this as their food source (Douglas, 2006). Sieve elements are elongated cells that are aligned end to end in order to form a conduit that will enable the phloem sap to flow continuously (Will *et al.*, 2007). After locating a suitable host, an aphid inserts its stylet, which perforates epidermal, mesophyll, and parenchyma cells (Goggin, 2007). Aphids produce two different types of saliva (Miles, 1999), which aid successful feeding. The first is dense and proteinaceous, and gels around the stylet while creating an intercellular pathway towards the phloem for feeding. In addition, this saliva isolates the plant tissues from contact with the stylet, which prevents a defence response at the site of feeding (Felton and Eichenseer, 1999). The second type of saliva is injected directly into the vascular system of the host and contains digestive enzymes. This mode of feeding enables the aphid to avoid eliciting a plant defence due to the lack of mechanical damage (Guerrieri and Digilio, 2008). Aphids are also capable of blocking sieve tube occlusion, a defence mechanism against puncturing, through injecting salivary proteins that bind to calcium in the host tissue (Will *et al.*, 2007). The primary purpose of phloem sap is the transportation of nutrients around the plant (Fisher and Cash-Clark, 2000) and by extracting phloem and essential nutrients from the sieve tubes the aphid weakens the defences of the host plant

(Will *et al.*, 2007). However, as phloem sap is high in carbohydrates (mainly sucrose) and is too low in free amino acids for animal requirements (Douglas, 2006), aphids synthesise amino acids and lipids from dietary sugars (Febvay *et al.*, 1999). This feeding pattern of aphids is made possible and enhanced by a bacterial endosymbiont e.g. *Buchnera aphidicola*, which is passed on from the parent aphid to the offspring. *B. aphidicola* is an obligate symbiont that has developed a mutualistic relationship with its host (Baumann, 2005). The majority of endosymbionts provide riboflavin and amino acids to the aphid that they cannot retrieve from their diet of phloem sap (Goggin, 2007). In the pea aphid, *Acyrthosiphon pisum, B. aphidicola* provides approximately 90% of the aphid's amino acids (Goggin, 2007), which are essential for the growth and development of the aphid.

1.3.1. Effects on aphid infestation on their hosts

Symptoms of aphid infestation can vary broadly depending on the species and the host plant. Symptoms caused by aphid attack can include chlorosis, necrosis, wilting and stunting of growth and development (Goggin, 2007). Changes in the host physiology caused by aphids are typically beneficial for the aphid and often damaging to the plant. The benefits to the aphid from plant damage such as chlorosis and necrosis are less clear however as damage caused to foliar parts of the host plant by the Russian wheat aphid (*Diuraphis noxia*) activated a breakdown of proteins in infected tissues, thereby increasing the nutritional quality of phloem sap (Sandström *et al.*, 2000). Furthermore, some species of aphids can cause acute physiological damage to the host plant, some of which are not as obvious as external damage (Girousse *et al.*, 2005). Aphids have been reported to alter resource allocation within the host plant. Girousse *et al.* (2005), demonstrated that nitrogen was re-allocated from the apical sites of alfalfa, to the feeding sites of the pea aphid, *Acyrthosiphon pisum*. Invertase activity was also enhanced in the galled tissues of the host plant by the gall aphid, *Hormaphis hamamelidis* and it was reported that sugars were transferred to the gall (Rehill and Schultz, 2003).

Host plants are also vulnerable to aphids as they cause an increased incidence of viral infection because they act as a vector for 50% of insect-transmissible viruses

(Brunt *et al.*, 1996) that can severely damage plants. Aphids facilitate the delivery of virions into plant cells due to their feeding strategy (section 1.5). In addition, due to the aphids ability to reproduce asexually, colonies can build up quickly in order for viruses to be transferred to new hosts quickly (Ng and Perry, 2004). *Myzus persicae*, the peach-potato aphid, is the most important virus vector in the United Kingdom (Harrington and Xia-Nian, 1984), as it is polyphagous and a vector for more than one hundred and twenty plant viruses including the harmful potato leaf-roll virus (PLRV) (Brault *et al.*, 2010). PLRV is a single-stranded RNA virus that belongs to the luteovirus group and replicates almost exclusively in the phloem tissue (van den Heuvel *et al.*, 1994). PLRV causes considerable losses to the economically important potato crop by causing speckling to occur on tubers and ultimately resulting in necrosis even after harvest (Roosen *et al.*, 1997).

1.4. Plant-mediated interactions between above-ground pests and below-ground pathogens

Organisms from both above and below the ground can impact on each other, either directly (e.g. attraction of above-ground predators to soil insects) or indirectly through their shard host plants (e.g. through changes in the nutritional quality of the host plant) (Bezemer *et al.*, 2005). Roots, in the past, were not considered to impact above-ground plant-herbivore activities; however, in recent years it has become apparent that roots in fact play a more direct role in above-ground plant defences (Van der Putten *et al.*, 2001). As leaves and roots are separated by space, the contribution roots make towards leaf defence and the resulting actions of above-ground herbivory are largely misunderstood (Kaplan *et al.*, 2008a, Johnson *et al.*, 2013, Johnson *et al.*, 2016). Because roots are below-ground and consequently protected from the majority of above-ground pathogens, they provide the perfect location for secondary plant compounds used in above-ground defences (Kaplan *et al.*, 2008a). Recently, reports have emerged of plants mediating interactions between above-ground pests and below-ground pathogens by the means of systemically-induced defences (Soler *et al.*, 2007, Erb *et al.*, 2008). A hypothesis has been formed that when secondary metabolites are produced below-ground, root feeders may impede the synthesis of

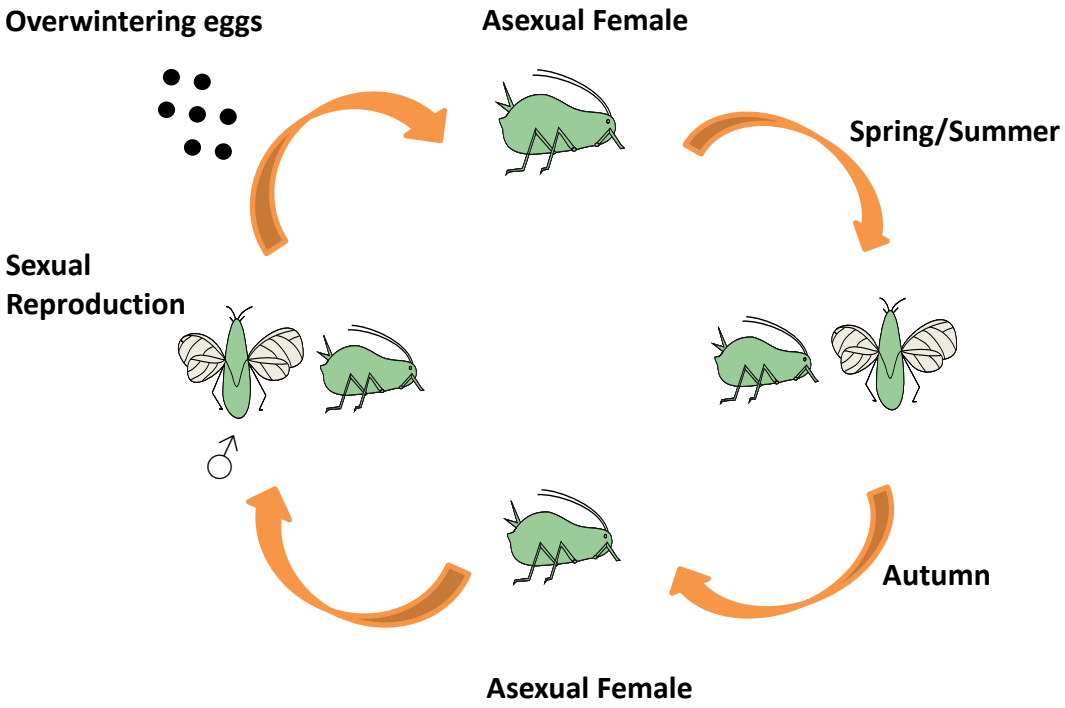

Figure 1.3 Life cycle of the peach-potato aphid, *Myzus persicae* (Shingleton, *et al*, 2003). Asexual apterous (wingless) females hatch from overwintering eggs in spring. As the temperature increases, colonies grow and nutrients deplete in the plant, asexual females gain the ability to produce winged (alate) aphids and to fly to a summer crop, for example the potato plant. As the temperature cools in Autumn females then gain the ability to produce males and sexual reproduction occurs. Overwintering eggs are then laid in the bark of trees, for example, the peach tree.

these compounds, and therefore decrease above-ground defences and benefit above-ground herbivory (Kaplan *et al.*, 2008b). Similarly, if a pathogen induces a defence response in above-ground tissues, plants may distribute essential nutrients below-ground. This re-allocation could therefore be beneficial to below-ground herbivory (Kaplan *et al.*, 2008b). It has been demonstrated that there can be increases in above-ground defences (in the absence of above-ground herbivory), following root attack, damage or application of exogenous chemicals (Erb *et al.*, 2008). Plant mediated interactions and defence compounds induced as a result of attack can also have a negative or positive impact on the plant ecosystem. For example, following pathogen attack, if a plant elicits a defence response such as volatile organic compounds (VOCs) or root exudates, neighbouring plants could be defended by inducing their defences or conversely compromised by attracting above-ground pests to a host plant that hasn't previously been attacked (Bezemer *et al.*, 2005).

Plants have a vast range of chemical compounds, called secondary metabolites that are unique and different from the primary metabolites that play roles in particular colours, odours and tastes of different species (Bennett and Wallsgrove, 1994). In the past, secondary metabolites were viewed as a by-product of primary metabolism with limited roles in plant growth and development (Fraenkel, 1959, Bennett and Wallsgrove, 1994). However, it is now known that these chemicals can be involved in protection against UV, osmotic and other environmental stresses and attraction of pollinating insects, in addition to playing a major role in host defence against pests and pathogens. Plant secondary metabolites include glucosinolates, non-protein amino acids, alkaloids and plant phenolics, all of which have functions for plant defence. Glucosinolates have defence properties against fungal infections and herbivory attack. Experiments were carried out on twenty-seven species of plants and the fecundity of aphids feeding on them was found to be negatively affected by glucosinolates content (Malik *et al.*, 1983). Non-protein amino acids have also been implicated in resistance to aphids. Holt and Birch (1984), demonstrated resistance to three species of aphid in *Vicia* species and varieties when non-protein amino acid contents were high in host tissues. Plant secondary

metabolites have been shown to be involved in resource allocation in order to prevent pathogen attack. Cotton, for example, has been shown to synthesise terpenoid aldehydes in roots (Smith, 1961) however, when these compounds are transported to the foliar parts of the plant, above-ground herbivory are adversely affected (Agrawal *et al.*, 2000). Similar experiments on roots have also shown an increase in phenolics in *Brassica nigra* (Van Dam *et al.*, 2005), phytoectosteroids in spinach (*Spinacia oleracea*) (Schmelz *et al.*, 1998), and proteinase inhibitors in *Nicotiana attenuata* (Van Dam *et al.*, 2001). Similarly, in reciprocal experiments, an increase in defence in roots following attack of aerial parts of the plant show an increase in certain compounds. Following treatment of aerial tissue with exogenous JA, an increase in nicotine and proteinase inhibitors in *N. attentua* was recorded (Baldwin *et al.*, 1994). In addition, an increase of glucosinolates in the roots of both *Brassica campestris* and *B. nigra* was observed when exposed to foliar herbivory (Soler *et al.*, 2007). An increase in the sink strength of roots has also been reported as a result of plant secondary metabolites being re-allocated below-ground following attack of the foliar parts of the plant (Fisher and Cash-Clark, 2000).

1.4.1. Plant-mediated interactions between aphids and nematodes

In recent years, a number of studies have been published on plant-mediated interactions between plant parasitic nematodes and aphids (Wurst and van der Putten, 2007, Hol *et al.*, 2010, Kabouw *et al.*, 2011). Although cyst nematodes and aphids may share the same host, their infection of the plant is temporally as well as spatially separated: nematodes infect plants soon after roots emerge, while aphids colonise plants later in the year, once there is sufficient biomass above ground (Van Emden *et al.*, 1969). This temporal separation may give rise to asymmetric interactions, whereby nematodes influence the performance of aphids, but aphids do not impact on nematodes. There is some evidence to support this in that there are more studies demonstrating that nematodes have an effect on the performance and fecundity of aphids than *vice versa* (Kutyniok and Müller, 2012). The mechanism underpinning this asymmetric interaction may be due to changes in primary and secondary metabolites. For example, a mixed nematode infection of *Pratylenchus, Meloidogyne* and *Heterodera* spp. has been reported to reduce the

fecundity of *Schizaphis rufula* without significantly affecting plant biomass (Vandegehuchte *et al.*, 2010). Similarly, an increase in phenolic content in foliar parts of plants has been reported following infection with plant parasitic nematodes (Kaplan *et al.*, 2008b, Van Dam *et al.*, 2005), which had a negative effect on the survival rate of above-ground herbivores. In a study of interactions between the soybean aphid and the soybean cyst nematode, alate aphids preferred plants without nematodes over nematode-infested plants, though the performance and population growth of aphids feeding on nematode-infested plants was either unaffected or even slightly improved (Hong *et al.*, 2010). Systemic changes to primary and secondary metabolites have been reported in *Arabidopsis thaliana* infected with the beet-cyst nematode *Heterodera schachtii* (Hofmann *et al.*, 2010). A similar response to *H. schachtii* in *Brassica oleracea* was subsequently reported to cause reduced aphid population growth and disturbed feeding relations between plants and aphids (Hol *et al.*, 2013). Plant-mediated interactions between plant parasitic nematodes and aphids studied to date have been variable: both neutral and negative impacts on aphid performance have been reported, with the outcome depending on the parasitic strategy of the nematode involved in the interaction. For example, a reduced aphid performance was reported when *Plantago lanceolata* (Wurst and van der Putten, 2007) was infected with the migratory nematode, *Pratylenchus penetrans*. Similarly, a decrease in the fecundity of aphids was observed when *Agrostis capillaris* was infected with a mixture consisting of ectoparasites and migratory endoparasites (Bezemer *et al.*, 2003). Reports using sedentary endoparasites have found negative or neutral impacts on aphids. An infection of *H. schachtii* on *B. oleracea* resulted in reduced growth and fecundity of a specialist aphid species, *Brevicoryne brassicae* as well as a generalist species, *M. persicae* (Hol *et al.*, 2010). However, in another study using a mix of different parasitic nematode species, no effect on the performance of *B. brassciae* was found (Kabouw *et al.*, 2011).

1.5. Project Objectives

The hypothesis on which the following studies were based is that a below-ground pathogen can indirectly influence the performance of an above-ground pest on a shared host.

Biological, molecular and biochemical studies were carried out on the potato crop to determine whether a sedentary plant parasitic nematode, which has a narrow host range (i.e. a specialist) affects a phytophagous phloem-sucking aphid, which has a broad host range (i.e. a generalist) while feeding on a shared host.

The specific objectives of this thesis were:

To characterise plant-mediated interactions between the potato cyst nematode, *Globodera pallida* and the peach-potato aphid, *Myzus persicae*.

- To investigate molecular and biochemical mechanisms in order to elucidate the relationship between the two pests that attack the potato plant and often simultaneously.

- To investigate whether or not the fecundity of aphids is altered on potato plants infected with nematodes.

- To assess whether or not aphid affect plant root diffusates and the subsequent hatching of second-stage juveniles.

- To investigate whether or not the developmental rates of nematodes is affected on plants that have been pre-infected with aphids.

- To subject plants to stress by *Globodera pallida* and *Myzus persicae* in isolation as well as a combination of both stressors and to analyse local and systemic tissues of these plants for molecular changes in hormonal signalling pathways involved in biotic signalling such as the salicylic acid and jasmonic acid signalling pathways.

- To perform a non-targeted metabolomics study to investigate the metabolome of the potato suffering dual biotic stress.

Chapter 2

General Materials and Methods

2.1. Biological material

2.1.1 Species used

Plants

- Potato - *Solanum tuberosum* cv. Désirée
- Potato - *Solanum tuberosum* cv. Maris piper
- Tomato - *Solanum lycopersicum* cv. Ailsa Craig

Nematodes

- White Potato Cyst Nematode - *Globodera pallida* Pa 2/3
- Golden Potato Cyst Nematode - *Globodera rostochiensis* Ro1

Aphids

- Peach Potato Aphid - *Myzus persicae* L. Sulzer (Clone O)

2.1.2 Growth of *Solanum* species in soil

Tuber cuttings of *S. tuberosum* with one chit present were planted in 18 cm pots containing compost (Petersfield No.2, Leicester, UK). Growth took place in a greenhouse at 20-22 °C under 16 h/8 h light/dark cycles for a period of three weeks. Potato plants for each experiment were grown under the same conditions unless otherwise specified. Potato plants were watered every second day. For tomato, two seeds of *S. lycopersicum* (cv. Ailsa Craig) were planted approximately 1 cm deep in compost in 4 cm pots. Once germinated the seedlings were transferred to 7 cm pots and left to grow for a period of 4 weeks.

2.1.3 Maintenance of cyst nematode populations

Cysts of *G. pallida* and *G. rostochiensis* were stored at 4 °C. Independent stocks were maintained for *Globodera* species by growing susceptible potato cultivar Désirée in 50:50 sand/loam containing cysts at a density of approximately 50

eggs/g. After approximately 3 months, aerial parts of the plants were removed, the soil was left to dry and then stored at 4 °C. Egg counts were performed on the infected soil to determine levels of infestation. This was carried out by extracting all the cysts from 100 g of soil (section 2.1.4), crushing them and re-suspending in water. The egg count per ml of water could then be determined using a Peter's Counting Slide.

2.1.4 Extraction of *G. pallida* cysts from infected soil samples

Cysts of *G. pallida* were extracted from infested soil stocks using the Fenwick can method (Fenwick, 1940), whereby the lighter cysts are separated by floatation from the heavier soil particles. Infested soil samples were washed through a 500 µm sieve into the can and the cysts were collected on a 150 µm sieve, then washed into a fast flow filter paper (Whatman, UK) in a funnel with a sealed bottom. The mixture was allowed to settle before the water was released to leave a ring of cysts at the top of the filter paper. The cysts were collected using fine tweezers under a stereo-binocular microscope and stored dry at 4 °C until hatching.

2.1.5 Preparation of potato and tomato root diffusate

Roots of three week old potato plants (cv. Désirée) grown in 50:50 sand/loam were excised from the bottom of the plant stem and washed to remove excess soil. Excised roots (80 g per litre) were soaked in water in darkness for 24 hours at 4 °C. Potato root diffusate, collected by straining the roots, was filter sterilised (0.22 µm) and stored at 4 °C. The above method was used to prepare tomato root diffusate from four week old tomato plants.

2.1.6 Hatching of *G. pallida* cysts for collection of second-stage juveniles

Cysts were prepared for hatching using a bleaching method (Heungens *et al.*, 1996). Cysts were transferred to a modified syringe with a 30 µm nylon mesh attached to the base and washed in 5 ml of 100 % ethanol for 30 seconds. Next, the cysts were treated with sodium hypochlorite (NaOCl) 1 % available chlorine aqueous solution for approximately twenty minutes or until the cysts appeared bleached. This was to chemically rupture the cyst wall and aid hatching of the second-stage juveniles (J2). The cysts were rinsed thoroughly five times in autoclaved tap water before being transferred to a hatching jar containing a ring with a 30 µm nylon mesh attached to

the base, which allows J2s to pass through but captures eggs. Potato root diffusate was added to the hatching jar in order to stimulate hatching and the jar was incubated in darkness at 20 °C for four days before daily collection of nematodes and replacement of root diffusate. Collection continued for a period of two weeks and nematodes that were not used immediately were stored in 6 ml of sterile tap water at 10 °C.

2.2. Infection of *Solanum tuberosum* with *Globodera* species

2.2.1 Infection with juvenile *G. pallida* and *G. rostochiensis*

Three week old potato plants were infected with hatched juveniles of *G. pallida* and were watered directly onto the potato roots in the soil. Four 1 ml pipette tips were inserted to a depth of 2 cm around the stem of each plant (Figure 2.1). A total of 10,000 *G. pallida* J2s in 6 ml of sterile water were applied through the tips to each plant and washed down with a further 1 ml of water. Control plants were mock-inoculated with 7 ml of water. The pots were kept at the conditions stated (section 2.1.2). After the infection period (7, 14 or 21 days post infection (dpi)) leaf samples were collected. Samples were snap frozen in liquid nitrogen and stored at -80 °C until analysis.

2.2.2 Staining of nematodes with acid fuchsin

In order to clearly visualise and count nematodes in the plant roots, acid fuchsin was used as a stain. After washing to remove soil, roots were soaked in sodium hypochlorite solution with 1 % available chlorine for three minutes. The roots were then washed thoroughly in tap water and transferred to boiling acid fuchsin (in 0.035 % glacial acetic acid) for two minutes. After rinsing again in tap water, the roots were left to de-stain in acidified glycerol in Petri dishes. Parasitising nematodes could then be visualised under a microscope.

Figure 2.1. Infection of *Globodera pallida* on *Solanum tuberosum* cv. Désirée.

A. Three week old potato plants were used for infection. **B.** Hatched juveniles were watered directly onto the roots of 3 week old potato plants growing in soil. Four 1 ml pipette tips were inserted to a depth of 2 cm around the stem of each plant. A total of 10,000 *G. pallida* J2s in 6 ml of sterile tap water were applied through the tips to each plant and washed down with a further 1 ml of water. Control plants were mock-inoculated with 7 ml of water.

2.3. Infestation of *Solanum tuberosum* with *Myzus persicae*

2.3.1 Maintenance of aphid culture

Nymphs of the peach-potato aphid (*Myzus persicae*) were obtained from the James Hutton Institute, Invergowrie, Dundee, Scotland. The aphids were clones of a wild population isolated in Scotland (Kasprowicz *et al.*, 2008). This population, known as Clone O, have a susceptibility to insecticide and a low tendency to form winged females except when feeding on the peach species. Aphids were maintained as asexual clones on a potato plant grown as described (section 2.1.2) kept inside a mesh cage. In order to avoid the development of winged females associated with high population density, every three weeks a new colony was started on a fresh potato plant with twenty nymphs from the old colony. Aphid colonies were kept in a greenhouse at 20-22 °C under 16 h/8 h light/dark cycles. Only apterous (wingless) aphids were used in experiments.

2.3.2 Aphid infection of potato plants

Depending on the corresponding nematode infection (7, 14 or 21 dpi), either four, five or six week old potato plants were used in aphid experiments to ensure all potato plants were the same age. Twenty apterous aphids of various life stages (first instar nymphs to adults) were transferred to the second fully expanded leaf with a fine paintbrush and confined to the abaxial leaf surface in a 2.5 cm diameter clip cage (Figure 2.2). Aphid-free clip cages were used in control experiments. Local leaf tissue was initially sampled at four different time-points (24 h, 48 h, 72 h and 96 h) and based on the highest level of *PR*-gene expression only at the time-point of 48 h thereafter. The corresponding leaf tissue was sampled from the uninfected control plants. Samples were immediately snap frozen in liquid nitrogen and stored at -80 °C until analysis.

2.3.3 Co-infection of potato plants with both nematodes and aphids

Co-infected plants were infected with nematodes first (7, 14 or 21 dpi). Following the nematode infection period 20 apterous aphids of various life stages (first instar nymphs to adults) were transferred to the second fully expanded leaf with a fine

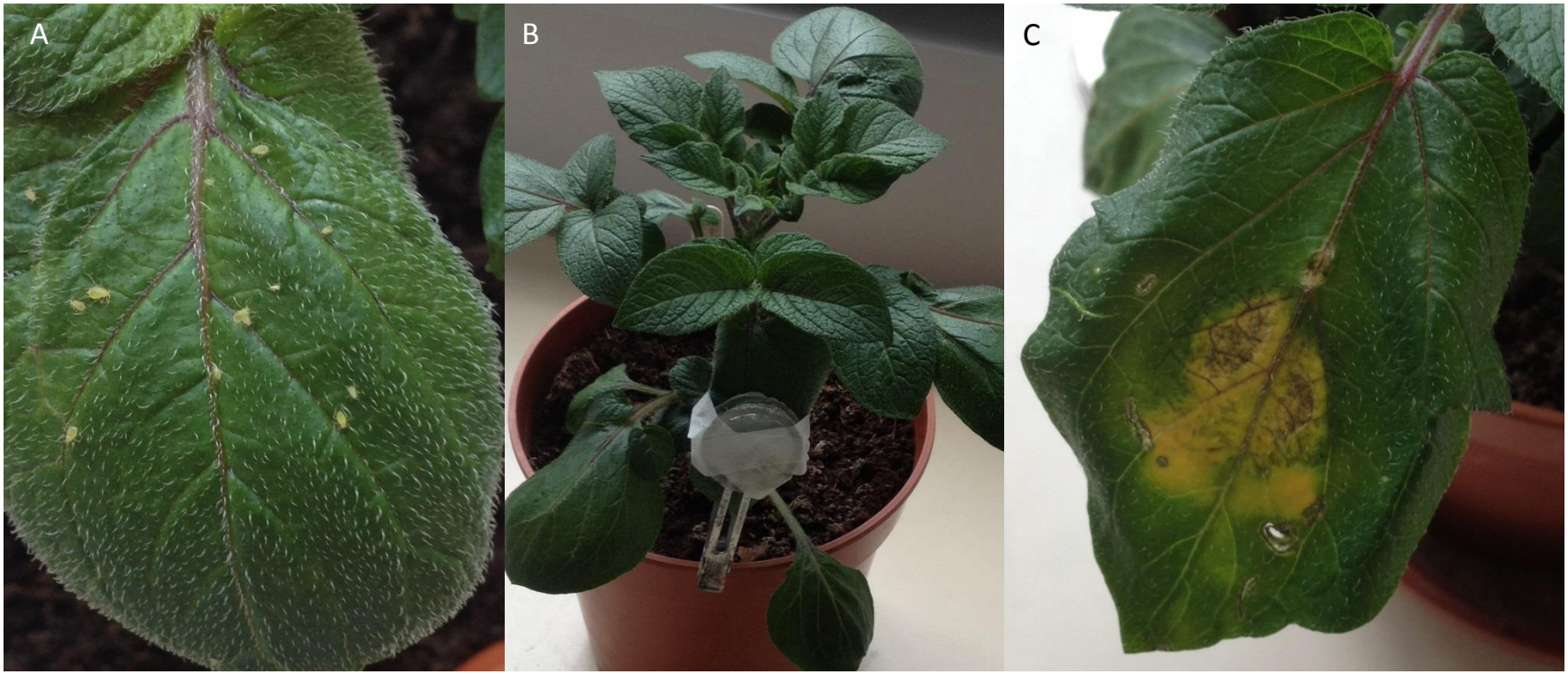

Figure 2.2 Infection of *Solanum tuberosum* cv. Désirée with the peach-potato aphid, *Myzus persicae*.

A. Potato leaf colonised by aphids of various life stages for maintenance. **B.** Experimental set up of potato plants infected *M. persicae*. Control plants received 2.5 cm clip-cages that contained no aphids. **C.** Damage caused to a potato leaf following the presence of sixty female apterous aphids inside a clip cage for 96 hours. Chlorosis of the leaf formed after presence of aphids for 72 hours.

paintbrush and confined to the abaxial leaf surface in a 2.5 cm diameter clip cage. Aphid-free clip cages were used in the control experiments.

2.4 Quantitative RT-PCR analysis of pathogenesis-related (*PR*) gene expression in the leaves of potato plants

2.4.1 Extraction of total RNA

Total RNA was prepared from frozen leaf tissue using an RNeasy Plant Mini Kit (Qiagen, UK) according to the manufacturer's protocol. Samples were ground to a powder whilst frozen using a sterile, RNase-treated pestle and mortar. Approximately 100 mg of powder was then used in the extraction protocol. RLT extraction buffer (450 µl) with 10 µl/ml β-mercapoethanol was added directly to the frozen plant material in a microcentrifuge tube. Disrupted tissue was centrifuged through a QIAshredder spin column to remove cell debris and reduce lysate viscosity. Ethanol was added to the supernatant, which was then applied to an RNeasy Spin Column. The optional on-column DNase I digestion was performed. The column was washed with the buffers RW1 and RPE to remove contaminants, and total RNA was eluted in 50 µl RNase-free water. The RNA was stored at -80 °C

2.4.2 Reverse transcription of RNA

A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) was used to estimate the concentration of RNA from a 1 µl sample, at a wavelength of 260 nm. The purity of the RNA was estimated by calculating the 260/280 nm and 260/230 nm ratios. Following quantification, RNA was used in a reverse transcription reaction to create cDNA using Superscript II Reverse Transcriptase (Invitrogen, UK). Approximately 1 µg of total RNA was added to 1 µl anchored oligo dT primers and dNTPs (10 Mm) and made up to a volume of 13 µl with sterile, distilled water. The RNA was heat denatured at 65 °C for 5 minutes and quickly chilled on ice. First Strand Buffer (5x) and DTT (0.1M) was added to the mixture and incubated at 42 °C for two minutes. Superscript II Reverse Transcriptase (200 units) was finally added to the mixture and incubated at 42 °C for 50 minutes, followed by a 15 minute incubation at 70 °C to inactivate the reaction. This produces single stranded cDNA complementary to the mRNA population as the oligo dT primers anneal to the poly (A)+tails.

2.4.3 qPCR primer design

qPCR primers were designed for four PR-genes of potato (*PR*-1, *PR*-2, *PR*-3 and *PR*-5), a gene involved in jasmonate signalling (*JAZ*-1) and *ELONGATION FACTOR* 1-α (Nicot *et al.*, 2005) for normalisation (Table 2.1). Each gene sequence was recovered using the BLAST tool available at the Solanaceae Genomics Resource website (Michigan State University) using the corresponding gene sequence from *Arabidopsis* as the search sequence. Primer3 software (http://primer3.wi.mit.edu/) was used to design primers. Where possible, primers were designed to span exon boundaries so that no genomic DNA would be amplified in the reaction. Two sets of primers were designed for each gene. Primer stocks were prepared by combining forward and reverse primers for the same gene and subsequently diluted to a concentration of 7.5 µM each with sterile distilled water.

2.4.4 Quantitative reverse transcriptase (RT)-PCR

qRT-PCR was carried out using a CFX Connect Real-Time PCR Detection System (Biorad, UK) and using SsoAdvanced™ Universal SYBR® Green Supermix (Biorad, UK). The supermix contains all the components necessary for the reactions, including buffer, $MgCl_2$, nucleotides, Sso7d fusion polymerase and a double-stranded DNA-binding dye SYBR Green I for detection. Reactions took place in 96-well polypropylene plates (Biorad, UK) sealed with optical quality sealing film. In each well the following components were combined: SsoAdvanced™ Universal SYBR® Green Supermix (12.5 µl), sterile distilled water (6.5 µl), combined primer pair (1 µl) and cDNA template (5 µl). Plates were mixed for 3 minutes at 600 rpm using a Mixmate (Eppendorf) and then centrifuged briefly to collect mixture at the bottom of the 96-well plate. During the reaction, plates underwent a two-step amplification profile. An initial denaturation step of 95 °C for 10 minutes was followed by 40 cycles of 95 °C for 30 seconds and 60 °C for 10 seconds. Fluorescence data was collected at the 60 °C annealing phase. A dissociation curve was constructed by subjecting the completed reaction products to a stepwise

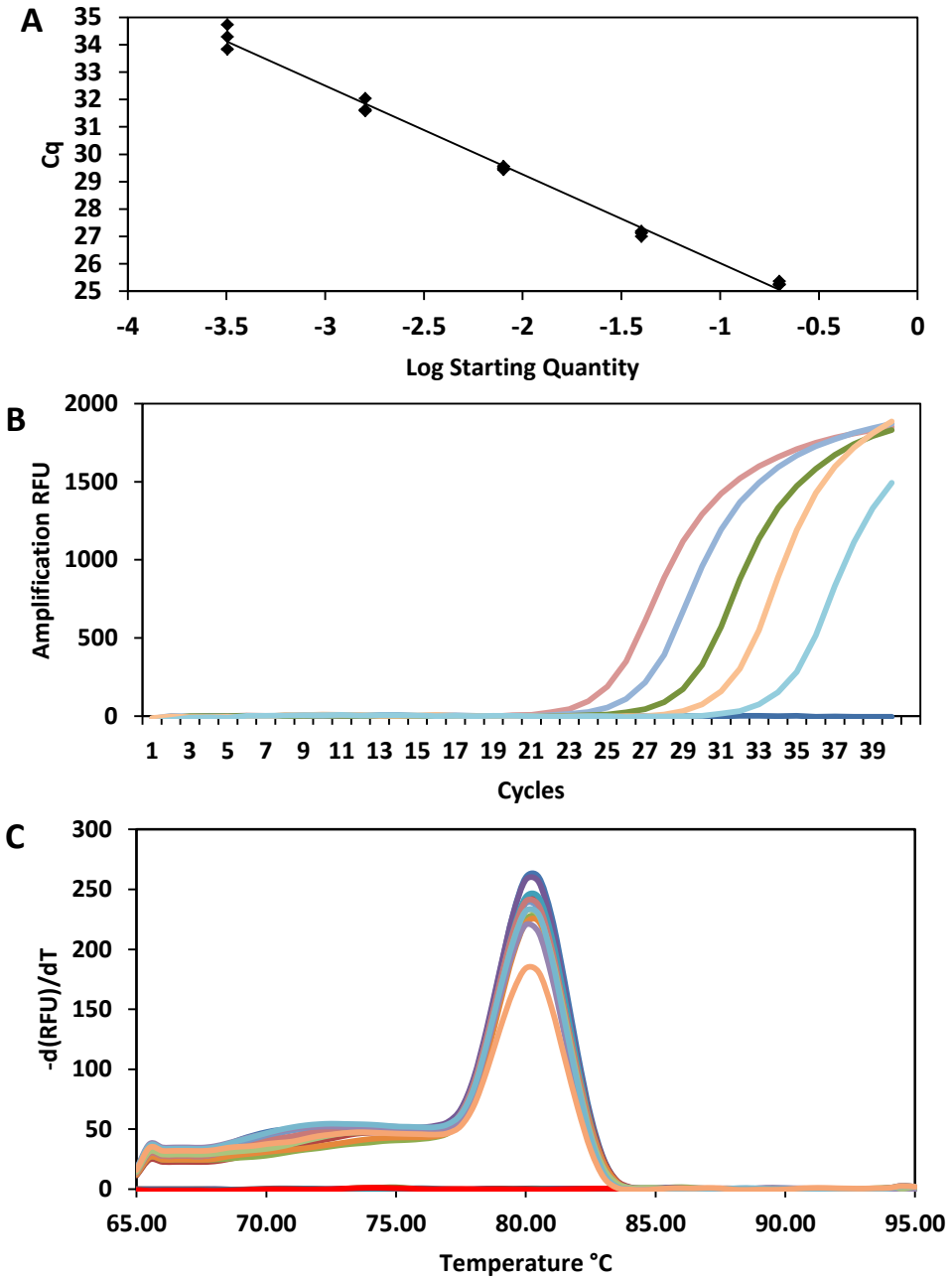

Figure 2.3 Standard curve, amplification plots and dissociation curve for the primer pair PR-5 used to amplify transcripts in the leaves of stressed potato leaves.

A. A standard curve was created for each primer pair using a 10-fold dilution series of potato cDNA. The *PR*-5 standard curve pictured here had an R-squared value of 0.994 and the primers had an efficiency of 93.7%. **B.** Raw data curves generated by qRT-PCR. Relative starting quantity of RNA was calculated by measuring the cycle number (Ct value) at which samples reached threshold fluorescence, compared to the normalising gene *ELONGATION FACTOR* 1-α. **C.** A dissociation curve illustrating the specificity of qRT-PCR primers. The specific melting temperature of a product is related to its size and base pair composition, so a pure product gives one single peak. The red line represents the no template control.

increase in temperature from 60 °C to 95 °C with fluorescent measurements taken throughout this range. The efficiency of each primer pair was first confirmed by generating a standard curve using cDNA known to contain detectable amounts of all genes to be tested (Figure 2.3 a). cDNA was diluted to create a 10-fold dilution series of five standards (Figure 2.3 b). Each standard was tested in duplicate for each primer pair. In addition, negative controls containing no cDNA were tested in order to check for contamination. The specificity of each primer pair was analysed using a dissociation curve; one single peak indicated a single product and amplification and an absence of primer dimers (Figure 2.3 c). For analysis of the samples, cDNA from leaves subjected to nematode stress, aphid stress or combined nematode and aphid stress was diluted 1:20 and each sample tested in technical triplicate. The expression levels of target genes were normalised to the housekeeping gene *ELONGATION FACTOR 1-α,* and cDNA from uninfected control plants was used as a calibrator to calculate the change in expression.

The CFX Manager™ Software (Biorad, UK) was used to determine Ct values and fold changes. The Ct value represents the cycle number at which threshold fluorescence is reached. A 1 Ct difference between samples represents two times as much transcript when primer efficiency is 100%. The default threshold fluorescence levels for determining Ct values were used. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method. The Ct value of *ELONGATION FACTOR 1-α* was subtracted from that of the gene of interest to give the ΔCt value. The control ΔCt value was then subtracted from the treatment ΔCt value to give the ΔΔCt. The log2 fold-change was then determined. The primer efficiency for each pair was manually adjusted in the CFX Manager™ Software, to account for any deviations from 100%. Between five and twelve biological replicates were used for the expression of each gene used in the study. Each of these biological replicates were carried out in triplicate to ensure there was no technical variation between samples.

2.5. Quantification of phytohormones in the leaves of potato plants

2.5.1 Quantification of endogenous salicylic acid

Salicylic acid (SA) extraction was performed using a modified protocol derived from Raskin *et al.* (1989). One millilitre (1 ml) of methanol (90%) was added to ground,

frozen leaf tissue, and the resulting mixture was vortexed for one minute followed by sonication in a bath for five minutes. After centrifugation for five minutes at 14,104 *g*, the supernatant was collected and the pellet was re-extracted with 500 µl methanol (100%), vortexed for one minute, re-sonicated for five minutes and re-centrifuged at 14,104 *g* for a further five minutes. Both supernatants were combined and dried using a GeneVac (EZ-2 series). For free SA quantification the dried samples were re-suspended in 250 µl of 5% trichloracetic acid (TCA) and vortexed. The sample was extracted twice in cyclohexane and ethyl acetate (1:1), vortexed vigorously and centrifuged at 14,104 *g* for one minute. The top organic phase was removed and dried using the GeneVac (EZ-2 series). The remaining phase was subjected to acid hydrolysis using 300 µl 8M hydrochloric acid (HCl) and incubated at 80 °C for one hour to quantify sugar-conjugated SA. The incubated sample was extracted twice in cyclohexane and ethyl acetate (1:1), vortexed vigorously and centrifuged at 14,104 *g* for one minute. The top organic phase was removed and dried using a GeneVac. Pooled SA extract was re-suspended in 600 µl of water and acetonitrile (95:5) and quantified by reverse-phase high-pressure liquid chromatography (RP-HPLC). Analysis was performed using a Supercosil™ LC-18 column (250 x 4.6 mm, 5 µm). An injection volume of 20 µl was separated under isocratic conditions using a mobile phase of water, acetonitrile (HPLC grade) and formic acid (60:40:0.1) at a flow rate of 1 ml/min. SA was detected using a Dionex RF 2000 Fluorescence Detector operated at an emission wavelength of 400 nm and an excitation wavelength of 303 nm respectively (Figure 2.2 a). SA was determined and quantified by comparing peaks of recovered SA using calibration standards. The efficiency of SA recovery was calculated by using a deuterium-labelled internal standard of SA-d_6. Between five and twelve biological replicates were used for the analysis of endogenous salicylic acid levels. Each of these biological replicates were carried out in duplicate to ensure there was no technical variation between samples.

2.5.2 Quantification of endogenous jasmonic acid

Leaf tissue was harvested as previously described. The samples were ground into a powder in a Tissue Lyser LT (Qiagen, Hilden, Germany) and 1 ml extraction solvent

(methanol/H_2O/formic acid; 80:19:1, v/v/v) was added and mixed. Samples were then sonicated at 4°C for 5 minutes, agitated for 30 minutes at 4°C and centrifuged at 12, 000 *g* for 10 minutes at 4°C. The extraction procedure was repeated with 500 µl solvent extraction and the supernatants were combined. Jasmonic acid was analysed on a UPLC AxION 2 TOF MS system coupled with an Altus SQ Detector (Perkin Elmer, UK). For the chromatographic separation the solvents were 0.1% HCO_2H in ultrapure water (A) and 0.1% HCO_2H in methanol (B), the column was a C18 100 x 1.2 mm (Perkin Elmer, UK) and the flow rate was set at 0.35 ml min^{-1}. The binary analytical gradient used was as follows: 0 min, 1% B; 20 min, 100% B; 22 min, 100% B; 25 min, 1 % B. The compound quantification was assured by calibration curve standards in the range of 5 – 50 ng/ml. The data analysis was performed using Empower 3 software (Waters, UK). Five biological replicates were used for the analysis of endogenous levels of jasmonic acid in this study.

2.6. General Primers

Table 2.1. Pathogenesis-related primers common to multiple chapters.

Primer Name	Sequence 5' – 3'	PCR Efficiency	Accession Number
EF1α_F	ATTGGAAACGGATATGCTCCA	96.5%	*AB061263*
EF1α_R	TCCTTACCTGAACGCCTGTCA		
*PR-1*_F	TGGTACCAACCAATGTGCAA	98.5%	AJ250136.1
*PR-1*_R	AATGAACCACCATCCGTTGT		
PR-2_F	CTGTTTATGCTGCGATGGAA	94.4%	U01901
PR-2_R	GTCTTGTGTGGCACCAAATG		
PR-3_F	GCTGCAGCTAACTCGTTTCC	98.3%	AF024537
PR-3_R	CGTGAGATGTTTGACCGAAA		
PR-5_F	GCATCTGGGCATCTTTGTTT	93.7%	AY737315.1
PR-5_R	GCACGGTTTTCAAGACGAAT		
JAZ1_F	AGCCAACAAACAGAACCCCA	101.8%	NM_001247954.1
JAZ1_R	TCACACCAGATTGATCAGCTGT		

2.7. Statistical Analysis

The effects of the nematode, aphid and co-infected treatments were determined using a Mann-Whitney U test to test the changes in gene expression, the levels of endogenous phytohormones JA and SA. A Mann-Whitney U test was carried out to compare the abundance of aphids on nematode infected plants against non-infected control plants independent of time. For non-targeted metabolite analysis, data was analysed using Progenesis QI® software: ANOVAs and mean fold change between groups were used to assess the difference in abundance of metabolites between PCN, aphid and co-infected plants and non-infected control plants. Compounds of interest were discovered by filtering on $p \leq 0.01$, fold-change ≥ 2 and percentage coefficient of variance between replicates <25%. Tentative identifications were assigned to compounds of interest using an in-house *m/z* database of plant metabolites and the Metlin: Metabolite MS Database (Scripps Centre for Metabolomics, California, USA).

Chapter 3

Multitrophic interactions between the potato cyst nematode, *Globodera pallida* and the peach potato aphid, *Myzus persicae*

3.1. Introduction

Plants are the primary source of nutrition for many pests and pathogens and therefore are exposed to simultaneous attack from a range of organisms, both above- and below-ground (Hong *et al.*, 2011). Due to these organisms being spatially and temporally separated, many studies have focussed on one organism alone, rather than combinations of species attacking plants. However, in recent years, reports have emerged describing how pests and pathogens can interact through their shared host plant, resulting in changes in their reproductive success (Gange, 2001, Masters *et al.*, 2001). The relationship between plant-parasitic nematodes and aphids is an interesting model to use to study plant-mediated multitrophic interactions because they are sensitive to the same plant resistance gene, *Mi-1.2* (Rossi *et al.*, 1998) and can attack crops at the same time. To date, plant-mediated interaction studies on plant parasitic nematodes and aphids have reported both positive and negative impacts on aphid reproductive success due to the presence of nematodes, with the final outcome largely depending on the parasitic strategy of the nematode involved in the interaction. A number of studies yielding a negative asymmetric interaction i.e. the negative impact of one pest on the reproductive success of a secondary arriving pest in one direction only, between nematodes and aphids have used species that have a migratory lifestyle as their nematode pathogen (Hol *et al.*, 2016, Wurst and van der Putten, 2007). However, migratory nematodes remain mobile throughout their life-cycle whilst feeding on cortex cells (Jones *et al.*, 2013) thus causing a distinct defence response (Wondafrash *et al.*, 2013) in the foliar parts of the plant (Nahar *et al.*, 2011); the

effects of sedentary nematodes, such as the *Heterodera* and *Globodera* spp. on aphids may be different.

In support of this, studies using *H. schachtii* as the below-ground pathogen to test the effects on the soybean aphid (*Aphis glycines*) found alate aphids preferred plants without nematodes compared to nematode-infested plants. However, the population growth of aphids feeding on nematode-infested plants was either unaffected or even slightly improved (Hong *et al.*, 2010). It is thought that the interaction between nematodes and aphids may be unidirectional as there have been more demonstrations of the effects of nematodes on the reproductive success of aphids (Bezemer *et al.*, 2005, Kaplan *et al.*, 2011, Wurst and van der Putten, 2007) compared to those on the effects that aphids may have on the development of nematodes (Kutyniok and Müller, 2012, Wardle *et al.*, 2012).

This chapter focuses on the relationship between the potato cyst nematode, *Globodera pallida* and the peach-potato aphid, *Myzus persicae*, two pests that can infect the potato plant and often do so simultaneously. In order to test the effects that the presence of aphids may have on the development of nematodes two separate experiments were carried out. Hatching assays of second stage juveniles (J2s) from cysts were carried out to investigate whether or not pre-infestation of the plant with aphids may alter the composition of potato root diffusate and subsequently affect the hatching rate of J2s. It was found that the sequence of arrival of each pest plays an important role in the outcome of the interaction between two pathogens as infection of the plant with one pest may be an important strategy in avoiding competition with a secondary arriving attacker (Erb *et al.*, 2011). Therefore, sequence of arrival assays were carried out to test if the presence of aphids had an effect on the infectivity and reproduction of nematodes. In addition, aphid abundance assays were carried out to investigate if the reproductive success of aphids was improved or worsened on potato plants pre-infected with nematodes.

3.2 Aims

1. Investigate whether or not the abundance of *Myzus persicae* was affected by the pre-establishment of *Globodera pallida* on potato plants at different time-points throughout the life-cycle of the nematode.

2. Investigate whether or not pre-establishment of aphids had an effect on potato root diffusate thus affecting the hatching rate of *G. pallida*.

3. Investigate if the sequence of arrival of *M. persicae* had an impact on the infectivity or development of *G. pallida*.

3.3. Materials and methods

3.3.1. Aphid abundance assays

Ten thousand nematodes were introduced around the roots of potato plants and control plants were mock-inoculated with water (section 2.2.1). At 14 and 21 days post inoculation (dpi) 10 adult, apterous aphids (*M. persicae*) were placed inside a 2.5 cm clip- cage and were placed on the abaxial surface of the leaf. After 24 hours all but five nymphs were removed from the clip-cage. This was to ensure that the generation of nymphs used during these experiments were accustomed to the phloem of either nematode-infected plants or non-infected control plants. Five biological replicates were used for the study. A Mann-Whitney U test was carried out to compare the abundance of aphids on nematode infected plants against non-infected control plants.

3.3.2. Aphid preference assays

Potato plants were grown and inoculated with nematodes or autoclaved tap water (section 2.2.1). At 14 dpi and 21 dpi a nematode infected plant and a non-infected control plant were placed inside a meshed cage. Ten apterous aphids were placed inside a 1.8 ml microcentrifuge tube and placed at equidistance at soil level of two plants. Twenty-four hours later the number of aphids present on the nematode infected plants and the non-infected control plants were counted. Four biological replicates were used for the study.

3.3.3. Hatching assays

Potato root diffusate (PRD) and tomato root diffusate (TRD) was prepared as previously described (section 2.1.5). For the pilot hatching assay potato plants were heavily infested with *M. persicae* (>100 aphids) ten days before the root harvesting time-point of three weeks. For the dose-dependent response hatching assays either 25 or 50 aphids were placed on potato plants or tomato plants ten days before the three week root harvesting time-point. Five cysts (*G. pallida*) were placed in polypropylene plates and one millilitre of PRD from aphid infested plants or control potato plants were placed into the wells. Initially the PRD was changed after four days as this was when J2s began to emerge from eggs contained within the cysts. PRD and TRD were changed every two days thereafter to ensure the cysts were

being incubated in fresh diffusate. Diffusate was collected from five separate whole root systems that were either infected with aphids or non-infected control potato plants. Hatching assays were carried out twelve times for each treatment.

3.3.4. Sequence of arrival assays

Tuber cuttings of *Solanum tuberosum* (cv. Désirée) with one potato chit present were planted in 13 cm pots containing a sand and loam mixture (50:50). Growth took place in a greenhouse at 20-22 °C under 16 h/8 h light/dark cycles for a period of ten days before application of pests. Five separate experiments were set up (Figure 3.1):

A) Nematodes applied to the plant first before subsequent aphid application,

B) Aphids applied to the plant first before subsequent nematode application,

C) Both nematodes and aphids applied to the plant at the same time,

D) Only nematodes were applied to the plant for 7 days and,

E) Only nematodes were applied to the plant for 14 days.

In the first experiment (A), 3000 nematodes suspended in 6 ml of autoclaved tap water were introduced around the roots of ten day old potato plants and left for a period of seven days. On day seven, ten apterous aphids were randomly placed on the foliar parts of the plants and left for a further seven days. A polypropylene cover with mesh was used to prevent aphids moving between biological replicates. In the second experiment (B), 10 aphids were placed on the foliar parts of the plant first and left for seven days. On day seven 3000 nematodes were introduced around the roots and left for a further 7 days. In experiment three (C), 3000 nematodes and ten aphids were applied to the plant at the same time and left for a period of 14 days. Experiments four and five were used as controls where only nematodes were applied to the plant for a period of seven or 14 days. At the end of the 14 day period roots were collected and stained with acid fuchsin (section 2.2.2). Nematodes in roots were analysed and counted using a stereo-binocular microscope. Five biological replicates were used for each treatment.

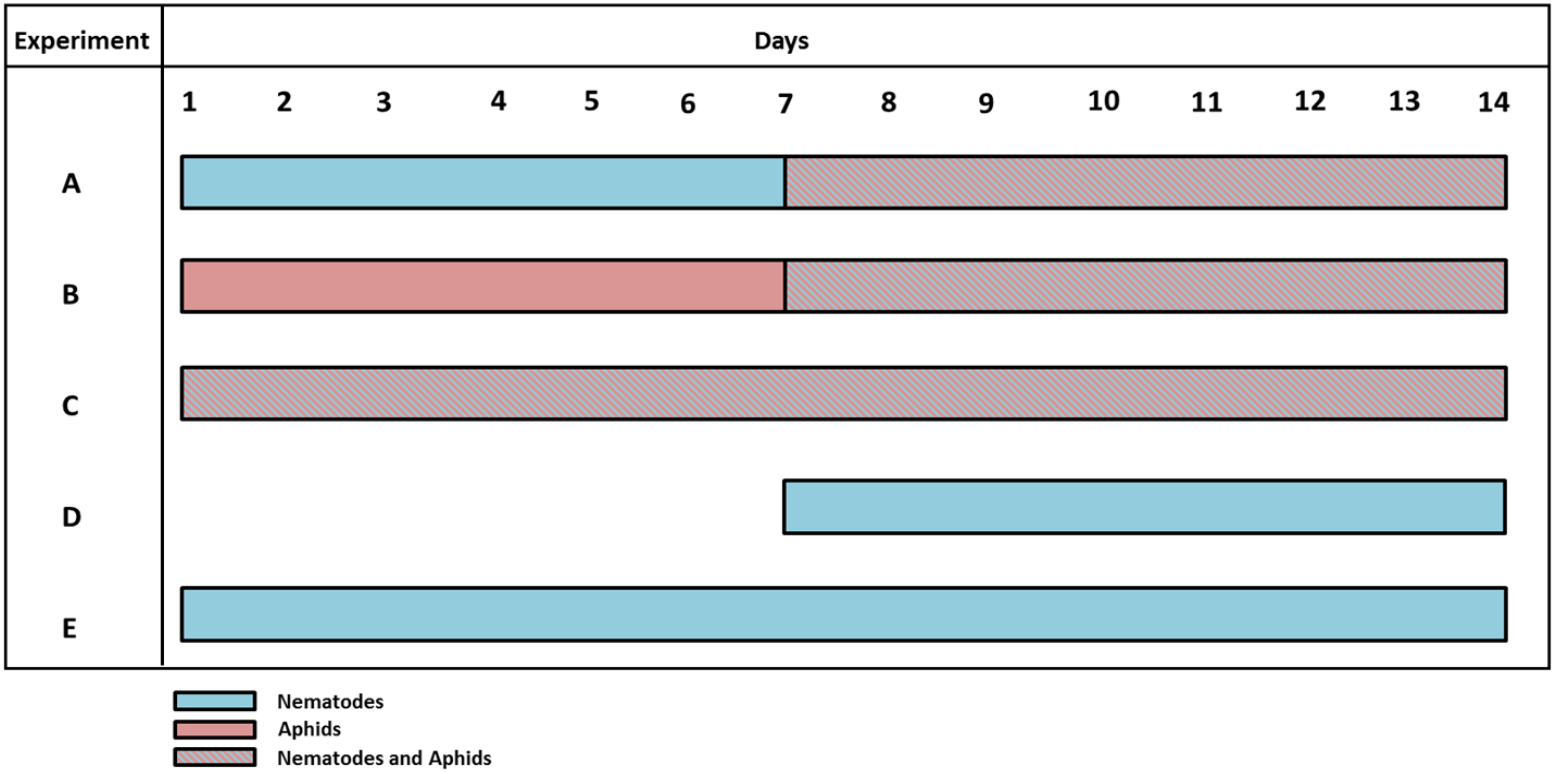

Figure 3.1. Sequence of pest arrival experimental set-up.

Five sequence of arrival experiments were set up as follows: **A.** Nematodes were applied to the plant first for 7 days before subsequent aphid application for 7 days; **B.** Aphids were applied to the plant first for 7 days before subsequent nematode application for 7 days; **C**. Both nematodes and aphids were applied to the plant at the same time for a period of 14 days; **D.** Only nematodes were applied to the plant for a period of 7 days; **E.** Only nematodes were applied to the plant for 14 days.

3.4. Results

3.4.1. Aphid abundance assays

To test if aphids had a higher abundance on potato plants that has been pre-infected with nematodes or mock-inoculated with water, the number of aphids were counted every 48 hours for a period of 8 days. There were significantly more aphids present on potato plants that had been pre-infected with 10,000 nematodes for a period of 14 days compared to control plants that had been mock-inoculated with water. At days two, four, six and eight post aphid application, there were more aphids present on nematode infected plants (P = 0.011) (Figure 3.2 a). These results may suggest that nematodes have the ability to alter molecular and biochemical properties systemically in the leaves of potato plants, which may have a positive effect on the reproductive success of aphids.

There was a significant difference in the abundance of aphids counted on potato plants that had been pre-infected with 10,000 nematodes for a period of 21 days at days two and four post aphid application (P = 0.043). However, there was no significant difference in the number of aphids present on nematode-infected plants compared to control plants on days six or eight post aphid application (P = 0.376) (Figure 3.2 b). These results indicate that nematodes continue to alter molecular and biochemical mechanisms in the leaves of potato plants until 26 days post inoculation (dpi), which subsequently are of benefit to the reproductive success of aphids. However, from 28 dpi with nematodes, the abundance of aphids does not continue to increase suggesting that molecular and biochemical mechanisms may have been altered for a second time in the leaves of potato plants.

3.4.2. Aphid preference tests

To assess if nematode infected plants or non-infected control plants were more attractive to aphids, preference assays were set-up. There was no significant difference between the number of aphids found on potato plants that had been infected with nematodes for 14 days compared to non-infected control plants (P = .627) (Figure 3.3 a) suggesting that *M*. *persicae* did not differentiate between potential volatile organic compounds released from either nematode-infected

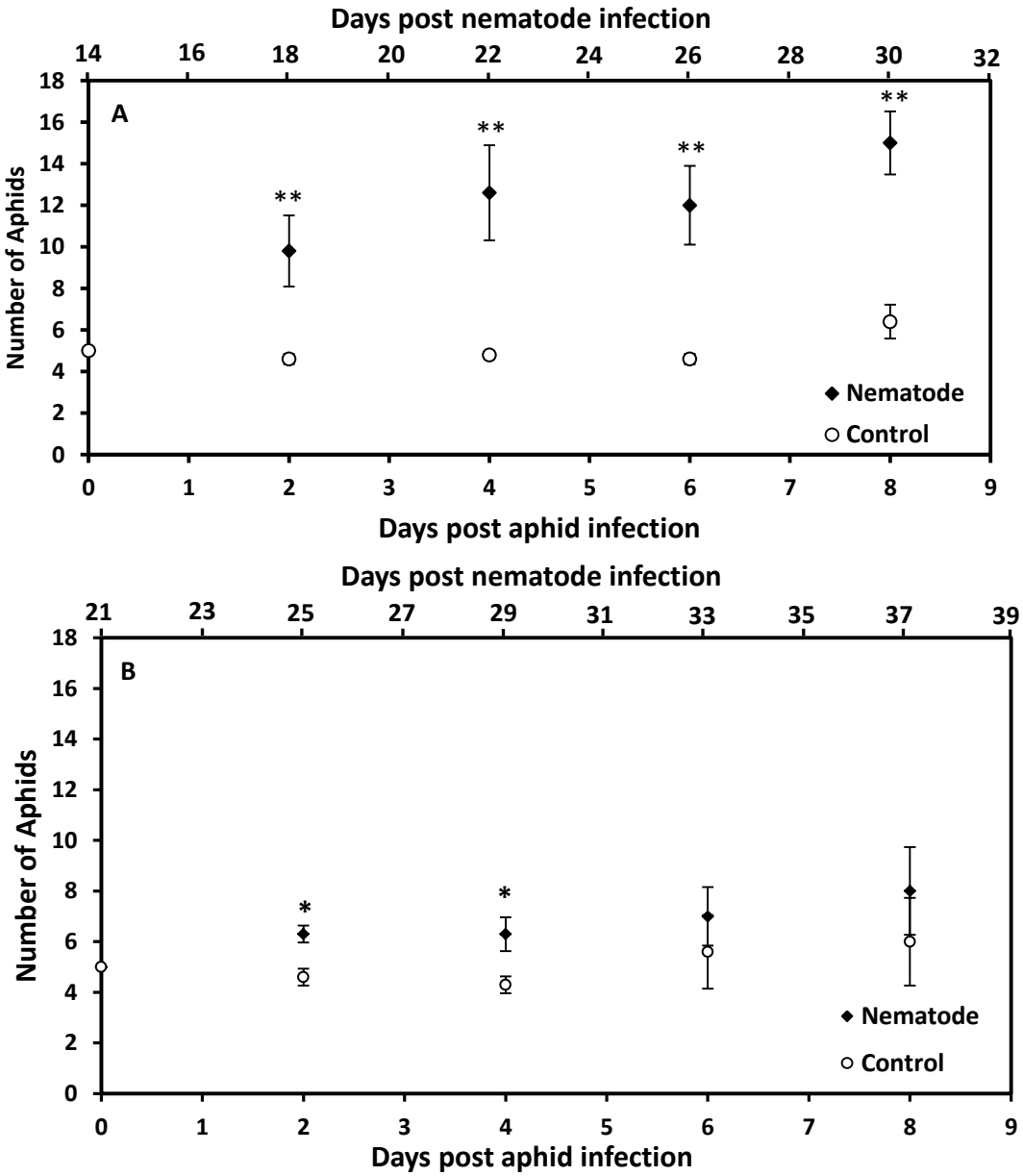

Figure 3.2. No choice performance assays of the peach-potato aphid, *Myzus persicae* on potato plants infected with the potato cyst nematode, *Globodera pallida.*

A. There were significantly more aphids present on potato plants that had been pre-infected with 10,000 nematodes 14 days prior to the arrival of aphids compared to non-infected control plants (n=5, *P* = 0.01). **B.** There were significantly more aphids present on potato plants that had been pre-infected with 10,000 nematodes for 21 days prior to the arrival of aphids compared to control plants at days 2 and 4 (n = 5, *P* = 0.05). There was no significant difference between the number of aphids present on nematode infected plants and control plants at days 6 and 8.

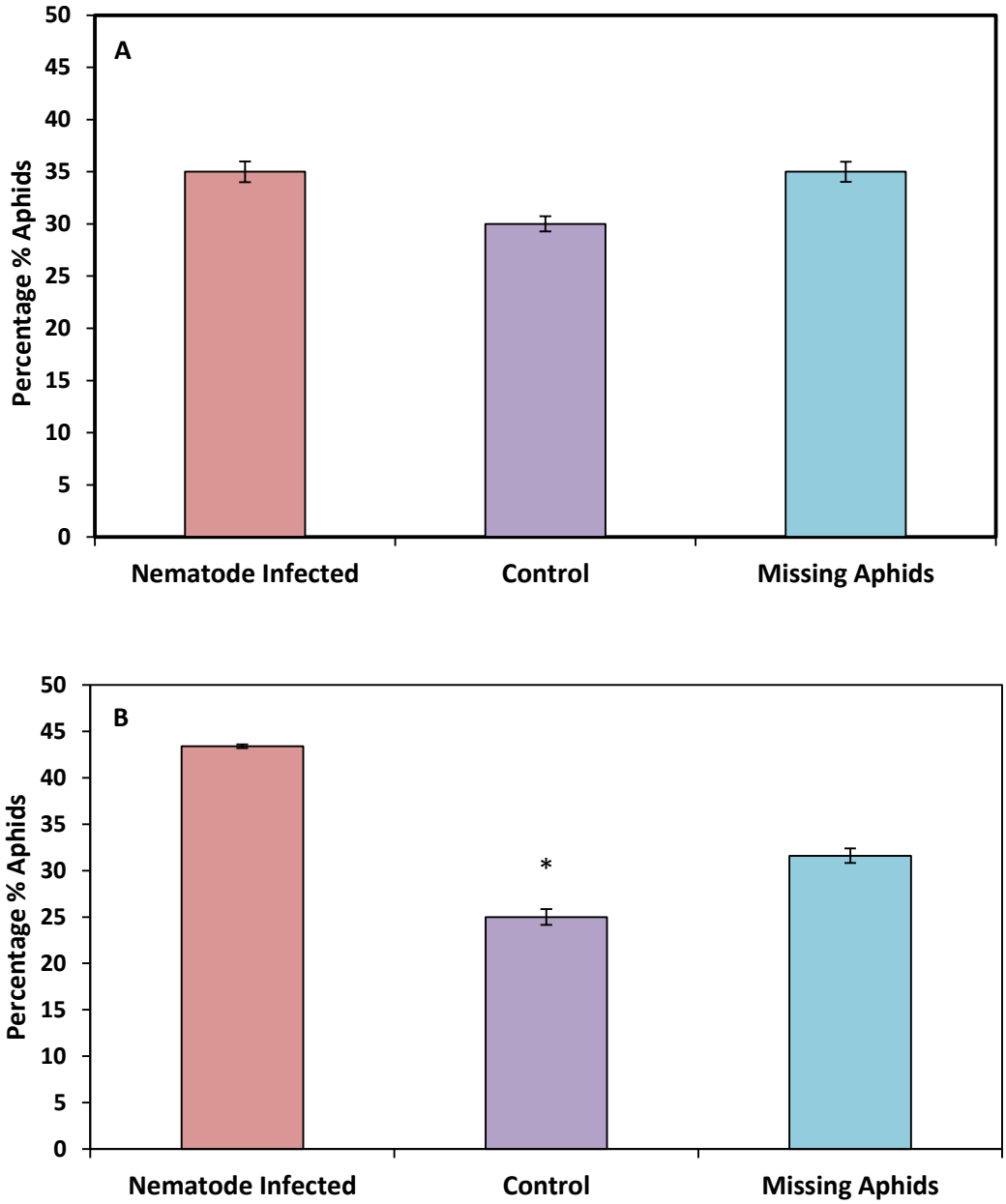

Figure 3.3. Aphid preference assays of the peach potato aphid, *Myzus persicae* on potato plants that had been pre-infected with 10,000 second stage juveniles of the potato cyst nematode, *Globodera pallida*.

A. There was no significant difference between the number of aphids recorded on potato plants that had been pre-infected with *G. pallida* for 14 days prior to positioning of aphids. **B.** There were significantly more aphids present on potato plants that had been pre-infected with *G. pallida* for 21 days prior to positioning of aphids (n=6, *P* = 0.05). Missing aphids are the percentage aphids that could not be accounted for during counting 24 hours post infection.

plants or control plants. However, there were more aphids counted on plants that had been pre-infected with nematodes for 21 days compared to control plants suggesting that the composition of potential volatile organic compounds from nematode-infected plants may change the longer nematodes are present. (P = .050) (Figure 3.3 b).

3.4.3. Hatching assays

In order to test the reciprocal experiments to assess whether aphid infection had an impact on the hatching factors in potato root diffusate (PRD) and tomato root diffusate (TRD) three different experiments were set up. First, potato plants were inoculated with a large number of aphids (>100) 10 days before the root harvest time-point of three weeks. Control plants received no aphids. Second-stage juveniles (J2s) did not begin to emerge from the cysts until day 4 of being incubated in PRD. From day 10 significantly more J2s emerged from cysts that had been incubated in PRD from potato plants that had no aphids present (P = 0.001) (Figure 3.4 a). This trend continued until day 17 of incubation. This may indicate the reduction of hatching stimulator in the PRD caused by the presence of aphids on the plant or alternatively it may suggest the presence of a hatching inhibitor in the PRD caused by the presence of aphids on the plant. From day 19 the cysts that were incubated in PRD from aphid infested plants were washed and re-incubated in PRD from non-infected plants. Normal emergence rates from these cysts were rescued and continued to emerge at a rate similar to the cysts which had been incubated in potato root diffusate from non-infected plants (Figure 3.4). By day 24 post incubation in either infected or non-infected PRD there was no significant difference between the numbers of J2s that had emerged from either set of cysts (Figure 3.4 b). This suggests that although the presence of aphids may reduce a hatching stimulant or activate a hatching inhibitor in PRD, this effect can be reversed and emergence rates of J2s can be rescued to normal rates once infected PRD is washed away.

A follow up dose dependent response assay was set up in order to assess how many aphids would have an effect on the emergence rate of J2s from cysts. Experiments were set up as previously described (section 3.3.3). Similar to the pilot hatching

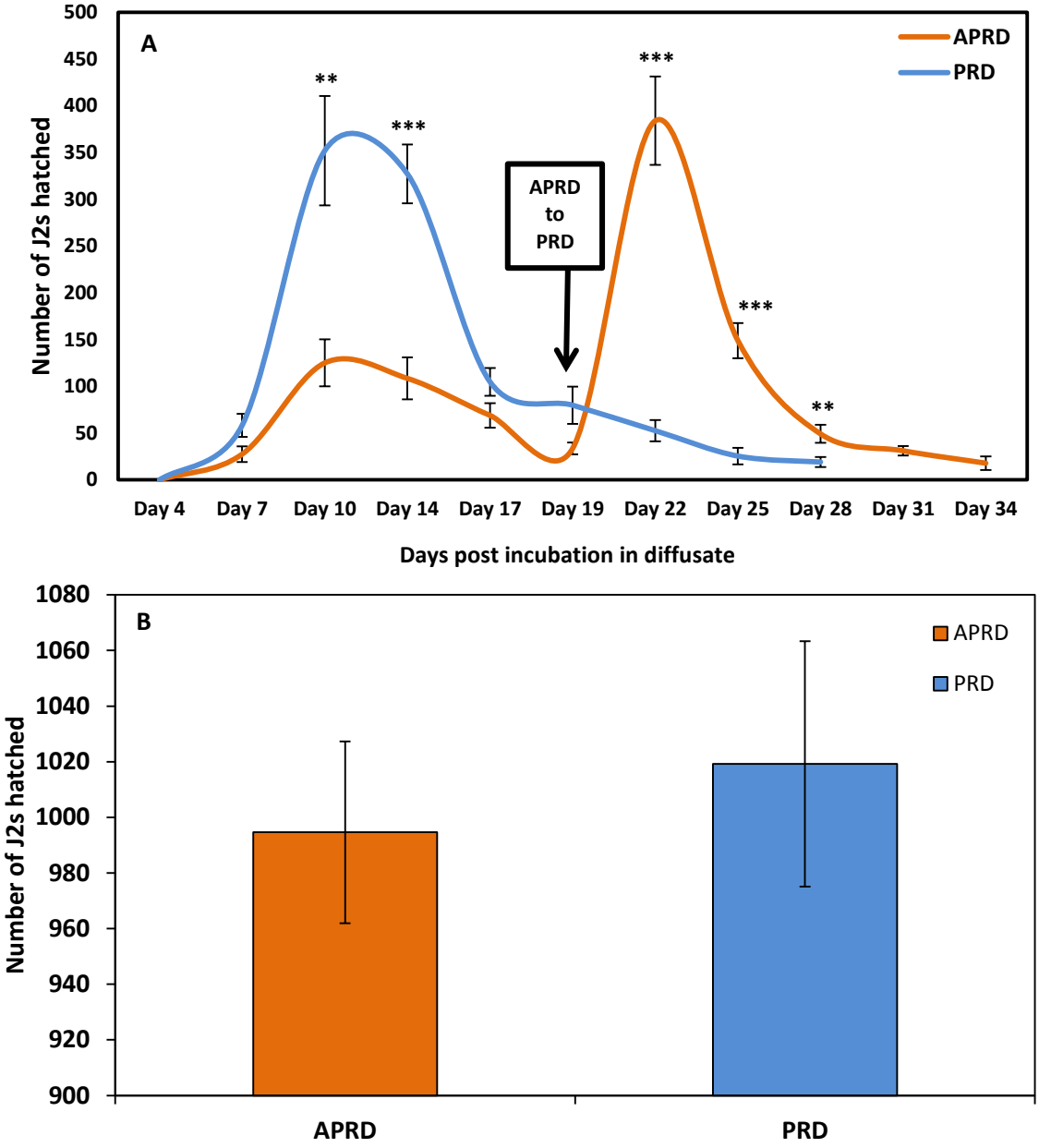

Figure 3.4. Effects of aphid-infected potato root diffusate on the hatching of second-stage juveniles from cysts of the potato cyst nematode, *Globodera pallida*.

A. There was a significant decrease in the amount of second-stage juveniles (J2s) hatching from cysts that were incubated in potato root diffusate (PRD), which had been pre-infected with aphids from day 10 (APRD) (n=12, *= 0.01, ** = 0.001). When cysts incubated in APRD were washed and re-incubated in non-infected PRD there was a significant increase in the amount of J2s hatching from the cysts (n=12, *** = 0.001, ** = 0.01). **B.** There was no significant difference between the amount of J2s hatched from cysts that had been incubated in either APRD or non-infected PRD at the end of the assay on day 34.

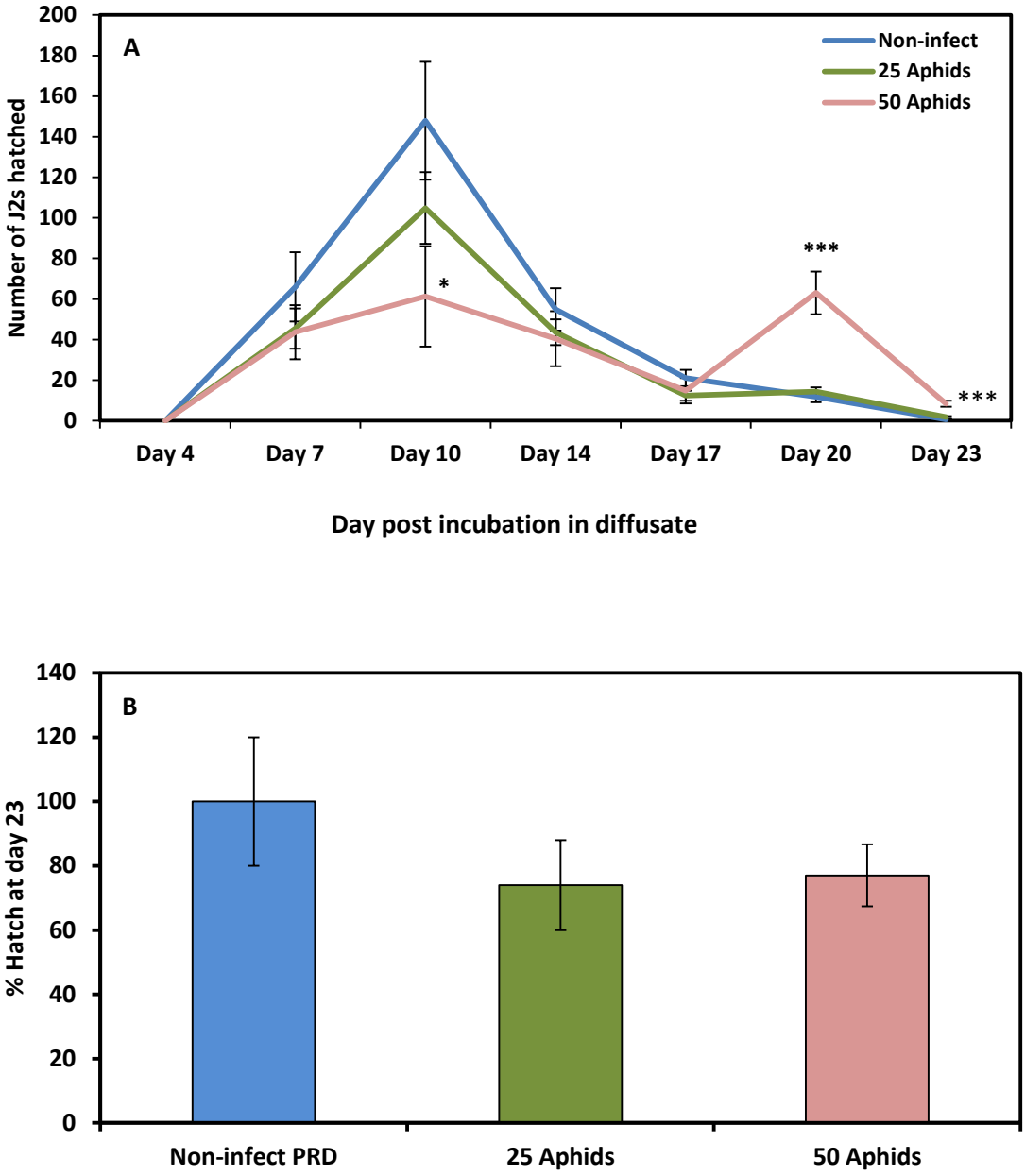

Figure 3.5. Effects of aphid infected potato root diffusate on the hatching of second-stage juveniles from cysts of the potato cyst nematode, *Globodera pallida* using a dose-dependent response.

A. There was a decrease in the in the amount of second-stage juveniles (J2s) hatching from cysts that were incubated in potato root diffusate (PRD) that had been pre-infected with 50 aphids from day 10 (n= 12, *** = 0.001). When cysts that had been incubated in APRD were washed and re-incubated in non-infected PRD there was a significant increase in the amount of J2s hatched from the cysts (P = 0.001). **B.** There was no significant difference in the total percentage hatch of J2s from cysts incubated in non-infected PRD or PRD that had been infected with 25 aphids or 50 aphids (n = 12).

assay no J2s emerged from cysts incubated in potato root diffusate from non-infected plants or plants that had aphids present until day 4 (Figure 3.5 a). From day 10 post incubation in either infected or non-infected PRD, there was a significant reduction in the number of J2s emerging from cysts incubated in PRD from plants infected with 50 aphids compared to those that had been incubated in PRD from non-infected plants (P = 0.05) (Figure 3.5 a). There was also a reduction in the number of J2s that emerged from cysts, which had been incubated in potato root diffusate from plants infected with 25 aphids, however this was non-significant. At day 17 post incubation, cysts that had been incubated in potato root diffusate from plants that had been infected with either 25 or 50 aphids were washed and re-incubated in potato root diffusate from non-infected plants. Again, normal emergence rates of J2s from cysts were restored. By day 20 post incubation the number of J2s that had emerged from re-incubated cysts was equivalent to the number of J2s that had emerged from cysts that had been incubated in non-infected potato root diffusate from the beginning of the experiment (Figure 3.5 b). This data indicates that the presence of aphids reduces hatching rates of J2s but the effect is dependent on the number of aphids that are present on the plant.

A dose-dependent response assay was also carried out using tomato root diffusate (TRD) from non-infected tomato plants and tomato plants that had been infected with 25 or 50 aphids. From day 10 post incubation in TRD there was a significant reduction in the number of J2s emerging from cysts that had been incubated in tomato root diffusate from plants that had been infested with 50 aphids 10 days prior to root harvesting (P = 0.05) (Figure 3.6 a). There was also a reduction in the number of J2s hatching from cysts incubated in tomato root diffusate from plants infected with 25 aphids, however this was not significant (Figure 3.6 a). This reduction in J2 emergence suggests that aphid infestation for 10 days prior to root harvesting does have an effect on hatching but is dependent on the number of aphids present on the plant. At day 17, cysts that had been incubated in tomato root diffusate from plants infected with either 25 or 50 aphids were washed and re-incubated in tomato root diffusate from plants that had no aphids present. However in this experiment there was no restoration of hatching as in the previous

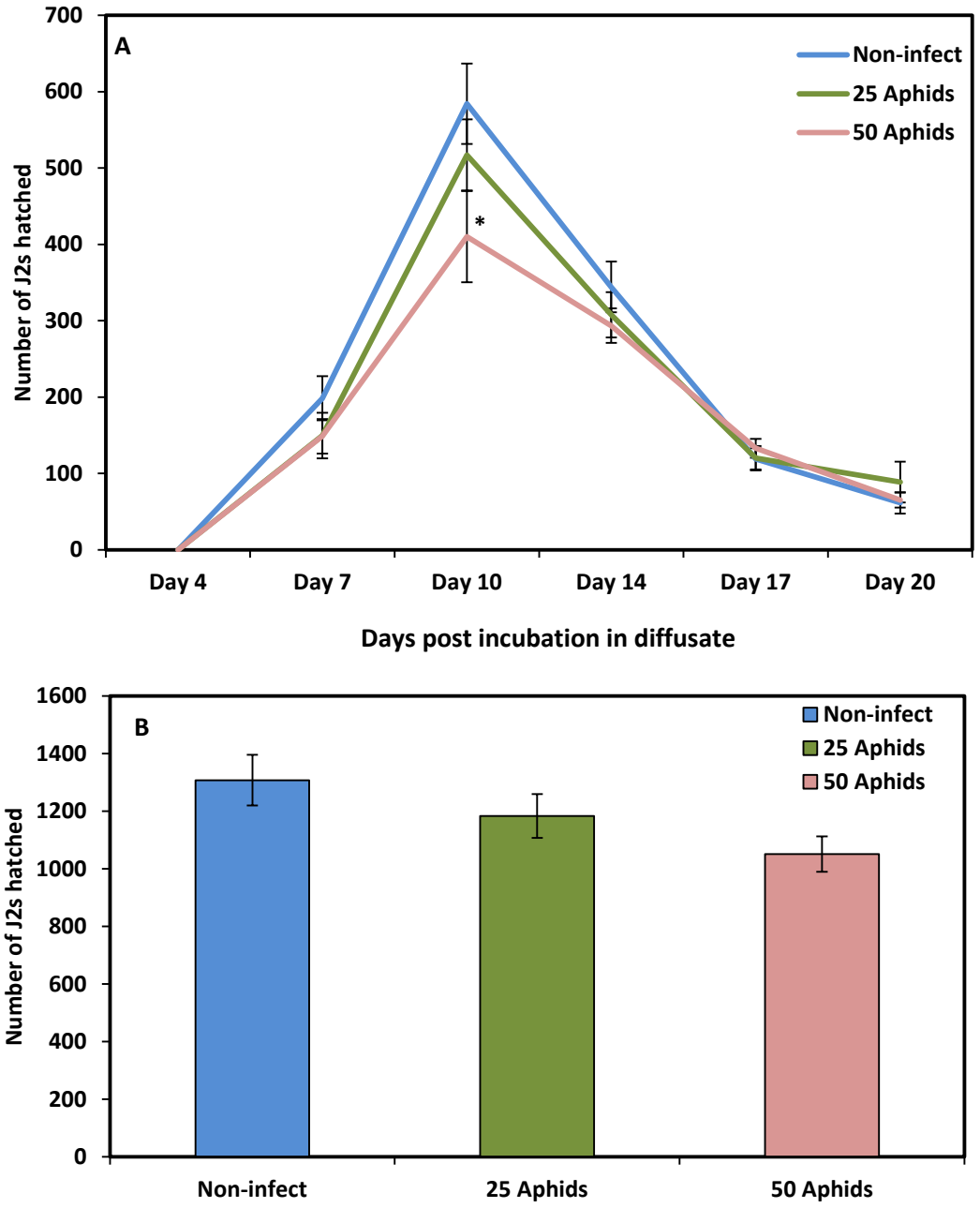

Figure 3.6. Effects of aphid infected tomato root diffusate on the hatching of second-stage juveniles from cysts of the potato cyst nematode, *Globodera pallida* using a dose-dependent response.

A. There was a decrease in the in the amount of second-stage juveniles (J2s) hatching from cysts that were incubated in tomato root diffusate (TRD) that had been pre-infected with 50 aphids from day 10 (n= 12, * = 0.05). When cysts that had been incubated in APRD were washed and re-incubated in non-infected PRD there was no significant increase in the amount of J2s hatched from the cysts. **B.** There was no significant difference in the total percentage hatch of J2s from cysts incubated in non-infected PRD or PRD that had been infected with 25 aphids or 50 aphids by day 20 (n = 12).

with potato root diffusate. The number of J2s emerging from both the infected and control experiments continued to decline until J2 emergence ceased on day 20 post incubation in TRD (Figure 3.6 a). There was no significant difference in the cumulative hatch between any of the hatching assays (25 aphids, 50 aphids or control) performed using TRD at day 20 post incubation (Figure 3.6 b).

3.4.4. Sequence of arrival assays

In order to further assess whether aphids had an effect on either the infection rate of nematodes or their development, sequence of arrival assays were set up (Figure 3.1). There was no significant difference between the total number of nematodes on potato plants that had been infected with nematodes for 7 days prior to the application of aphids for 7 days and the number of nematodes on plants that had been infected with nematodes only for 14 days (Figure 3.7). This was also the case for potato plants that had both aphids and nematodes applied simultaneously for a total time period of 14 days (Figure 3.7). There was also no significant difference in the number of nematodes in each developmental stage across all treatments (Figure 3.7). These results indicate that aphids do not affect the infectivity or development of J2s when aphids infest the plant after nematodes or if aphids infest the plant at the same time as nematodes infect the plant. However, when aphids were applied to the plant 7 days prior to the arrival of nematodes there was a significant reduction in the number of J2s found in the root system of potato plants compared to potato plants that had only nematodes introduced around the root system for a period of 7 days (P = .049) (Figure 3.8). This suggests that aphids arriving on the plant first has an effect on the reproduction of nematodes when they infect the plant, possibly due to a defence mechanism that may have been elicited in the roots of the plant due to the presence of aphids 7 days before the arrival of nematodes. Alternatively, phloem feeding by the aphids may reduce resources being translocated from the leaves to the roots for nematodes to utilise.

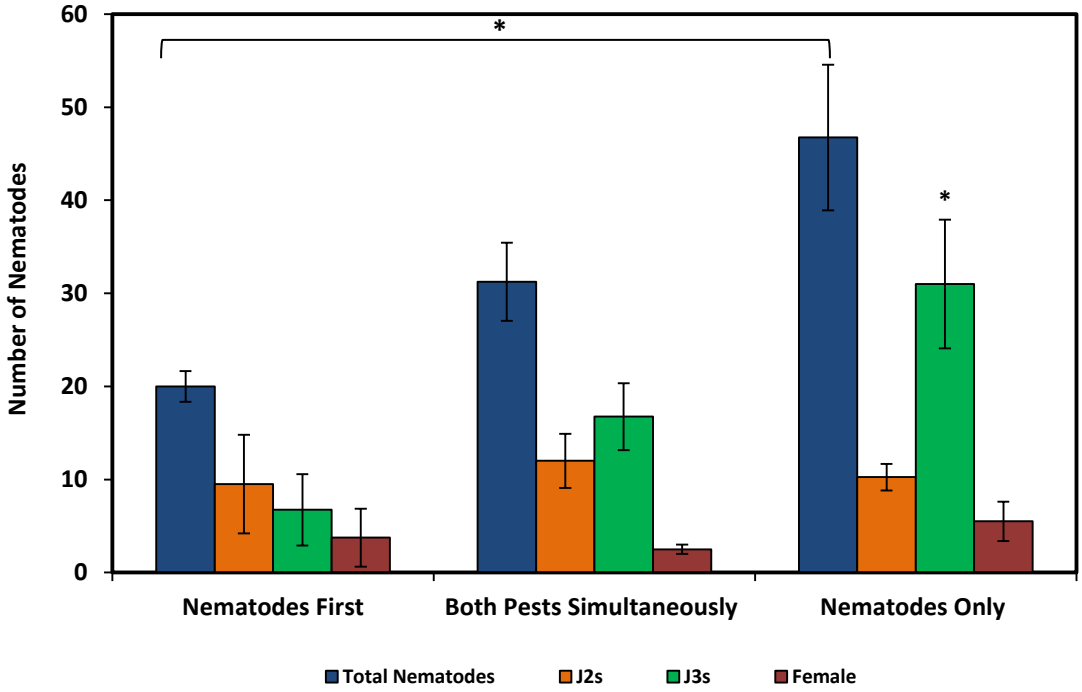

Figure 3.7. Sequence of pest arrival assays.

Assays were set up to determine how the development and infectivity of second-stage juveniles (J2s) of the potato cyst nematode were affected when potato plants were infected with *Globodera pallida* first, *Myzus persicae* first, or when both pests were applied simultaneously. Nematodes were present on plants for a total of 14 days across all experiments. There was a significant difference in the total number of nematodes that infected potato plants that had been infected with nematodes first before the arrival of aphids for seven days compared to those plants that received just nematodes (n = 4, *P* = 0.43). There was also a significantly higher number of nematodes that had developed into J3s on plants that received nematodes only compared to those plants that had aphids present (n = 4, *P* = 0.43).

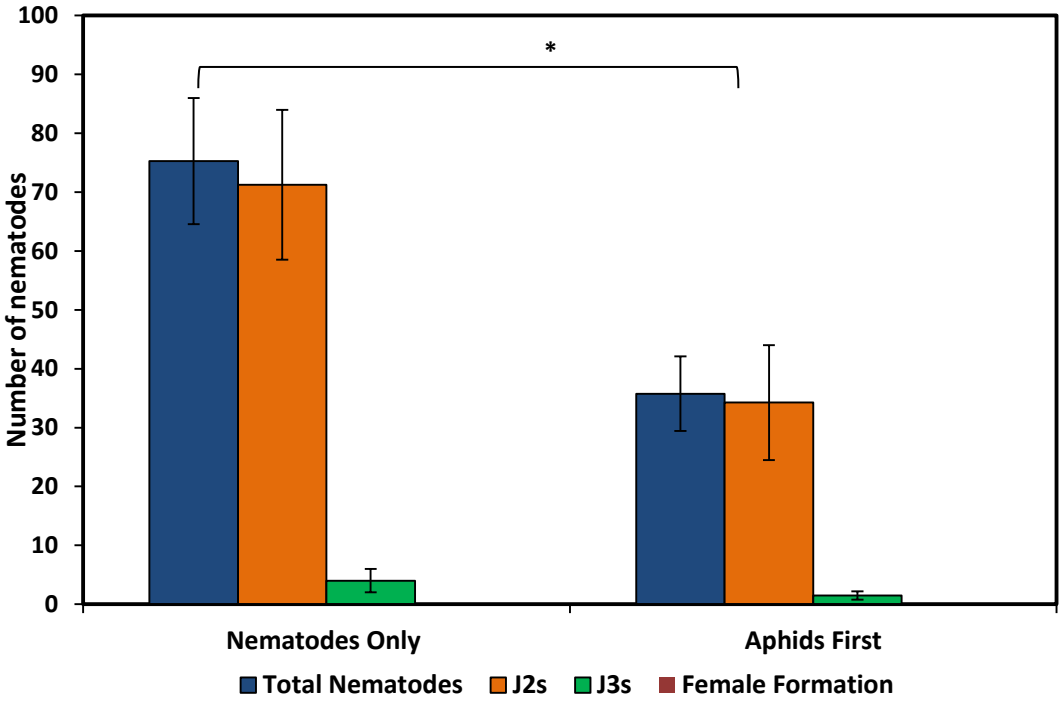

Figure 3.8. Sequence of pest arrival assays.

Assays were set up to determine how the development and infectivity of second-stage juveniles (J2s) of the potato cyst nematode were affected when potato plants were infected with *Globodera pallida* first, *Myzus persicae* first, or when both pests were applied simultaneously. Nematodes were present on plants for a total of seven days across both experiments. There were significantly fewer second stage juveniles found in the roots of potato plants that had been infected with aphids seven days prior to the arrival of nematodes compared to potato plants that received only nematodes (n=8, $P = 0.05$).

3.5 Discussion

3.5.1. Aphid abundance assays

There is increasing evidence that above-ground pests and below-ground pathogens on a shared host can interact with one another despite their spatial separation (Gange and Brown, 1989, Masters *et al.*, 1993, Erb *et al.*, 2008, Johnson *et al.*, 2013). Such interactions are diverse and five possible outcomes have been described (Arthur and Mitchell, 1989): competition (where both species are competing and have a detrimental effect on one another), amensalism (where there is a negative effect on only one of the attackers), contramenalism (where there is a positive effect on one of the attackers and an opposing negative effect on the second attacker), commensalism (where there is a positive effect on only one of the attackers) and mutualism (where there is a positive effect on both attackers). Below-ground pathogens can directly or indirectly affect the growth of the host plant by chewing or sucking essential nutrients from the roots, which in turn can negatively affect the performance of an above-ground herbivore. Alternatively, infection of the roots by a below-ground herbivore can cause an increase in soluble amino acids and carbohydrates in the foliar parts of the host plant thus increasing the reproduction of an above-ground pest (Masters *et al.*, 1993, Rowntree *et al.*, 2014, Johnson *et al.*, 2016).

Previous studies have focussed on the interactions between different species of plant parasitic nematodes and aphids; however the results have been conflicting. In this study we found a positive interaction (commensalism) between the potato cyst nematode, *Globodera pallida* and the peach potato aphid, *Myzus persicae* in which more aphids were present on plants infected with nematodes compared to control plants. Plant parasitic nematodes can affect host plants in a number of ways including systemically altering the growth, reproduction and physiology of foliar parts of the plant (Trudgill, 1991), thus affecting the abundance of an above-ground pest. The majority of studies report nematodes as having a negative impact (amensalism) on aphid fecundity (Bezemer *et al.*, 2005, Wurst and van der Putten, 2007, Kaplan *et al.*, 2009, Hol *et al.,* 2010) with changes in primary and secondary metabolites playing a decisive role in this negative interaction between the two

pests. Either aphid fecundity, aphid performance (ability to survive) or both have been negatively correlated with changes in phenolics and amino acid changes (Bezemer *et al.*, 2005), iridoid glycosides (Wurst and van der Putten, 2007) and amino acid changes and glucosinolate levels (Hol *et al.*, 2013) in response to nematode infection in the host plant.

There are only a small number of studies that report nematodes having a neutral effect on the performance or fecundity of aphids. However, the studies did not focus on the possible molecular mechanisms behind this interaction (Hong *et al.*, 2010). There are reports to suggest that the host plants defence pathway may play a role in commensalism between two pests from different kingdoms. The plant defence pathway is regulated by three main phytohormones: salicylic acid, jasmonic acid and ethylene, all of which can act antagonistically or synergistically with each other. The phytohormone elicited by a particular pathogen can be partly predicted from their feeding strategy, for example biotrophic pathogens primarily elicit an effective salicylic acid-mediated defence pathway, whereas necrotrophic and piercing/sucking pathogens primarily elicit the jasmonic acid and ethylene defence pathway. Depending on which invading pathogen arrives first, the resulting phytohormone that is activated may impact the fecundity or performance of the secondary arriving pest positively or negatively. The contribution and timing of these phytohormones are crucial to plant-mediated interactions between two or more pests on a shared host (Solano and Gimenez-Ibanez, 2013). To date there has been no clear effect on below-ground pathogens on aphids. In three different studies, which used different biotrophic pathogens the results showed both facilitation and inhibition of aphids conflicting generalisations on how biotrophic pathogens may affect the performance of secondary arriving pests (Al-Naemi and Hatcher, 2013, Johnson *et al.*, 2003, Lee *et al.*, 2012).

Metabolomics is becoming a widely used scientific tool to assess changes in pathogen infected plants and this growing technology has been the main methodology used to better understand plant-mediated interactions between different pests and pathogens. Secondary arriving pests have been reported to be sensitive to a range of secondary metabolites (Bezemer *et al.*, 2005, Wurst and van

der Putten, 2007, Hol *et al.*, 2013), therefore this technique coupled with an investigation into the profile of potential hormonal crosstalk upon infection with nematodes and aphids singly as well as simultaneously may elucidate our findings of a positive asymmetric interaction between *G. pallida* and *M. persicae*.

3.5.2. Aphid Preference Assays

Although recent studies on plant-mediated interactions have focussed on the molecular and biochemical response of the host plant to primary infection and how this may affect the secondary arriving attacker, the identification and quantification of volatile organic compounds (VOCs) is another set of biochemical experiments that are of importance in elucidating a relationship between two or more pests. VOCs are defined as any organic compound with vapour pressure high enough to be vapourised into the atmosphere (Yuan *et al.*, 2009). Although it is difficult for a human to detect VOCs, these compounds can be an attractant or warning to potential secondary arriving pests. To date, there is no evidence to suggest that infection with nematodes releases VOCs in the shoots of plants, however the release of these chemicals cannot be ruled out in plant-mediated interactions between aphids and nematodes because there have been studies that report the release of VOCs caused by other biotic stressors that can influence the performance of aphids (Hong *et al.*, 2010, Ferreres *et al.*, 1989, Medina-Ortega *et al.*, 2009, Werner *et al.*, 2009). Viruses including barley yellow dwarf virus (BYDV) (Bosque-Pérez and Eigenbrode, 2011), the turnip mosaic virus (TMV) (Casteel *et al.*, 2014) and the potato leaf roll virus (PLRV) (Bosque-Pérez and Eigenbrode, 2011) are stressors that have been reported to influence the performance of aphids. These viruses manipulate host physiology in order to attract aphids, which in turn act as their vectors and thus can transmit the viruses to a new host.

In this study the preference of *M. persicae* to either nematode-infected plants or control plants was tested. There was no significant difference found between the number of *M. persicae* on plants infected with nematodes for 14 days or control plants. This suggests that *M. persicae* were not influenced by VOCs or that potato plants infected by nematodes did not release VOCs at 14 days post infection (dpi). However, at 21 dpi there were significantly more aphids found on plants that had

been pre-infected with nematodes suggesting that nematode infected plants may be releasing VOCs that influenced the settling of aphids. As VOCs were not measured in this experiment, we cannot be sure if it is this mechanism underpinning these findings.

3.5.3. Potato root diffusate assays

Second-stage juveniles (J2) of the golden potato cyst nematode, *Globodera rostochiensis* and the white potato cyst nematode, *G. pallida* hatch from eggs when they are exposed to root diffusate from host plants containing hatching factors (Perry, 1989, Devine *et al.*, 1996). A change in permeability of the eggshell (Clarke *et al.*, 1978) and movement of trehalose from the periotic fluid followed by an influx of water into the egg rehydrates the J2 and thus allows it to become mobile and cut its way out of the egg (Perry, 1978). Identification of potato root diffusate hatching factors responsible for the stimulation of J2 hatching has resulted in a number of chemical formulas for hatching factors being presented however, due to the high specific activity of these hatching factors only trace amounts were available thus making their purification and identification difficult (Devine *et al.*, 1996). To date, only nine hatching factors have been identified including the potato glycoalkaloids solanine and α-chaconine (Devine and Jones, 2000).

We observed a decrease in the percentage hatching rate of J2s from eggs encapsulated inside cysts that were incubated in potato root diffusate collected from potato plants that had been infected with a large number of *M. persicae* for ten days before the three week harvesting date of roots. In addition, there was a delay in hatching of J2s from eggs that had been incubated in potato root diffusate from aphid infected plants. However, once these cysts were washed and re-incubated in potato root diffusate that was collected from non-infected control plants normal hatching rates of J2s was rescued. These findings led to further but more controlled experiments. Hatching assays were repeated to assess whether the number of aphids present on the plant played a role in the slower emergence of J2s from cysts. It was noted that as little as 25 aphids reduced the percentage of J2s emerging from cysts compared to those incubated in potato root diffusate collected from control plants. It was further observed that potato root diffusate collected

from plants that had 50 aphids present significantly reduced the percentage hatching of J2s compared to control plants. Similarly, when cysts were washed and re-incubated in potato root diffusate that had been collected from non-infected potato plants normal hatching rates of J2s was rescued. An identical experiment was set up, however with tomato root diffusate to test whether these hatching observations made with potato root diffusate could be made in a different plant. The same trend of delayed hatching of J2s was observed when cysts were incubated in tomato root diffusate collected from plants infected with 25 aphids and 50 aphids, however when the cysts were washed and re-incubated in tomato root diffusate from control plants normal hatching rates were not rescued. This may suggest that effects on tomato root diffusate caused by the presence of aphids are irreversible.

These observations of a delayed hatching rate that can be rescued once reintroduced to root diffusate from non-infected plants could indicate that aphids may affect hatching factors in root diffusates in two possible ways: decreasing hatching stimulants or increasing hatching inhibitors. However, as no research on the effects of aphids on plant root diffusates can be currently found in the literature no assumptions can be made until further assays across different plant, aphid and nematode species are carried out.

3.5.4. Sequence of arrival assays

Following attack by a pathogen the host plant activates a range of signal transduction cascades that help it tolerate damage and protect itself from further damage (Jones and Dangl, 2006). This increased defence is not only triggered locally but also in undamaged systemic tissues (Heil and Ton, 2008, Schwachtje and Baldwin, 2008). Such systemic effects can have a profound effect in shaping the relationship between spatially and temporally separated pathogens (Van der Ent *et al.*, 2008, Poelman *et al.*, 2008) and in recent years it has been noted that below-ground pathogens can affect plant physiology and subsequently impact the performance of above-ground attackers and *vice versa* (Erb *et al.*, 2008, Erb *et al.*, 2011). Research has indicated that it is not just changes in plant physiology and changes in host quality that affects the outcome of plant-mediated interactions but

it's also the combination of attackers in addition to the sequence of arrival of each pest that plays a role (Blossey and Hunt-Joshi, 2003, Erb *et al.*, 2011, Johnson *et al.*, 2012).

Plant-mediated interaction studies between plant-parasitic nematodes and aphids have mainly focussed on the effect of nematodes on the performance of aphids (Bezemer *et al.*, 2005, Wurst and van der Putten, 2007, Kaplan *et al.*, 2009, Hol *et al.*, 2010). There are only a small number of investigations into the effects that aphids may have the reproduction of nematodes (Kaplan *et al.*, 2009, Kutyniok and Müller, 2012). There was no significant difference between the number of nematodes found in the roots of potato plants that were introduced to the plant for seven days before the introduction of aphids for a period of seven days (Experiment A) and potato plants that were infected with nematodes only for 14 days (Experiment E - see figure 3.1 for experimental design set-up). However, when aphids were present on the plant for seven days before the introduction of nematodes for 7 days (Experiment B) there were significantly less nematodes present on those dual infected plants compared to potato plants that had been infected with nematodes only for seven days (Experiment D). This suggests that aphids may have an effect on the infectivity of second-stage juveniles by either activating a cascade of defence signalling events in the roots in the 7 days prior to the arrival of nematodes or perhaps altering hatching factors in root exudates therefore deterring nematodes from entering host roots. Similar results were previously found in a study involving the leaf herbivore, *Spodoptera frugiperda* and the root-feeding herbivore, *Diabrotica virgifera virgifera* where *S.frugiperda* had a negative effect on *D. virgifera* but only when *S. frugiperda* arrived on the plant before the root feeder (Erb *et al.*, 2011). Foliar-feeding organisms, like below-ground organisms have been shown to influence plant defence responses, albeit not as extensively. Reduced alkaloid concentrations in ragwort root were observed following caterpillar feeding above-ground (Hol *et al*., 2004), however in other studies using foliar-feeding insects (Bezemer et al., 2004), shoot hormone applications (Van Dam *et al*., 2004) or moderate artificial defoliation (Collantes *et al*., 1998) no significant difference could be found between experimental and

control plants (Bezemer and van Dam, 2005). The lack of defence response in the roots of plants may be attributed to the source-sink relationship within a plant i.e. carbon translocated to the roots in response to above-ground feeding may be used towards growth and reproduction of the plant (Bardgett *et al*., 2005). No literature could be found to suggest that aphids induce a defence response in the roots of plants therefore more research needs to be carried out. A further study needs to be carried out in order to investigate whether the presence of aphids on the plant 7 days prior to the infestation has an effect on the infectivity of nematodes or the reproduction of nematodes when the aphids are removed before the introduction of nematodes. In nature, however, below-ground pathogens such as plant parasitic nematodes may be able to avoid the negative effects on their infectivity and reproduction from above-ground pests, as nematodes infect the plant soon after roots emerge and above-ground pests usually infect the host much later in the year once a sufficient foliage is formed (Van Emden *et al.*, 1969). Still, even though in reality nematodes may avoid negative effects in the plant caused by above-ground pests, aphids were found to have an effect on the emergence of J2s from cysts and also reduce the infectivity of J2s. Therefore, these findings could be exploited by biotechnology in the future to develop resistance against plant parasitic nematodes. If a specific defence mechanism in the roots can be triggered by aphids to reduce the infectivity of plant parasitic nematodes, resistant crops against nematodes can be produced.

These results show that the sequence of arrival is important in the outcome of plant-mediated interactions between above- and below-ground pests. However, more research needs to be carried out in order to elucidate the molecular and biochemical mechanisms involved in the negative effect that an above-ground pest has on a below-ground pathogen when it arrives first. Gene expression, phytohormone quantification and metabolomics studies on the roots of the potato plant would reveal much data in order to decode the relationship between *Globodera pallida* and *Myzus persicae*.

Summary

- The presence of *Globodera pallida* on potato plants (cv. Désirée) for 14 days has a positive effect on the performance of *Myzus persicae* whereby the abundance of aphids is increased.

- The presence of nematodes on potato plants for 21 days influences the settling of aphids on nematode-infected plants compared to non-infected control plants.

- The presence of aphids on potato plants reduces the hatching rate of *G. pallida* second-stage juveniles (J2s).

- A reduction in the emergence rate of J2s from cysts incubated in root diffusate collected from potato plants pre-infected with aphids is dependent on the infection level of aphids.

- A dose-dependent reduction in the emergence rate of J2s from cysts incubated in root diffusate from tomato plants pre-infected with aphids was observed, however once the cysts were washed and re-incubated in non-infected tomato root diffusate original emergence rates or rescuing of emergence rates was not observed.

- The reduction in the emergence rate of J2s from cysts incubated in infected root diffusate is reversible.

- The infectivity and development of J2s is compromised in roots of potato plants that have been pre-infected with aphids.

Chapter 4

Characterising the molecular response of susceptible and resistant cultivars of *Solanum tuberosum* to attack by *Globodera pallida* and *Myzus persicae*, alone and in combination

4.1. Introduction

To protect themselves from invading pathogens, plants have evolved basal defence mechanisms that include chemical and physical barriers and a highly specialised resistance (R) gene-mediated defence (see section 1.1.2). In recent years it has been revealed that plants use similar *R*-gene-based resistant mechanisms to protect themselves from parasitic nematodes (Williamson and Kumar, 2006). Resistance to nematodes is expressed in many forms, and is conditioned by a wide variety of genetic systems. The resistance response can be determined by a single dominant gene (vertical resistance), which has a major effect such as a hypersensitive response by affected plant cells. It may also be determined by multiple genes (horizontal resistance), which may also present a hypersensitive response or just have a less dramatic response by not supporting well-developed giant cells or syncytia, therefore reducing the developmental rate of the nematode (Starr *et al.*, 2013).

Plants are defined as resistant to nematodes when they support reduced levels of nematode reproduction (Trudgill, 1991). A number of nematode resistant genes have been mapped to chromosomal regions and a few have been cloned (Williamson, 1999). *HS1*$^{pro-1}$ was reported to be the first nematode resistant gene to be cloned from a wild relative of sugar beet that confers resistance against *Heterodera schachtii* (Cai *et al.,* 1997), however subsequent studies performed in different plant species suggested that this group of proteins has a more general role

in the regulation of plant responses to biotic and abiotic stresses (Puthoff *et al.*, 2003, Baena-González and Sheen, 2008). The most extensively studied nematode resistant gene is *Mi-1.2.*, which was introgressed into cultivated tomato, *Solanum lycopersicum* and is now widely used in commercial tomato production. The gene has many unique characteristics including the resistance it confers to the potato aphid and whiteflies (Milligan *et al.*, 1998, Nombela *et al.*, 2003, Rossi *et al.*, 1998). Although the gene confers resistance against both nematode and aphids in tomato, this was not the case for other species within the Solanaceae: when the gene was transformed into eggplant, *Mi*-1.2 only conferred resistance to root-knot nematodes but not to the potato aphid (Goggin *et al.*, 2006). In addition, the resistance of *Mi-1.2* was tested against other species of aphids, but it did not confer resistance to the generalist species *Myzus persicae* (Goggin *et al.*, 2001). There have been a number of genes that have been identified as conferring resistance against potato cyst nematodes. The broad-spectrum nematode resistant gene, *Hero*, was identified in wild tomato (*Lycopersicon pimpinellifolium*) and confers resistance to all pathotypes of the golden potato cyst nematode, *Globodera rostochiensis* and partially resistant to *G. pallida* pathotype Pa2/3 in tomato (Ellis and Smith, 1971, Ellis *et al.*, 2002). Many genes have been cloned from potato including the *Gro1-4* gene, which confers resistance against *G. rostochiensis* pathotype Ro1 (Paal *et al.*, 2004). The potato *Gpa2* gene was also cloned and is resistant to certain isolates of *G. pallida* (Van Der Vossen *et al.*, 2000). Potato cultivars that harbour the gene for H_1 resistance, which is derived from *Solanum tuberosum* ssp. *andigena,* confer resistance to the golden potato cyst nematode, *Globodera rostochiensis* (Forrest *et al.*, 1986).

Shortly after root invasion, sedentary nematodes elicit two basic defence mechanisms in resistant hosts. The first is a hypersensitive response (HR) in which the second-stage juvenile becomes surrounded by necrotised cells upon initiation of a feeding structure and is therefore unable to create a feeding site in order to complete reproduction (Sobczak *et al.*, 2005). This has been the described defence response to root-knot nematodes such as *Meloidogyne incognita* in tomato which harbours the *Mi-1.2* gene (Bleve-Zacheo *et al.*, 1998). The second type of response

occurs following infection with potato cyst nematodes (*Globodera* spp.) and is termed a "hypersensitive-like" response, and has been observed for the *H1* and *Hero* genes in resistant potato and tomato plants, respectively (Rice *et al.*, 1985). Unlike the defence mechanism in response to root-knot nematodes, potato cyst nematodes (PCN) are able to initiate a feeding site, however development is abnormal or the syncytium slowly deteriorates due to the formation of necrotised cells rendering it impossible for the syncytium to expand (Grymaszewska and Golinowski, 1998, Holtmann *et al.*, 2000). A resistant response can also occur in plants whereby cyst nematodes are able to form a small syncytium, but due to a lack of sufficient nutrients the nematodes are only able to develop into males and therefore cannot complete reproduction (Goverse and Smant, 2014).

Although many nematode resistant genes have been identified, to date, there has been no gene identified that confers full resistance to both potato cyst nematodes. Currently there is an overuse of resistant potato cultivars such as Maris Piper, which confers resistance to *G. rostochiensis*, in the UK. As there is no cultivar fully resistant to *G. pallida*, this species remains a major pathogen of the potato crop. In this study, potato plants (cv. Désirée) susceptible to PCN infection were infected with *G. pallida* and *M. persicae* singly as well as infected with both pests simultaneously. Phytohormones are increasingly being recognised as important signalling molecules that play a key role in mediating interactions between different organisms, which share the same host (see section 1.1.3). Therefore, endogenous levels of salicylic acid and jasmonic acid were quantified and the expression of three SA-mediated genes and two JA-mediated genes were measured in order to elucidate the molecular response to dual biotic stress in potato. Potato plants (cv. Maris Piper) resistant to PCN infection were also infected with *G. rostochiensis* and *M. persicae* singly as well as infected with both pests simultaneously in order to elucidate the effects of simultaneous attack by both pests on the plant. These resistant responses were then compared to those found in potato cultivars susceptible to nematode infection.

4.2. Aims

The following experiments were carried out in order to investigate the molecular responses of different cultivars of potato in response to nematode and aphid infection singly as well as in combination.

1. To quantify the endogenous phytohormones salicylic acid and jasmonic acid in potato cultivars susceptible to the potato cyst nematode, *Globodera pallida* and the peach potato aphid singly as well as simultaneously with both pests.

2. To measure the expression of salicylic acid- and jasmonic acid-mediated genes in potato cultivars susceptible to the potato cyst nematode, *Globodera rostochiensis* and the peach potato aphid singly as well as simultaneously with both pests.

3. To investigate potential hormonal crosstalk between molecular pathways upon co-infection of the potato plant with both *Globodera pallida* and *Myzus persicae* simultaneously.

4.3. Results

4.3.1. Salicylic acid- and jasmonic acid-mediated defence responses in susceptible potato cultivars infected with *Globodera pallida*

Salicylic acid- and jasmonic acid-mediated defence responses were measured in the leaves of susceptible potato plants (*Solanum tuberosum* cv. Désirée) infected with the potato cyst nematode, *Globodera pallida* 14 days post inoculation (dpi) (Figure 4.1). There was a significant difference in the levels of free (571 ± 70 ng, $P < 0.002$), stored (3996 ± 231 ng, $P < 0.003$) and total endogenous salicylic acid (4541 ± 268 ng, $P < 0.007$) in plants infected with *G. pallida* for 14 days compared to non-infected control plants (231 ± 27 ng, 1901 ± 761 ng, 2132 ± 758 ng, respectively) (Figure 4.1 a). This suggests that infection with *G. pallida* elicits the salicylic acid defence pathway systemically in potato plants. The levels of endogenous jasmonic acid were quantified from the same leaf as where the salicylic acid was quantified. There was no significant difference found between the levels of jasmonic acid in plants infected with nematodes for 14 days (348 ± 21 ng) compared to control plants (328 ± 32 ng) (Figure 4.1 b). This suggests that *G. pallida* does not elicit jasmonic acid-mediated defences in the leaves of the host plant. Phytohormones lead to the downstream activation of defence genes (Cao, *et al*, 1997; van Loon, 2006) therefore the expression of genes mediated by salicylic acid (*PR*-1, *PR*-2 and *PR*-5) and jasmonic acid (*PR*-3 and *JAZ*-1) were analysed. There was no significant difference found in the expression levels of *PR*-1 or *PR*-2 in plants infected with nematodes for 14 days, however there was a significant increase in *PR*-5 suggesting an activation of salicylic acid-mediated genes ($P < 0.027$) (Figure 4.1 c). There was no significant difference in the expression of *PR*-3 or *JAZ*-1 in infected plants compared to control plants (Figure 4.1 c), which correlated with the endogenous jasmonic acid results (Figure 4.1 b).

To complement data collected for the time-point of 14 dpi with *G. pallida*, a full time course analysis of the levels of endogenous salicylic acid was conducted, with measurements at 3, 7 and 21 dpi with *G. pallida* (Figure 4.2), alongside an analysis of the expression of defence genes for the same time-points (Figure 4.1 c). There

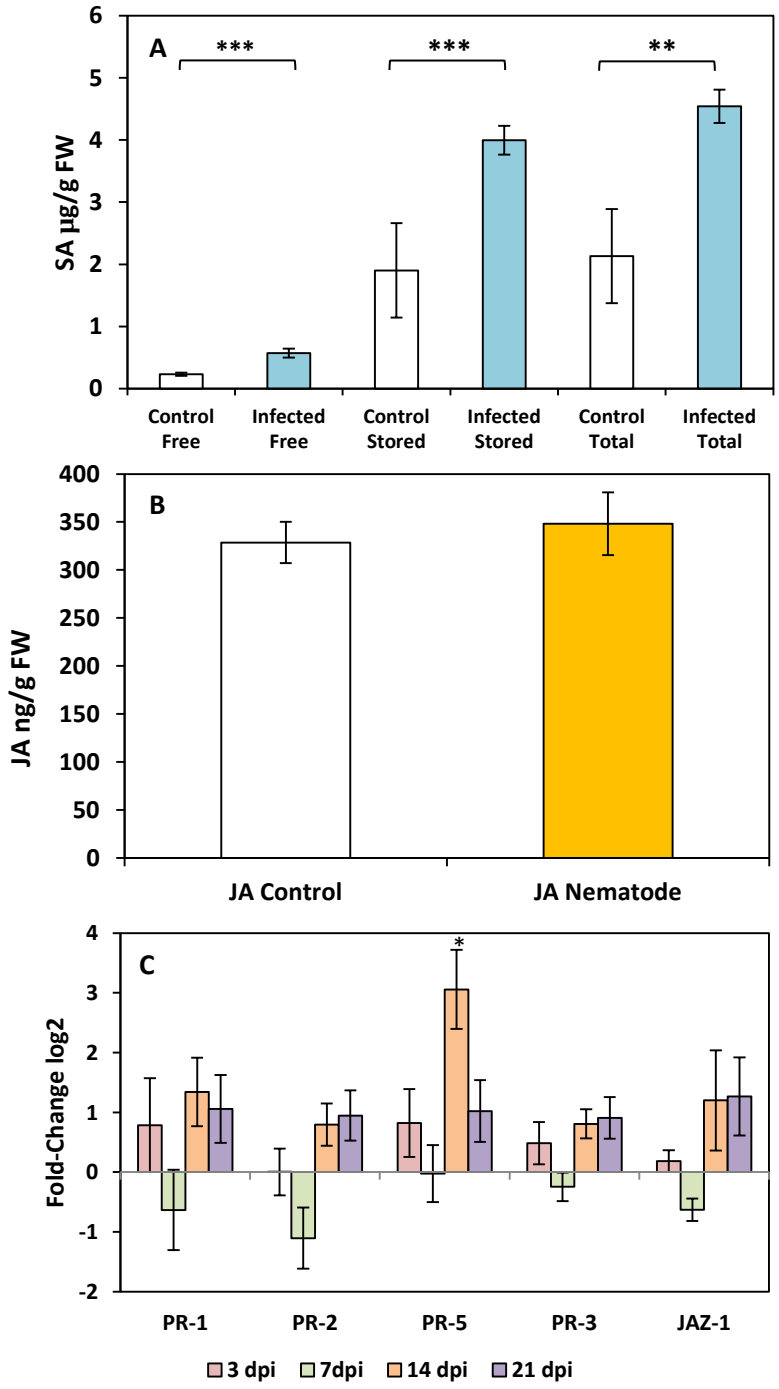

Figure 4.1. Quantification of endogenous salicylic acid and jasmonic acid and analysis of PR-gene expression by qRT-PCR in leaves of potato plants (*Solanum tuberosum* cv. Désirée) infected with the potato cyst nematode, *Globodera pallida*.

A. Levels of endogenous salicylic acid in leaves of potato plants infected with *G.* pallida 14 days post inoculation (dpi). **B.** Levels of endogenous jasmonic acid in leaves of potato plants infected with *G. pallida* 14 dpi. **C.** Expression levels of *PR*-genes in the leaves of potato plants infected with *G. pallida* at 3, 7, 14 and 21 dpi. The presented data are the mean fold changes ± standard errors of biological replicates in both graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from different biological replicates (Mann-Whitney U, * $P < 0.05$, n = 5 (qPCR and JA analysis), n=12 (Endogenous SA)).

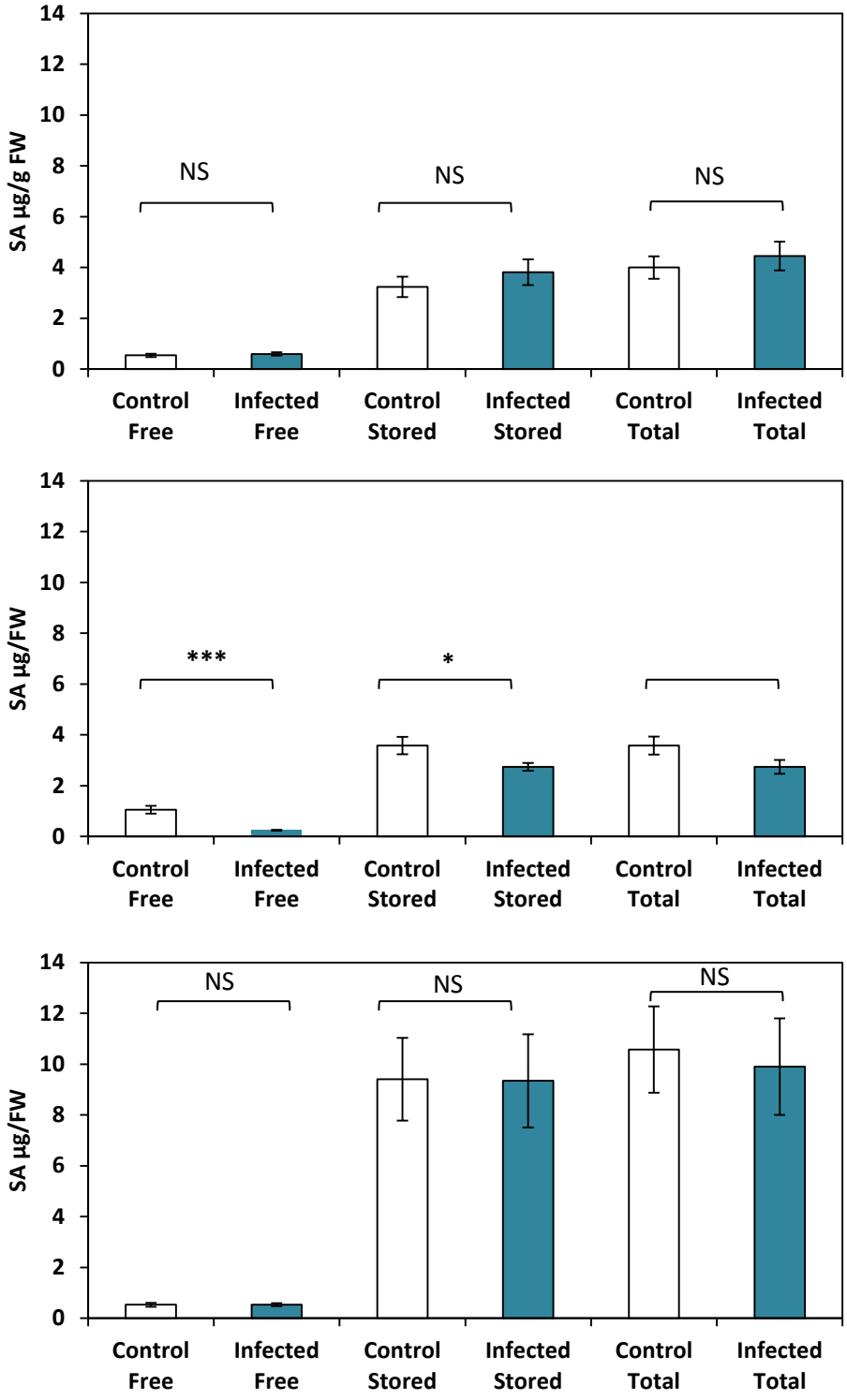

Figure 4.2. Quantification of endogenous salicylic acid in leaves of potato plants (*Solanum tuberosum* cv. Désirée) infected with the potato cyst nematode, *Globodera pallida*.

A. Levels of endogenous salicylic acid in leaves of potato plants infected with *G.* pallida 3 days post inoculation (dpi). **B.** Levels of endogenous salicylic acid in leaves of potato plants infected with *G. pallida* 7 dpi. **C.** Levels of endogenous salicylic acid in leaves of potato plants infected with *G. pallida* 21 dpi. The presented data are the mean fold changes ± standard errors of biological replicates in all graphs (Mann-Whitney U, * < 0.05, ** < 0.01, *** < 0.001, n = 7 (SA analysis 3 and 7 dpi), n = 9 (SA analysis 21 dpi).

was no significant difference in the levels of free (598 ± 69 ng), stored (3814 ± 509 ng) or total (4446 ± 565 ng) endogenous salicylic acid found in the leaves of potato plants infected with *G. pallida* for 3 days when compared to control plants (Free: 540 ± 63 ng, Stored: 3233 ± 403 ng, Total: 3997 ± 443 ng, respectively) (Figure 4.2 a). This mirrored the data collected for the expression of salicylic acid- and jasmonic acid-mediated genes as there was no significant difference in their expression between infected and control plants (Figure 4.1 c). Collectively, this suggests that salicylic acid-mediated defences are not activated by *G. pallida* 3dpi.

In contrast, plants that had been infected with the same number of nematodes but for a period of seven days, levels of free (245 ± 18 ng, $P < 0.000$), stored (2738 ± 157 ng, $P < 0.012$) and total (2738 ± 275 ng, $P < 0.001$) endogenous salicylic acid were significantly down-regulated compared to control plants (Free: 1052 ± 153 ng, Stored: 3579 ± 343 ng, Total: 3579 ± 350 ng, respectively) (Figure 4.2 b). Although expression levels of salicylic acid defence-related genes did not significantly change between nematode infected and control plants following infection for 7 days (Figure 4.1 c) there was an indication that these genes showed a trend similar to that of the levels of endogenous salicylic acid (Figure 4.2 b). These results could suggest that *G. pallida* is able to suppress salicylic-acid mediated defences 7 dpi.

There was no significant difference in the levels of endogenous salicylic acid in the leaves of potato plants infected with nematodes for 21 days (Free: 533 ± 61 ng, Stored: 9344 ± 1835 ng, Total: 9901 ± 1896 ng) compared to non-infected control plants (Free: 530 ± 73 ng, Stored: 9400 ± 1628 ng, Total: 10574 ± 1700 ng) (Figure 4.2 c). There was also no significant increase in the expression of salicylic acid- and jasmonic acid-mediated genes at 21 dpi with nematodes (Figure 4.1 c). This suggests that salicylic acid mediated defences are not activated at this time-point.

4.3.2. Salicylic acid- and jasmonic acid-mediated defence responses in susceptible potato cultivars with the peach potato aphid, *Myzus persicae*.

It should be noted that although potato plants were only infected with aphids for a period of 48 hours the time-points are annotated as 3, 7, 14 and 21 dpi to correspond with the time-points used for nematode infection (section 4.3.1). All experimental groups being compared (nematode only infection, aphid only

infection or co-infection) were the same age for phytohormone and gene expression analysis.

Five week old potato plants infected with aphids (equivalent to infection with *G. pallida* for 14 days) were analysed for levels of the endogenous hormones salicylic acid and jasmonic acid. In addition, the expression of the defence genes mediated by these phytohormones was measured. There was a significant increase in the levels of free (686 ± 76 ng, $P < 0.000$), stored (7010 ± 547 ng, $P < 0.000$) and total (8046 ± 555 ng, $P < 0.000$) endogenous salicylic acid in the leaves of plants infected with aphids compared to control plants (Free: 276 ± 32 ng, Stored: 3581 ± 392 ng, Total: 4055 ± 396 ng) (Figure 4.3 a). There was also a significant increase in salicylic acid-mediated defence genes, *PR*-1 ($P < 0.000$) and *PR*-5 ($P < 0.000$), however there was no significant increase in the expression of *PR*-2 (Figure 4.3 c). There was a significant increase in the endogenous levels of jasmonic acid in leaves locally infected with aphids (729 ± 22 ng) compared to control plants (356 ± 88 ng) ($P < 0.025$) (Figure 4.3 b) and this was also the case for the expression levels of the jasmonic acid-mediated defence genes, *PR*-3 ($P < 0.000$) and *JAZ*-1 ($P < 0.000$). Collectively, this data indicates that both the salicylic acid- and jasmonic acid-signalling pathways are elicited in the leaves of potato plants following infection with aphids.

A time-course analysis using the time-points of 3, 7 and 21 dpi with aphids was carried out for the measurement of endogenous levels of salicylic acid and the expression analysis of salicylic acid-mediated defence genes. There was a significant increase in the levels of free (1222 ± 204 ng, $P < 0.014$) salicylic acid but no significant increase in stored (8526 ± 1117 ng) or total (10586 ± 1307 ng) salicylic acid in three week old potato plants infected with aphids compared to control plants (Free: 657 ± 74 ng, Stored: 6001 ± 1196 ng, Total: 7294 ± 1257 ng) (equivalent to infection with *G. pallida* for 3 days) (Figure 4.4 a). The expression of all the salicylic acid-mediated defence genes (*PR*-1, *PR*-2 and *PR*-5) was significantly increased at 3 dpi with aphids. The expression of *PR*-1 increased 4-fold in comparison to control plants ($P < 0.001$), the expression of *PR*-2 increased almost

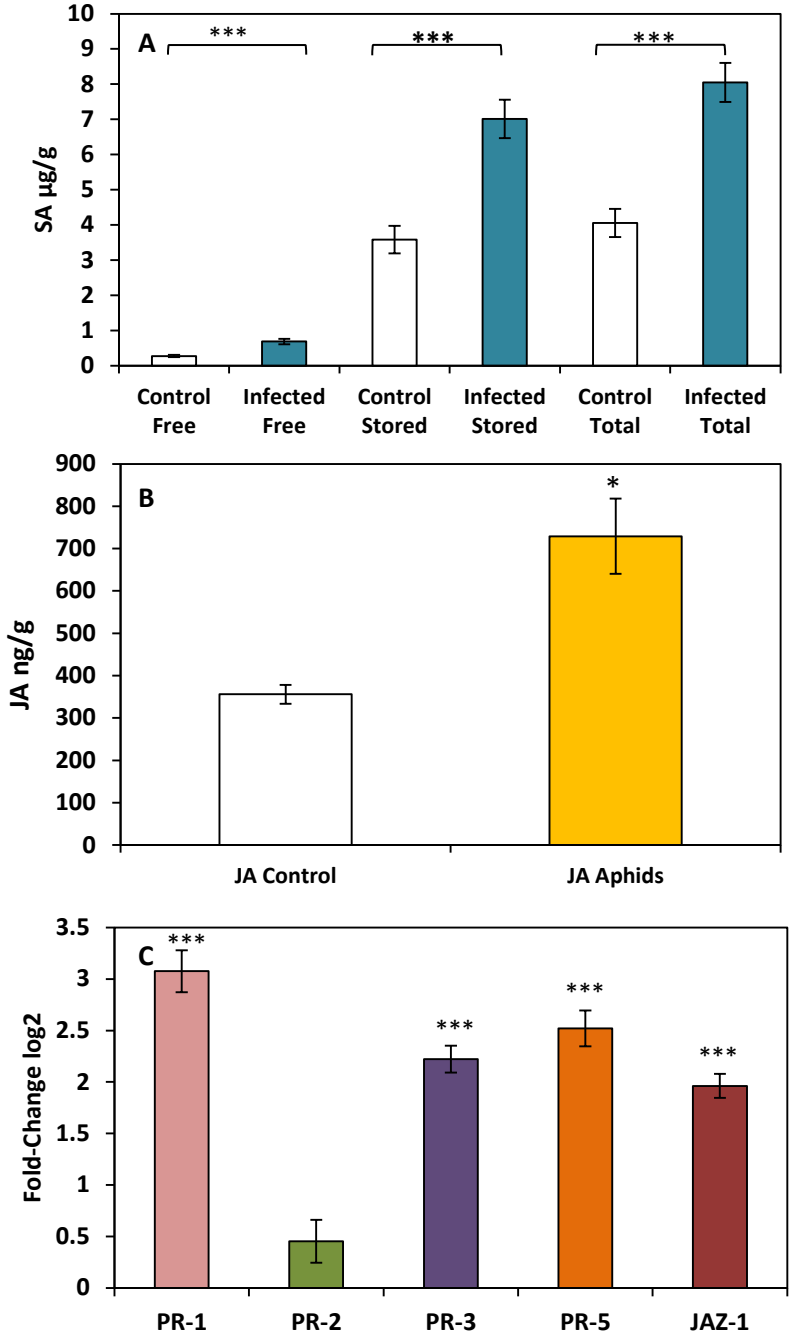

Figure 4.3. Quantification of endogenous salicylic acid and jasmonic acid and analysis of PR-gene expression by qRT-PCR in leaves of potato plants (*Solanum tuberosum* cv. Désirée) infected with the peach potato aphid, *Myzus persicae*.

A. Levels of endogenous salicylic acid in leaves of potato plants infected with *M. persicae* for 48 hours on 5 week old plants (equivalent to 14 dpi with *G.pallida*). **B.** Levels of endogenous jasmonic acid in leaves of potato plants infected with *M. persicae* for 48 hours on 5 week old plants. **C.** Expression levels of *PR*-genes in the leaves of potato plants infected with *M. persicae* for 48 hours on 5 week old plants. The presented data are the mean fold changes ± standard errors of biological replicates in all graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from different biological replicates (Mann Whitney U, *** $P < 0.001$, n = 12).

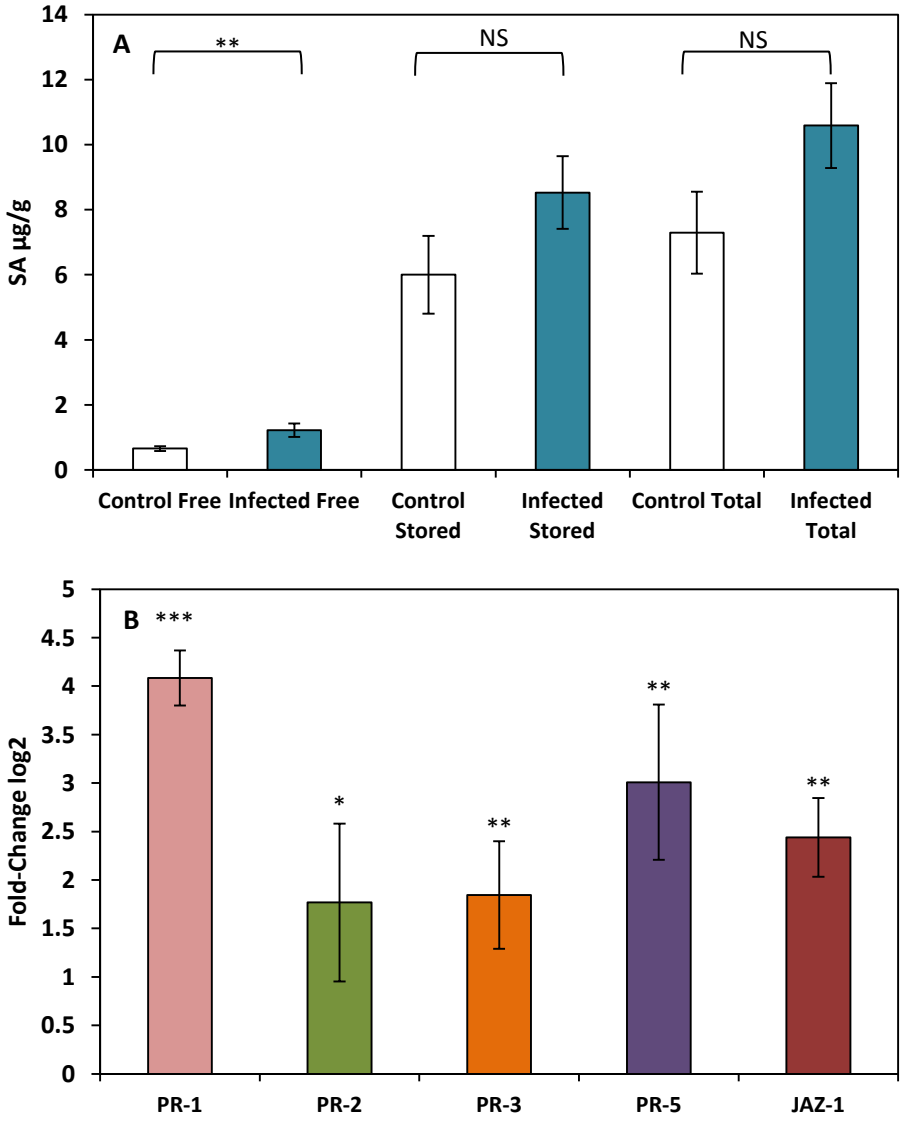

Figure 4.4. Quantification of endogenous salicylic acid and analysis of PR-gene expression by qRT-PCR in leaves of potato plants (*Solanum tuberosum* cv. Désirée) infected with the peach potato aphid, *Myzus persicae*.

A. Levels of endogenous salicylic acid in leaves of potato plants infected with *M. persicae* for 48 hours on 3 week old plants (equivalent to 3 dpi with *G.pallida*). **B.** Expression levels of *PR*-genes in the leaves of potato plants infected with *M. persicae* for 48 hours on 3 week old potato plants. The presented data are the mean fold changes ± standard errors of biological replicates in both graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from different biological replicates (Mann-Whitney U, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n = 12).

2-fold ($P < 0.02$) and the expression of *PR*-5 increased 3-fold ($P < 0.011$) in comparison to control plants in potato plants 3 dpi with aphids (Figure 4.4 b). Similarly, there was a significant increase in the expression of *PR*-3 ($P < 0.003$) and *JAZ*-1 ($P < 0.002$) (Figure 4.4 b). These results suggest that aphids elicit both the salicylic acid and jasmonic acid defence pathways in the leaves of potato plants.

There was no significant difference in free (1156 ± 272 ng), stored (8302 ± 1703 ng) or total (10151 ± 1954 ng) endogenous salicylic acid in potato plants that were infected with aphids for 48 hours on four week old plants (equivalent to plants infected with *G. pallida* for 7 days) compared to non-infected control plants (Free: 874 ± 347 ng, Stored: 7868 ± 2649 ng, Total: 9861 ± 2993 ng) (Figure 4.5 a). However, when both salicylic acid- and jasmonic acid-mediated gene expression was measured there was a significant increase in all genes tested (Figure 4.5 b).

The expression of *PR*-1 increased almost 5-fold in comparison to the control ($P < 0.001$) and the expression of both *PR*-2 and *PR*-5 increased 3-fold in comparison to the control ($P < 0.001$). The increase in the expression of salicylic acid mediated genes may suggest that they were activated independent of salicylic acid levels because there was no significant increase in endogenous salicylic acid. The expression of *PR*-3 and *JAZ*-1 increased by 100% in comparison to the control (Figure 4.5) suggesting an activation of the jasmonic acid pathway 7 dpi with aphids.

When six week old plants (equivalent to infection with *G. pallida* 21 dpi) were infected with aphids there was only a significant increase in free (2023 ± 355 ng) endogenous salicylic acid ($P < 0.036$). There was no difference in stored (21457 ± 2170 ng) or total (22663 ± 2398 ng) levels of endogenous salicylic acid compared to control plants (Free: 1878 ± 339 ng, Stored: 17406 ± 1168 ng, Total: 20068 ± 1172 ng) (Figure 4.6 a). When expression levels of salicylic acid-mediated genes were measured there was a significant increase in the expression of *PR*-1 and *PR*-5 ($P < 0.001$), however there was no significant increase in the expression of *PR*-2 compared to the control. This may suggest that aphids activate salicylic acid defence responses in potato plants but do not elicit β-1,3-glucanases as a defence

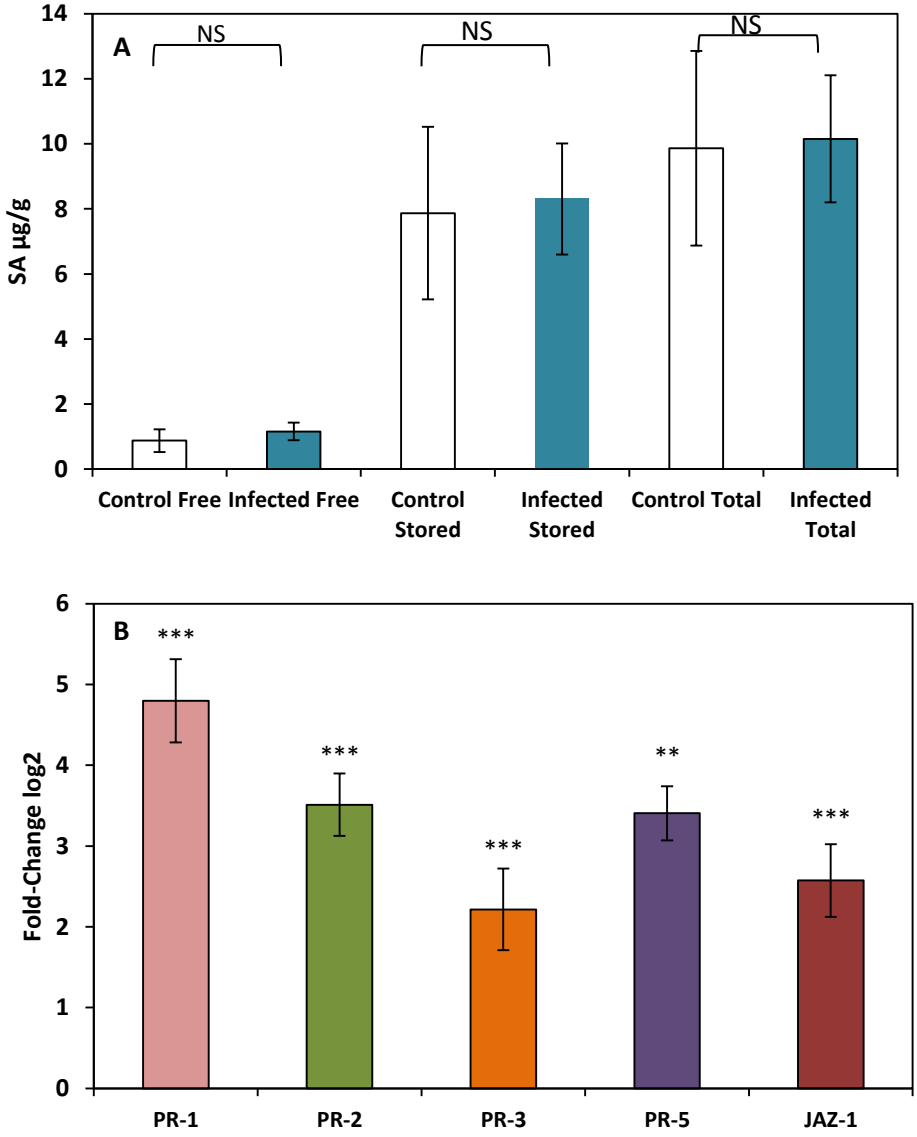

Figure 4.5. Quantification of endogenous salicylic acid and analysis of PR-gene expression by qRT-PCR in leaves of potato plants (*Solanum tuberosum* cv. Désirée) infected with the peach potato aphid, *Myzus persicae*.

A. Levels of endogenous salicylic acid in leaves of potato plants infected with *M. persicae* for 48 hours on 4 week old plants (equivalent to 7 dpi with *G.pallida*). **B.** Expression levels of *PR*-genes in the leaves of potato plants infected with *M. persicae* for 48 hours on 4 week old potato plants. The presented data are the mean fold changes ± standard errors in both graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from different biological replicates (Mann-Whitney U, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n = 6 (endogenous SA levels) n = 12 (gene expression analysis)).

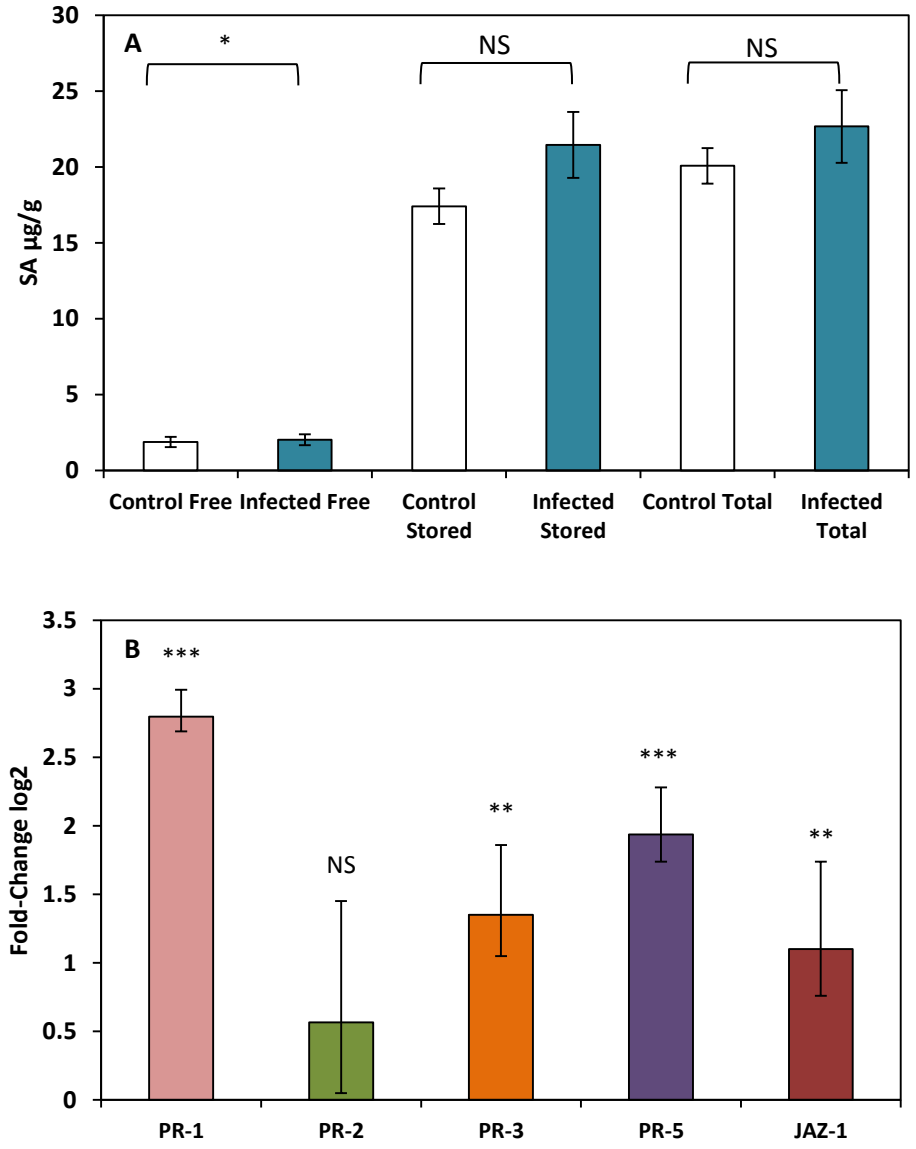

Figure 4.6. Quantification of endogenous salicylic acid and analysis of PR-gene expression by qRT-PCR in leaves of potato plants (*Solanum tuberosum* cv. *Désirée*) infected with the peach potato aphid, *Myzus persicae*.

A. Levels of endogenous salicylic acid in leaves of potato plants infected with *M. persicae* for 48 hours on 6 week old plants (equivalent to 21 dpi with *G.pallida*). **B.** Expression levels of *PR*-genes in the leaves of potato plants infected with *M. persicae* for 48 hours on 6 week old potato plants. The presented data are the mean fold changes ± standard errors in both graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from different biological replicates (Mann Whitney U, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n = 6 (endogenous SA levels) n = 12 (gene expression analysis).

mechanism. There was also a significant increase in the expression of jasmonic acid-mediated genes ($P < 0.01$) (Figure 4.6 b).

4.3.3. Salicylic acid- and jasmonic acid-mediated defence responses in susceptible potato cultivars when co-infected with the potato cyst nematode, *Globodera pallida* and the peach potato aphid, *Myzus persicae*.

Leaves of five week old plants that had been pre-infected with *G. pallida* for a period of 14 days and *Myzus persicae* for a period of 48 hours were tested for levels of the endogenous phytohormones salicylic acid and jasmonic acid. In addition, the expression of salicylic acid- and jasmonic acid-mediated defence genes was measured. There was no significant difference in the levels of free (691 ± 45 ng) endogenous levels of salicylic acid when compared to the control (743 ± 146 ng), however there was a significant increase in the levels of stored (9943 ± 1522 ng) and total (10750 ± 1557 ng) salicylic acid when compared to the control (Stored: 4665 ± 906 ng, Total: 5409 ± 930 ng, $P < 0.012$) (Figure 4.7 a). The increase in stored salicylic acid may indicate that salicylic acid defences are being increased in the plant due to co-infection with both *G.pallida* and *M. persicae*, however these reserves of salicylic acid have not yet been converted into free salicylic acid. There was no change in the levels of endogenous jasmonic acid plants when co-infected with both pests (372 ± 73 ng) compared to control plants (392 ± 64 ng, $P < 0.855$) (Figure 4.7 b) suggesting that there may antagonistic suppression of the jasmonic acid defence pathway by salicylic acid. There was no significant difference in either salicylic acid- or jasmonic acid-mediated defence genes in co-infected plants at 14 dpi (Figure 4.7 c). This suggests that there was a suppression of jasmonic acid mediated defences in the potato plant following co-infection with *G. pallida* and *M. persicae* for 14 days.

To complement data collected for potato plants co-infected 14 dpi with *G. pallida* and *M. persicae* a full time-course analysis for the levels of endogenous salicylic acid were measured at 3, 7 and 21 dpi with both pests (Figure 4.8) alongside an analysis of the expression of defence genes for the same time-points (Figure 4.7 c). At 3 dpi with both pests there was a significant increase in free (1307 ± 92 ng, $P < 0.015$), stored (9954 ± 822 ng, $P < 0.047$) and total (11680 ± 892 ng, $P < 0.031$)

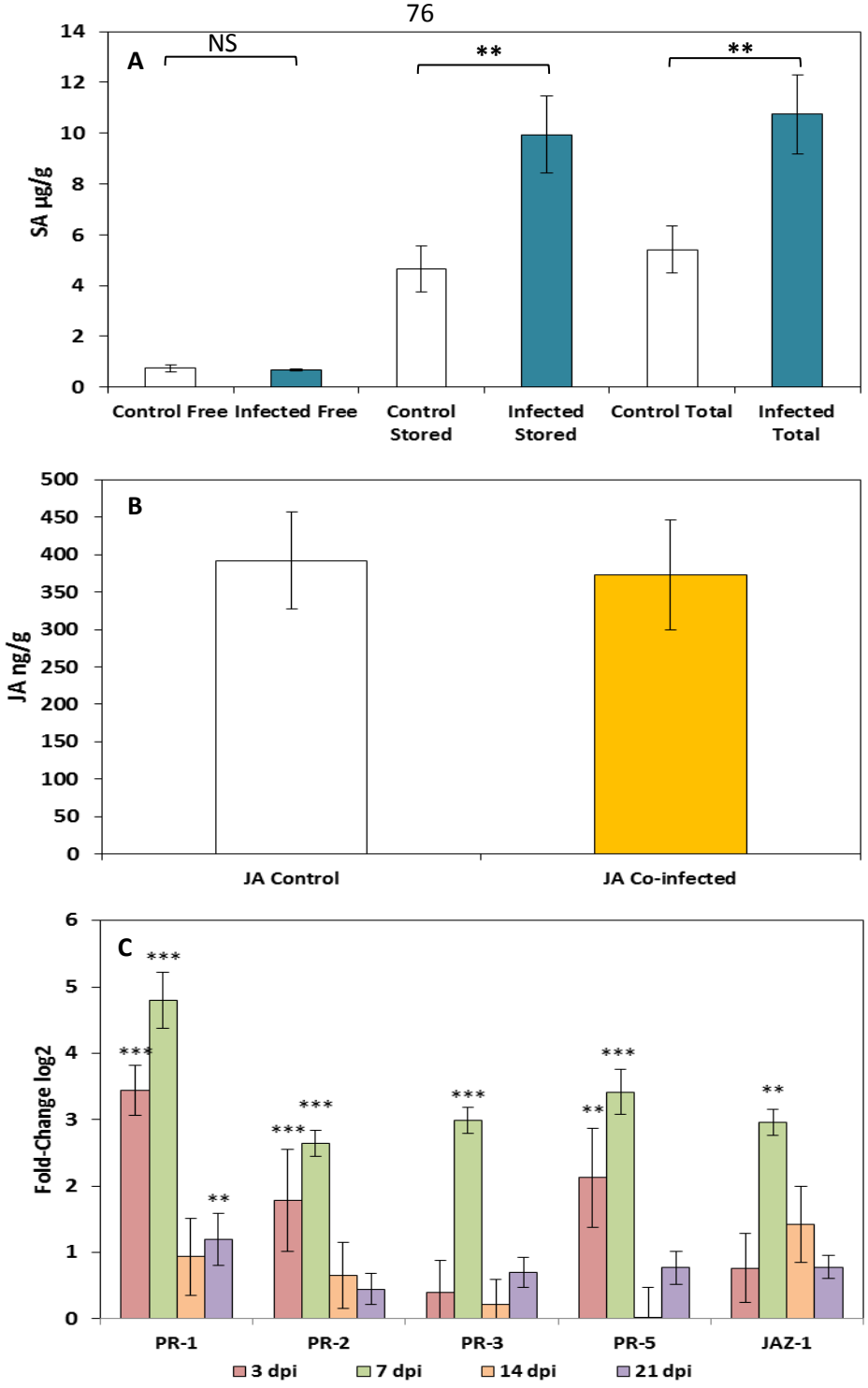

Figure 4.7. Quantification of endogenous salicylic acid and jasmonic acid and analysis of PR-gene expression by qRT-PCR in leaves of potato plants (*Solanum tuberosum* cv. Désirée) infected with the potato cyst nematode, *Globodera pallida* and the peach potato aphid, *Myzus persicae.*

A. Levels of endogenous salicylic acid in leaves of potato plants infected with both pests for 14 dpi on 5 week old plants. **B.** Levels of endogenous jasmonic acid in leaves of potato plants infected with both pests for 14 dpi on 5 week old plants. **C.** Expression levels of *PR*-genes in the leaves of potato plants infected with both pests for 14 dpi on 5 week old plants. The presented data are the mean fold changes ± standard errors in all graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from different biological replicates (Mann-Whitney U, *** $P < 0.001$, n = 12).

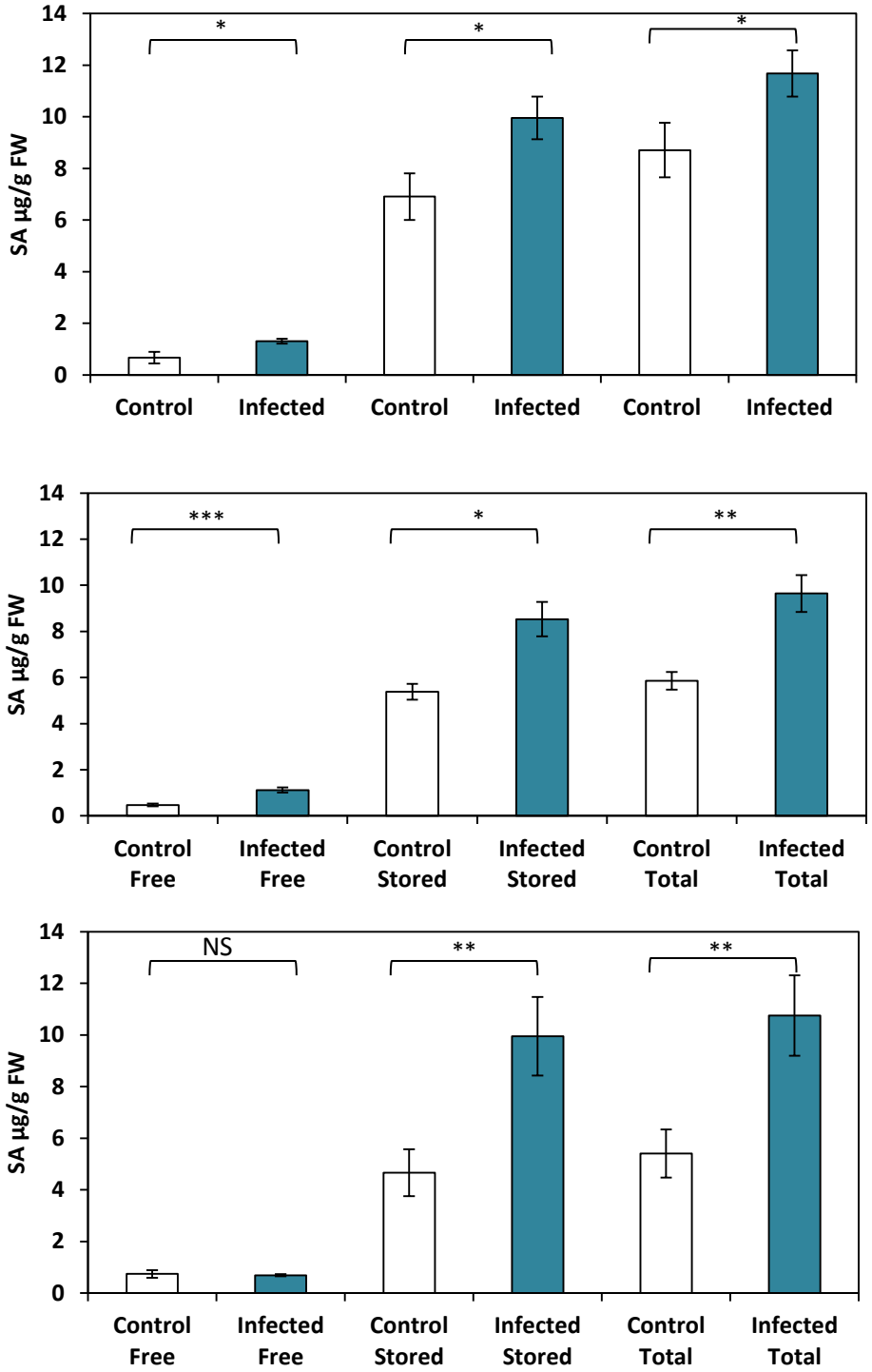

Figure 4.8. Quantification of endogenous salicylic acid in leaves of potato plants (*Solanum tuberosum* cv. Désirée) infected with the potato cyst nematode, *Globodera pallida* and the peach potato aphid, *Myzus persicae*.

A. Levels of endogenous salicylic acid in leaves of potato plants infected with both pests 3 days post inoculation (dpi). **B.** Levels of endogenous salicylic acid in leaves of potato plants infected with both pests 7 dpi. **C.** Levels of endogenous salicylic acid in leaves of potato plants infected with both pests 21 dpi. The presented data are the mean fold changes ± standard errors of biological replicates in all graphs (Mann-Whitney U, * < 0.05, ** < 0.01, *** < 0.001; n=7 (endogenous SA 3 dpi), n=9 (endogenous SA 7 and 21 dpi).

compared to control plants (Free: 669 ± 220 ng, Stored: 6907 ± 905 ng, Total: 8710 ± 1051 ng) (Figure 4.8 a). The expression of *PR*-1, *PR*-2 and *PR*-5 were measured for the same time-point and there was a significant increase in all three compared to the control (Figure 4.7 c). The expression of *PR-1* increased 3-fold in comparison to the control while there was a two-fold increase in the expression of *PR*-2 and *PR*-5 (Figure 4.7 c). There was no significant increase in the expression of either *PR*-3 or *JAZ*-1 in the leaves of the same plants (Figure 4.7 c). This indicates that salicylic acid-mediated defences were initiated in potato plants 3 dpi with *G. pallida* and *M. persicae,* but jasmonic acid-mediated defences were not.

When three-week old plants were co-infected with both pests for seven days (equivalent to infection with *G. pallida* for seven days) there was a significant increase in free (1116 ± 113 ng, $P < 0.002$), stored (8530 ± 742 ng, $P < 0.018$) and total (9645 ± 798 ng, $P < 0.012$) endogenous levels of salicylic acid compared to control plants (Free: 472 ± 63 ng, Stored: 5380 ± 339 ng, Total: 5852 ± 381 ng) (Figure 4.8 b) as well as a significant increase in the expression of *PR*-1 ($P < 0.002$), *PR*-2 ($P < 0.002$) and *PR*-5 ($P < 0.002$) compared to control plants. The expression of both jasmonic-acid mediated genes *PR*-3 ($P < 0.002$) and *JAZ*-1 ($P < 0.008$) was also significantly up-regulated in co-infected plants at seven days post inoculation (Figure 4.7 c).

There was no significant increase in free (620 ± 112 ng), stored (6069 ± 650 ng) or total (6993 ± 675 ng) endogenous salicylic acid in leaves of potato plants 21 dpi with both *G. pallida* and *M. persicae* compared to the control (Free: 1327 ± 71 ng, Stored: 5623 ± 1000 ng, Total: 5719 ± 1029 ng) (Figure 4.8 c). The expression of *PR*-1 increased significantly approximately 1.5 fold compared to control plants ($P < 0.013$), however there was no significant increase in the expression of *PR*-2 or *PR*-5. There was also no significant change in the expression of *PR*-3 or *PR*-5 when compared to control plants suggesting that jasmonic acid defences are not elicited in co-infected plants at this time-point (Figure 4.7 c).

4.3.4. Salicylic acid- and jasmonic acid-mediated defence responses in resistant potato cultivars when infected with the golden potato cyst nematode, *Globodera rostochiensis*.

Potato plants (*Solanum tuberosum* cv. Maris Piper), which confer resistance to the golden potato cyst nematode were infected with *Globodera rostochiensis* to elucidate salicylic acid- and jasmonic-acid mediated defence responses. In parallel, potato plants (*S. tuberosum* cv. Désirée), which are susceptible to potato cyst nematodes (both *G. pallida* and *G. rostochiensis*) were infected with *G. rostochiensis* so defence responses to nematode infection could be compared against those defence responses found in the resistant cultivar. There was no significant difference in levels of free (813 ± 107 ng), stored (2915 ± 463 ng) or total (4052 ± 521 ng) endogenous salicylic acid in the leaves of potato plants susceptible (cv. Désirée) to *G. rostochiensis* when compared to control plants 3 dpi with nematodes (Free: 608 ± 99 ng, Stored: 3892 ± 467 ng, Total: 4585 ± 502 ng) (Figure 4.9 a). In contrast, when resistant potato plants (cv. Maris Piper) were infected with *G. rostochiensis* there was a significant increase in the levels of free (20124 ±2498 ng, $P < 0.000$), and total (26009 ± 2712 ng, $P < 0.000$) levels of endogenous salicylic acid (Figure 4.9 b). This may indicate that defence responses are elicited earlier in resistant cultivars compared to susceptible cultivars following nematode infection. There was no significant difference in the expression of *PR*-1 or *PR*-2 in susceptible potato cultivars, however there was a significant increase in the expression of *PR*-5 ($P < 0.021$). There was no significant difference in the expression of jasmonic acid-mediated defence genes, *PR*-3 or *JAZ*-1 (Figure 4.9 c) suggesting that jasmonic acid is not elicited in the leaves of potato plants following infection with *G. rostochiensis*. When resistant potato plants were infected with *G. rostochiensis* there was only a significant increase in the expression of *JAZ*-1 ($P < 0.015$) suggesting jasmonic acid is involved in the resistant defence response to *G. rostochiensis*. There was no significant difference in the expression of the other defence genes measured at 3dpi with *G. rostochiensis* (Figure 4.9 c).

An analysis of salicylic acid- and jasmonic acid-mediated defence responses was carried out on susceptible and resistant cultivars of potato at seven days post inoculation with *G. rostochiensis* (Figure 4.10). There was no significant difference

in the levels of endogenous salicylic acid in susceptible cultivars when infected with *G. rostochiensis* for 7 days (Free: 569 ± 45 ng, Stored: 7182 ± 441 ng, Total: 7834 ± 466 ng) compared to control plants (Free: 577 ± 36 ng, Stored: 5993 ± 481 ng, Total: 6428 ± 498 ng) (Figure 4.10 a). Similarly, there was no difference found between the levels of endogenous salicylic acid in the leaves of resistant potato cultivars (cv. Maris Piper) when infected with *G. rostochiensis* for 7 days (Free: 535 ± 197 ng, Stored: 1254 ± 192 ng, Total: 1871 ± 356 ng) compared to control plants (Free: 197 ± 32 ng, Stored: 1354 ± 112 ng, Total: 1574 ± 101 ng) (Figure 4.10 b). There was no change in the expression of any defence genes, salicylic acid- or jasmonic-acid mediated, in potato plants susceptible to *G. rostochiensis* infection at 7 dpi (Figure 4.10 c). However, in potato plants resistant to *G. rostochiensis* there was a significant decrease in the expression of *PR*-1 when infected with nematodes for 7 days ($P < 0.018$). There was no significant difference in expression of the other defence related genes in potato plants resistant to *G. rostochiensis* at 7 dpi (Figure 4.10 c). Together these results imply that Maris Piper, which harbours the H1 gene, is resistant to *G. rostochiensis.*

4.3.5. Salicylic acid- and jasmonic acid-mediated defence responses in different potato cultivars when infected with the peach potato aphid, *Myzus persicae*.

Salicylic acid- and jasmonic acid-mediated defence responses were analysed in different potato cultivars when they were infected with the peach potato aphid, *Myzus persicae* at the time-points of 3 and 7 dpi. It should be noted that although potato plants were only infected with aphids for a period of 48 hours the time-points are annotated as 3 and 7 dpi to correspond with the time-points used for nematode infection (section 4.3.4). All experimental groups being compared (nematode only infection, aphid only infection or co-infection) were the same age for phytohormone and gene expression analysis.

There was a significant increase in the levels of free (1222 ± 204 ng, $P < 0.014$) salicylic acid but no significant increase in stored (8526 ± 1117 ng) or total (10586 ± 1307 ng) salicylic acid in susceptible plants 3 dpi with aphids compared to control

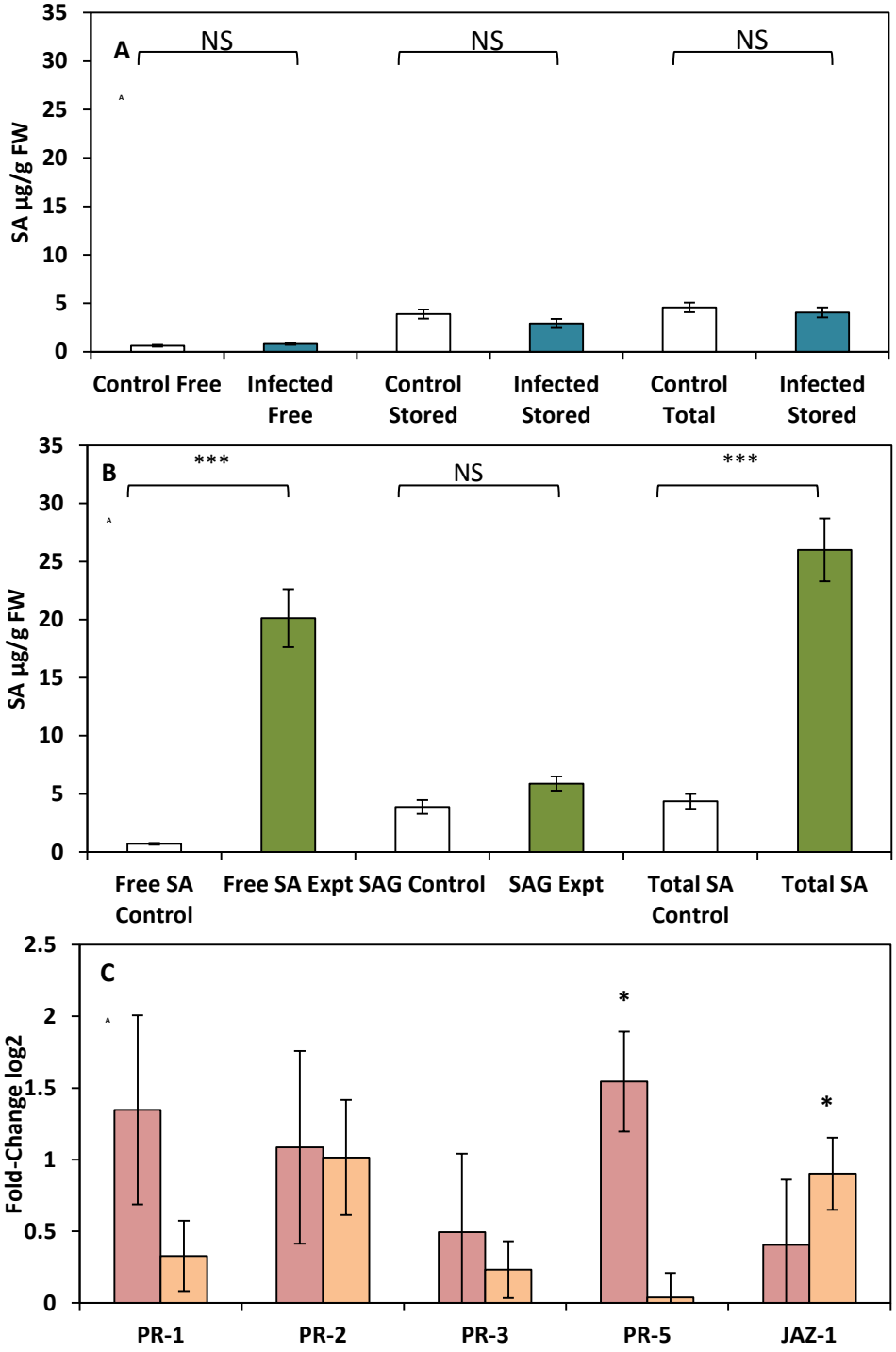

Figure 4.9. Quantification of endogenous salicylic acid and analysis of pathogen-related PR-gene expression by qRT-PCR in leaves of potato plants susceptible to the golden potato cyst nematode, *Globodera rostochiensis* (*Solanum tuberosum* cv. Désirée) and resistant potato cultivars (*Solanum tuberosum* cv. Maris Piper)

A. Levels of endogenous salicylic acid in leaves of potato plants (cv. Désirée) infected with *G. rostochiensis* 3 days post inoculation (dpi). **B.** Levels of endogenous salicylic acid in leaves of potato plants (cv. Maris Piper) infected with *G. rostochiensis* at 3 dpi. **C.** Expression levels of *PR*-genes in the leaves of potato plants (cv. Désirée and Maris Piper) infected with *G. rostochiensis* at 3 dpi. The presented data are the mean fold changes ± standard errors in all graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from different biological replicates (Mann-Whitney U, * < 0.05, *** < 0.001, n=12).

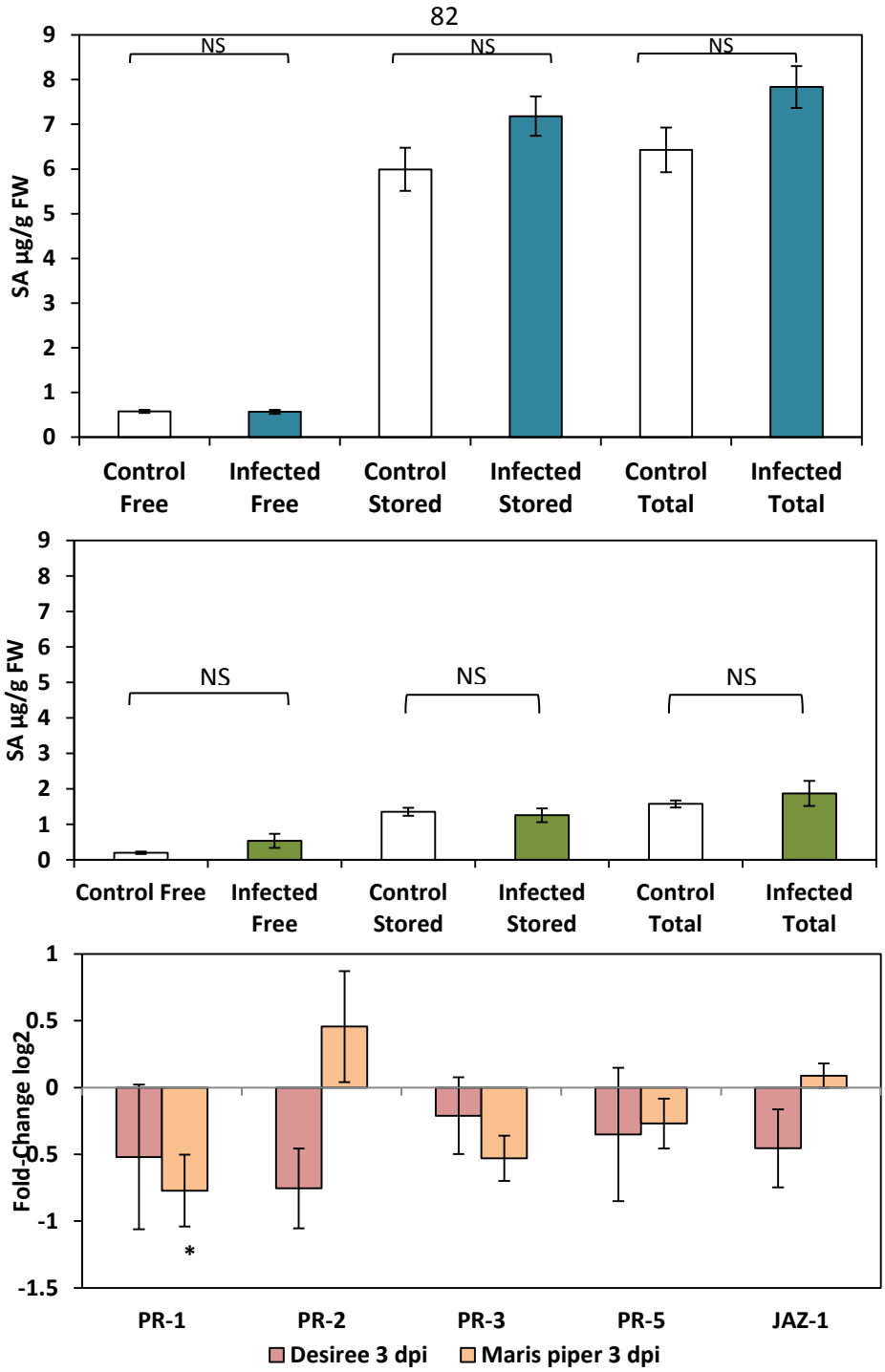

Figure 4.10. Quantification of endogenous salicylic acid and analysis of pathogen-related PR-gene expression by qRT-PCR in leaves of potato plants susceptible to the golden potato cyst nematode, *Globodera rostochiensis* (*Solanum tuberosum* cv. Désirée) and resistant to G. rostochiensis (*Solanum tuberosum* cv. Maris Piper)

A. Levels of endogenous salicylic acid in leaves of potato plants (cv. Désirée) infected with *G. rostochiensis* 7 days post inoculation (dpi). **B.** Levels of endogenous salicylic acid in leaves of potato plants (cv. Maris Piper) infected with *G. rostochiensis* at 7 dpi. **C.** Expression levels of *PR*-genes in the leaves of potato plants (cv. Désirée and Maris Piper) infected with *G. rostochiensis* at 7 dpi. The presented data are the mean fold changes ± standard errors in all graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from different biological replicates (Mann-Whitney U, * < 0.05, n=12).

plants (Free: 657 ± 74 ng, Stored: 6001 ± 1196 ng, Total: 7294 ± 1257 ng) (Figure 4.11 a). When the expression of salicylic acid-mediated defence genes was measured, there was a significant increase in all three genes *PR*-1, *PR*-2 and *PR*-5. The expression of *PR*-1 increased 4-fold compared to that measured in the control plants ($P < 0.001$), the expression of *PR*-2 increased almost 2-fold ($P < 0.02$) and *PR*-5 increased 3-fold ($P < 0.011$) in comparison to control plants (Figure 4.11 c). Similarly, there was a significant increase in the expression of *PR*-3 ($P < 0.003$) and *JAZ*-1 ($P < 0.002$) (Figure 4.4 c). This suggests that both salicylic acid and jasmonic acid-mediated defence responses are elicited in potato plants susceptible (cv. Désirée) to aphid infection. There was a more sensitive response to aphid infection in potato cultivars resistant to *G. rostochiensis* (*S. tuberosum* cv. Maris Piper). There was a significant increase in free (910 ± 61 ng, $P < 0.004$), stored (5533 ± 701 ng, $P < 0.006$) and total (6361 ± 749 ng, $P < 0.004$) endogenous levels of salicylic acid 3 dpi with *M. persicae* compared to control plants (Free: 340 ± 13 ng, Stored: 2311 ± 400 ng, Total: 2629 ± 403 ng) (Figure 4.11 b). The expression of defence genes also significantly increased in the leaves of the cultivar Maris piper following aphid infection. The expression of *PR*-1 increased almost 8-fold in comparison to the control ($P < 0.000$), while the expression of *PR*-2 ($P < 0.000$) and *PR*-3 ($P < 0.000$) increased 6-fold in comparison to the control (Figure 4.11 c). The expression of *PR*-5 ($P < 0.000$) and *JAZ*-1 ($P < 0.000$) increased 5-fold and 3-fold respectively (Figure 4.11 c). Collectively, these results indicate involvement of both salicylic acid- and jasmonic acid-mediated defences in response to aphid infection in the potato cultivar Maris Piper.

Phytohormone analysis was also carried out on both resistant and susceptible potato cultivars 7 dpi with aphids. There was a significant increase in the level of free (8302 ± 1703 ng, $P < 0.014$) salicylic acid but no significant increase in stored (1156 ± 272 ng) or total (10151 ± 1954 ng) salicylic acid in susceptible plants 7 dpi with aphids compared to control plants (Free: 874 ± 347 ng, Stored: 7868 ± 2649 ng, Total: 9861 ± 2993 ng) (Figure 4.12 a). There was a significant increase in the expression of all three salicylic acid-mediated defence genes. The expression of *PR*-1 increased 4-fold compared to control plants ($P < 0.001$), the expression of *PR*-2

increased almost 2-fold ($P < 0.02$) and the expression of *PR*-5 3-fold ($P < 0.011$) compared to control plants (Figure 4.12 b). Similarly, there was a significant increase in the expression of *PR*-3 ($P < 0.003$) and *JAZ*-1 ($P < 0.002$) (Figure 4.12 b) suggesting an involvement of jasmonic acid signalling in response to aphid infection.

When potato plants (cv. Maris Piper) 7 dpi with aphids were measured for phytohormones a significant reduction in the levels of free (379 ± 40 ng, $P < 0.001$) endogenous salicylic acid was observed, however there was no difference found in the levels of stored (3007 ± 1118 ng) or total (3842 ± 1137 ng) salicylic acid between infected or control plants (Free: 3987 ± 327 ng, Stored: 2184 ± 461 ng, Total: 6396 ± 588 ng) (Figure 4.12 b). Although there was a significant reduction in the free levels of endogenous salicylic acid, when the expression levels of salicylic acid-mediated genes were tested, there was a significant increase in the expression of all three genes. The expression of *PR*-1 increased 3.5-fold compared to the control ($P < 0.009$), while the expression of *PR*-2 and *PR*-5 increased almost 2-fold compared to the control (Figure 4.12 c). The expression of *PR*-3 was significantly up-regulated 2-fold compared to the control ($P < 0.027$).

4.3.6. Salicylic acid- and jasmonic acid-mediated defence responses in different potato cultivars when infected with both the potato cyst nematode, *Globodera rostochiensis* and the peach potato aphid, *Myzus persicae*.

Salicylic acid- and jasmonic acid-mediated defence responses were analysed in different potato cultivars 3 dpi and 7 dpi with the golden potato cyst nematode, *Globodera rostochiensis* and the peach potato aphid, *Myzus persicae*.

At 3 dpi there was a significant increase in the levels of free (2251 ± 230 ng, $P < 0.006$), stored (16829 ± 1012 ng, $P < 0.018$) and total (19476 ± 1213 ng, $P < 0.011$) endogenous salicylic acid in the leaves of susceptible potato plants (cv. Désirée) infected with both pests when compared to control plants (Free: 757 ± 98 ng, Stored: 8945 ± 1538 ng, Total: 10309 ± 1612 ng) (Figure 4.13 a). Similar results were found when resistant potato plants (cv. Maris Piper) were infected with both pests (Figure 4.13 b). There was a significant increase in the amount of free, stored and

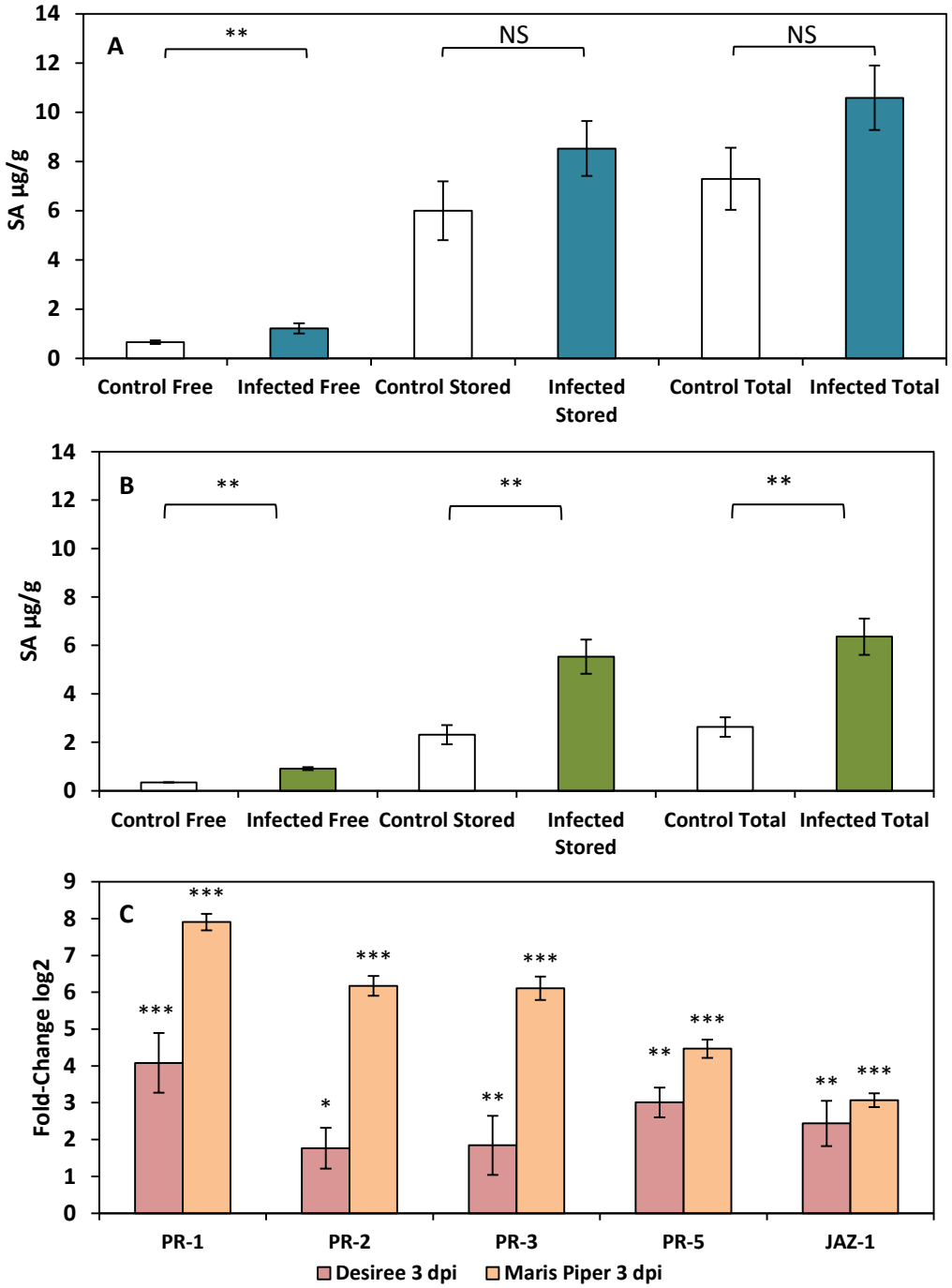

Figure 4.11. Quantification of endogenous salicylic acid and analysis of PR-gene expression by qRT-PCR in leaves of potato plants infected with the peach potato aphid, *Myzus persicae* (*Solanum tuberosum* cv. Désirée and *S. tuberosum* cv. Maris Piper).

Levels of endogenous salicylic acid in leaves of potato plants (cv. Désirée) infected with *M. persicae* 3 days post inoculation (dpi) (equivalent to 3dpi with *Globodera rostochiensis*). **B.** Levels of endogenous salicylic acid in leaves of potato plants (cv. Maris Piper) infected with *M. persicae* at 3 dpi. **C.** Expression levels of *PR*-genes in the leaves of potato plants (cv. Désirée and Maris Piper) infected with *M. persicae* at 3 dpi. The presented data are the mean fold changes ± standard errors in all graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from different biological replicates (Mann-Whitney U, * < 0.05, *** < 0.001, n=12).

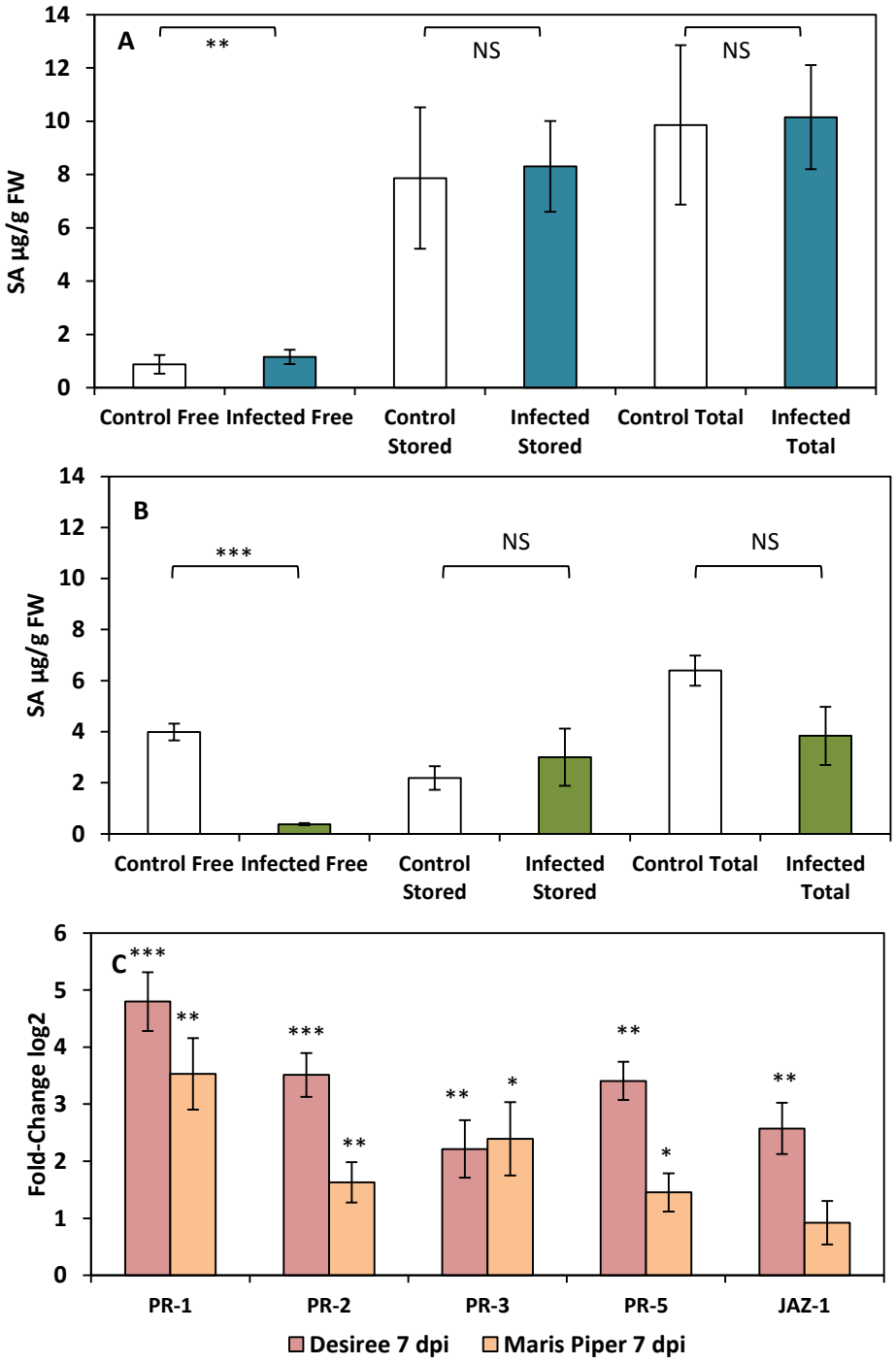

Figure 4.12. Quantification of endogenous salicylic acid and analysis of PR-gene expression by qRT-PCR in leaves of potato plants infected with the peach potato aphid, *Myzus persicae* (*Solanum tuberosum* cv. Désirée and *S. tuberosum* cv. Maris Piper).

A. Levels of endogenous salicylic acid in leaves of potato plants (cv. Désirée) infected with *M. persicae* 7 days post inoculation (dpi) (equivalent to 7 dpi with *Globodera rostochiensis*). **B.** Levels of endogenous salicylic acid in leaves of potato plants (cv. Maris Piper) infected with *M. persicae* at 7 dpi. **C.** Expression levels of *PR*-genes in the leaves of potato plants (cv. Désirée and Maris Piper) infected with *M. persicae* at 7 dpi. The presented data are the mean fold changes ± standard errors in all graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from different biological replicates (Mann-Whitney U, * < 0.05, *** < 0.001, n=12).

total (1222 ± 181 ng, 13424 ± 2293 ng, 15832 ± 2450 ng) salicylic acid compared to control plants (Free: 590 ± 30 ng, Stored: 2738 ± 390 ng, Total: 3394 ± 411 ng), however there was more salicylic acid present in infected leaves in the resistant cultivars compared to the susceptible cultivars (Figure 4.13 b), potentially suggesting a faster defence response to nematodes and aphids in resistant cultivars compared to susceptible cultivars. There was a significant increase in the expression of all salicylic acid- and jasmonic acid-mediated defence genes in both susceptible (cv. Désirée) and resistant (cv. Maris Piper) cultivars apart from *PR*-2, which was not up-regulated in the resistant cultivar (Figure 4.13 c). In susceptible cultivars, the expression of *PR*-1 increased 4-fold compared to the control ($P < 0.006$), while the expression of *PR*-2 and *PR*-5 increased approximately 3-fold compared to the control ($P < 0.006$). The expression of *PR*-3 and *JAZ*-1 increased 3-fold compared to the control ($P < 0.006$) (Figure 4.13 c). In the resistant cultivar Maris Piper, *PR*-3 increased almost 7-fold compared to the control ($P < 0.001$) suggesting that perhaps jasmonic acid plays an important defence role against simultaneous attack by *G. rostochiensis* and *M. persicae* in resistant cultivars of potato (Figure 4.13 c).The expression of *PR*-genes was higher (Figure 4.13 c) in resistant cultivars compared to that measured in susceptible cultivars, which correlates with the higher levels of endogenous salicylic acid measured in the resistant cultivar compared to susceptible cultivar (Figure 4.13 b). Endogenous phytohormones were measured in resistant and susceptible cultivars of potato when co-infected with both pests 7 dpi (Figure 4.14). There was no significant increase in free (4334 ± 953 ng) endogenous salicylic acid when the susceptible cultivar (cv. Désirée) was infected with both pests for 7 days, however there was a significant increase in stored (24199 ± 4207 ng, $P < 0.016$) and total (33319 ± 4599 ng, $P < 0.016$) salicylic acid compared to control plants (Free: 2944 ± 939 ng, Stored: 11713 ± 1507 ng, Total: 15520 ± 2362 ng). The significant increase in stored salicylic acid may suggest that the plant is increasing its stored defences in order to convert it into free salicylic acid so it can be actively used against co-infection of *G. pallida* and *M. persicae* (Figure 4.14 a). There was a significant increase in free (1565 ± 161 ng, $P < 0.004$), stored (29727 ± 5829 ng, $P < 0.025$) and total (31100 ± 5979 ng, $P < 0.025$) salicylic acid in the resistant cultivar (cv. Maris Piper) compared to control plants

(Free: 604 ± 49 ng, Stored: 10821 ± 3296 ng, Total: 11201 ± 3332 ng) suggesting an increase in salicylic acid-mediated defences (Figure 4.14 b). The expression of both salicylic acid- and jasmonic acid-mediated genes were significantly up-regulated compared to the control in both the susceptible and resistant cultivar (Figure 4.14 c), suggesting that both defence responses are activated upon co-infection with potato with both *G. pallida* and *M. persicae* 7 dpi.

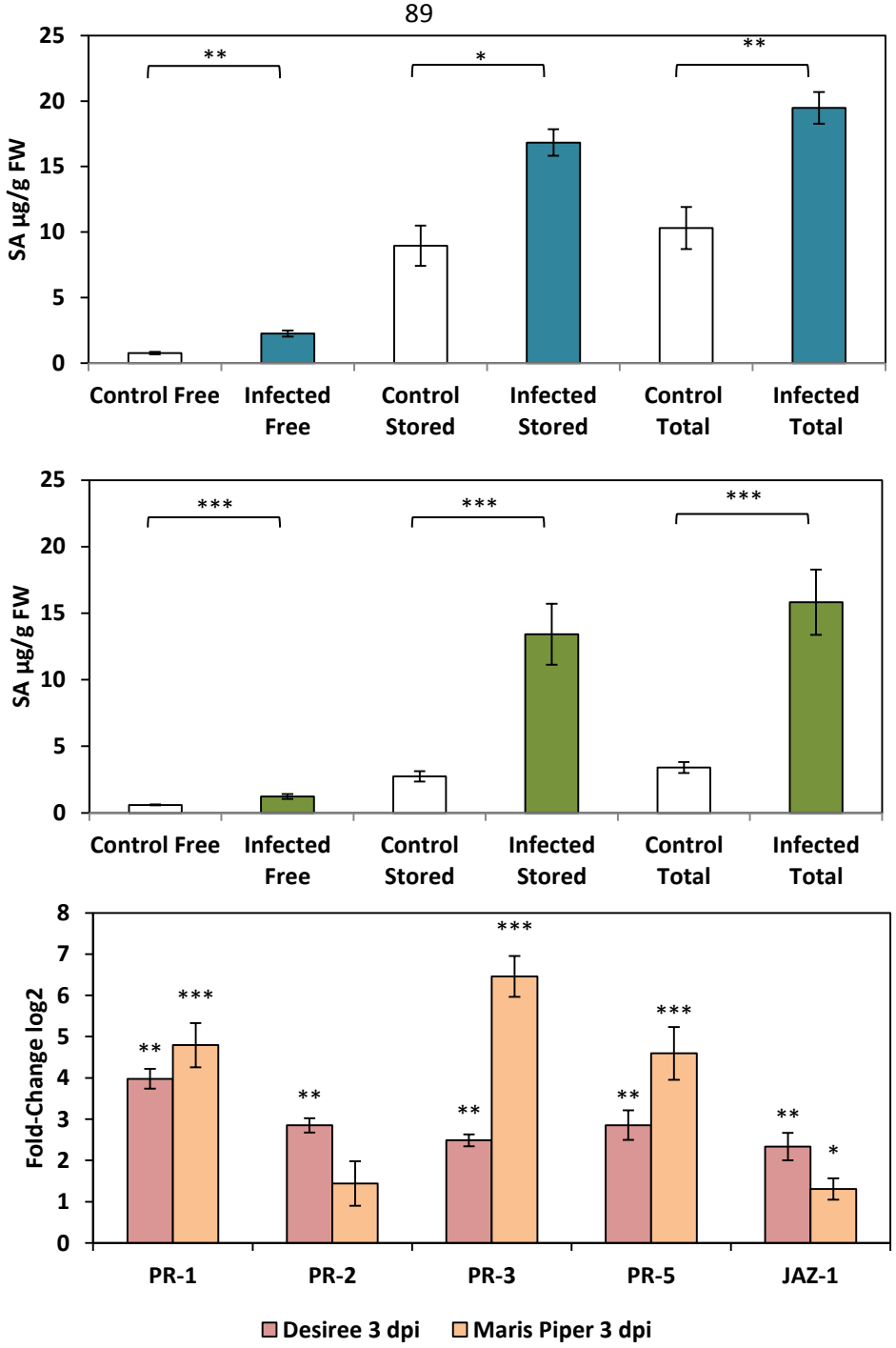

Figure 4.13. Quantification of endogenous salicylic acid and analysis of PR-gene expression by qRT-PCR in leaves of potato plants susceptible to the golden potato cyst nematode, *Globodera rostochiensis* (*Solanum tuberosum* cv. Désirée) and resistant to *G. rostochiensis* (*Solanum tuberosum* cv. Maris Piper).

A. Levels of endogenous salicylic acid in leaves of potato plants (cv. Désirée) infected with *G. rostochiensis* and *Myzus persicae* 3 days post inoculation (dpi). **B.** Levels of endogenous salicylic acid in leaves of potato plants (cv. Maris Piper) infected with *G. rostochiensis* and *M. persicae* 3 dpi. **C.** Expression levels of *PR*-genes in the leaves of potato plants (cv. Désirée and Maris Piper) infected with *G. rostochiensis* and *M. persicae* 3 dpi. The presented data are the mean fold changes ± standard errors in all graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from different biological replicates (Mann-Whitney U, * < 0.05, ** < 0.01, *** < 0.001, n=12).

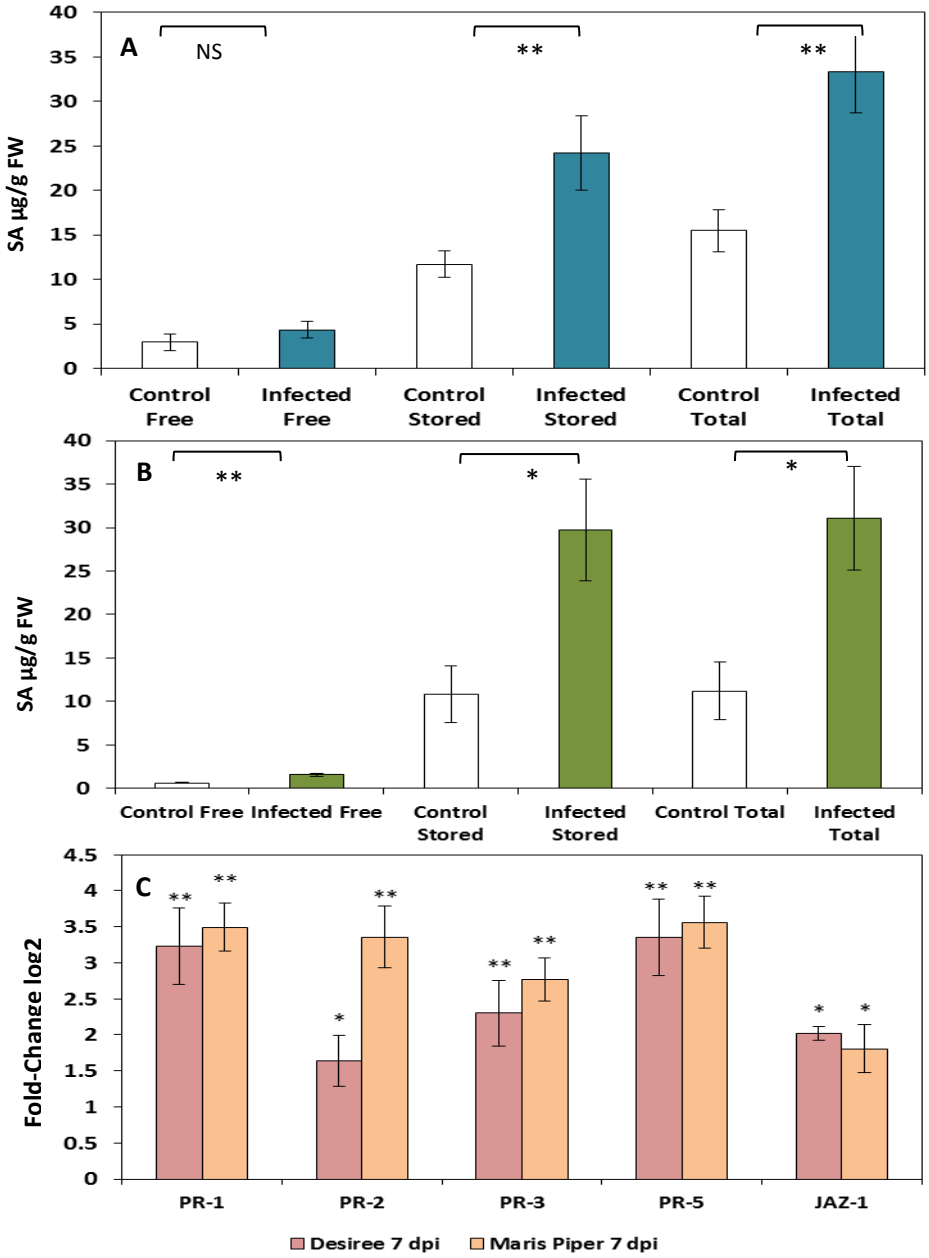

Figure 4.14. Quantification of endogenous salicylic acid and analysis of PR-gene expression by qRT-PCR in leaves of potato plants susceptible to the golden potato cyst nematode, *Globodera rostochiensis* (*Solanum tuberosum* cv. Désirée) and resistant to *G. rostochiensis* (*Solanum tuberosum* cv. Maris Piper).

A. Levels of endogenous salicylic acid in leaves of potato plants (cv. Désirée) infected with *G. rostochiensis* and *Myzus persicae* 7 days post inoculation (dpi). **B.** Levels of endogenous salicylic acid in leaves of potato plants (cv. Maris Piper) infected with *G. rostochiensis* and *M. persicae* 7 dpi. **C.** Expression levels of *PR*-genes in the leaves of potato plants (cv. Désirée and Maris Piper) infected with *G. rostochiensis* and *M. persicae* 7 dpi. The presented data are the mean fold changes ± standard errors in all graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from different biological replicates (Mann-Whitney U, * < 0.05, ** < 0.01, *** < 0.001, n=12).

Table 4.1. Composite table of signalling compounds changing over time in the leaves of potato plants in response to both nematode and aphid infection singly and in combination. A. Composite table of SA and JA compounds changing over a time-course analysis in the leaves of *S. tuberosum* (cv. Désirée), which is susceptible to the potato cyst nematode, *Globodera pallida*. **B.** Composite table of SA and JA compounds changing over a time-course analysis in the leaves of *S. tuberosum* (cv. Maris Piper), which is susceptible to *G. pallida* but resistant to the golden potato cyst nematode, *G. rostochiensis*.

A

	3dpi		7dpi		14dpi		21dpi	
	SA	JA	SA	JA	SA	JA	SA	JA
Nematode Only	Endogenous Levels		Endogenous Levels		Endogenous Levels		Endogenous Levels	
	–	✖	↓	✖	↑	–	–	✖
	Expression of genes		Expression of genes		Expression of genes		Expression of genes	
	–	–	–	–	↑	–	–	–
Aphids Only	Endogenous Levels		Endogenous Levels		Endogenous Levels		Endogenous Levels	
	↑	✖	–	✖	↑	↑	↑	✖
	Expression of genes		Expression of genes		Expression of genes		Expression of genes	
	↑	↑	↑	↑	↑	↑	↑	↑
Co-infection	Endogenous Levels		Endogenous Levels		Endogenous Levels		Endogenous Levels	
	↑	✖	↑	✖	↑	–	–	✖
	Expression of genes		Expression of genes		Expression of genes		Expression of genes	
	↑	–	↑	↑	–	–	–	–

B

	3dpi		7dpi	
	SA	JA	SA	JA
Nematode Only	Endogenous Levels		Endogenous Levels	
	↑	✖	–	✖
	Expression of genes		Expression of genes	
	–	↑	↓	–
Aphids Only	Endogenous Levels		Endogenous Levels	
	↑	✖	–	✖
	Expression of genes		Expression of genes	
	↑	↑	↑	↑
Co-infection	Endogenous Levels		Endogenous Levels	
	↑	✖	↑	✖
	Expression of genes		Expression of genes	
	↑	↑	↑	↑

↑ Significantly up-regulated compared to the control

↓ Significantly down-regulated compared to the control

– No significant difference compared to the control

✖ Not measured

4.4. Discussion

4.4.1. Molecular defence responses in the leaves of susceptible and resistant potato cultivars to nematode infection

4.4.1.1. Susceptible potato cultivar (cv. Désirée)

Complex changes in plant gene expression occur when plant parasitic nematodes infect a suitable host plant. Root-knot nematodes move intercellularly through the root tissue, therefore avoiding the elicitation of strong defence responses. In contrast, cyst nematodes migrate intracellularly straight through the cortical cells causing destruction from the site of entry to their settling point (Wyss, 2002). Changes to infected host cells enable the successful establishment of a permanent feeding site called the syncytium (Williamson and Gleason, 2003), which in turn can induce defence and stress responses systemically, in uninfected parts of the plant (Wubben *et al.*, 2008, Hamamouch *et al.*, 2011). This is termed systemic acquired resistance (SAR) and as a result plants may be rendered more prepared for attack by secondary arriving nematodes (Hamamouch *et al.*, 2011) or above-ground pests such as leaf herbivores. It has been well established that salicylic acid is a key player in plant defence signalling and has been implicated in cyst nematode parasitism (Wubben *et al.*, 2008).

It has been reported that plant parasitic nematodes can elicit defence responses in host plants soon after invasion. Just 12 hours post inoculation of tomato roots with root knot nematodes, there was an up-regulation of general defence genes in the plant (Williamson and Hussey, 1996). Common time-points that have been used in published studies include 3, 7, 14 and 21 days post inoculation (dpi) with plant parasitic nematodes (Wubben *et al.*, 2008, Hamamouch *et al.*, 2011), therefore these time-points were used in this study. Our analysis of phytohormones and corresponding pathogenesis-related (*PR*) defence genes in the leaves of potato plants revealed that salicylic acid is present and *PR*-genes are expressed in plants infected with *G. pallida*, albeit induced at different time-points and at varying levels. In this study, endogenous levels of salicylic acid were not altered in plants 3 dpi with *G. pallida* and the expression of *PR*-genes was not up-regulated compared to control plants. Salicylic acid has been reported to be effective against biotrophic

pathogens (Delaney *et al.*, 1994, Reuber *et al.*, 1998) and in *Arabidopsis* elicits the response of these three genes (Thomma *et al.*, 1998). Our results show no up-regulation of endogenous salicylic acid or in the expression of salicylic acid-mediated defence genes in the leaves of potato plants and this is in contrast to previous reports for cyst nematode infection. The expression of salicylic acid related defence genes were strongly up-regulated following soybean cyst nematode (*Heterodera glycines*) infection in soybean (Ithal *et al.*, 2007), however these changes were detected locally in the roots. Salicylic acid mediated defence responses were also up-regulated in the leaves of *Arabidopsis* following infection with the sugarbeet cyst nematode, *Heterodera schachtii,* however the expression of only one pathogenesis-related gene was measured. A dramatic increase in *PR*-1 was found in the shoots of the plant 3 dpi with 300 nematodes. However, when the total levels of endogenous salicylic acid was measured in shoots of *Arabidopsis* plants 4 dpi with *H. schactii*, no significant difference was found between infected and control plants (Wubben *et al.*, 2008). JA plant defence genes have also been reported to be strongly up-regulated in response to cyst nematode infection, albeit, these were again, measured locally in the roots (Ithal *et al.*, 2007). *PR*-3 is commonly used as a molecular marker for jasmonic acid SAR (Hamamouch *et al.*, 2011) and in one particular study *H. schactii* was found to potentially alter the jasmonic acid SAR pathway in the leaves of *Arabidopsis* due to a significant increase in the expression of *PR*-3 at 5 dpi with the nematode (Hamamouch *et al.*, 2011). However, in our study there was no up-regulation in the expression of either *PR*-3 or *JAZ*-1 suggesting that *G. pallida* does not alter the jasmonic acid pathway in the leaves of potato plants at the time-point of 3 days despite the destructive intracellular migration pattern of cyst nematodes.

Endogenous levels of salicylic acid were significantly down-regulated in plants 7 dpi with *G. pallida* and although not significantly different to control plants the expression of salicylic acid-mediated defence genes showed a trend of down-regulation suggesting that nematode infection may cause a suppression of salicylic acid mediated defences systemically in potato plants. Seven days post invasion by cyst nematodes marks the beginning of the initiation phase whereby the nematode

selects a cell to form a permanent feeding site called a syncytium. The formation of the syncytium is characterised by an induction of specific developmental pathways and in order for the biotrophic pathogen to survive plant defence responses need to be supressed (Kyndt *et al.*, 2013). Many hormonal pathways are influenced by the formation of the syncytium and this may be attributed to the secretion of effector protein molecules from the nematodes oesophageal glands. The oesophageal glands are made up of the subventral glands, which are more active during hatching and invasion, and the dorsal glands, which become active when the nematode reaches moults to a J3 and remains active until the life cycle of the nematode is completed (Hussey and Mims, 1990). Chorismate mutase, an effector protein secreted by *G. pallida*, is also an enzyme in the shikimate pathway of plants and bacteria and until recently had not been described in animals (Lambert *et al.*, 1999, Jones *et al.*, 2003). Chorismate mutase catalyses the conversion of chorismate into prephenate, which is the final stage in the shikimate pathway and which also is a precursor for salicylic acid (Jones *et al.*, 2003). Furthermore, chorismate has been shown to be metabolised to salicylic acid through an isochorismate intermediate (Wildermuth *et al.*, 2001), therefore one potential effect of chorismate mutase being secreted into a plant by *G. pallida* may be to reduce the plants ability to mount a defence response to early infection by the nematode (Jones *et al.*, 2003). More recently, when over-expressed in *Arabidopsis* plants, the effector *10A06* from *H. schachtii* was shown to cause down-regulation in expression of *PR*-1, *PR*-2 and *PR*-5 with *PR*-1 being the most responsive (Hewezi *et al.*, 2010). This further supports a hypothesis that the secretion of effector proteins from cyst nematodes may be causing a down-regulation of salicylic acid in the host plant. Suppression of phytohormone pathways has been reported previously in response to sedentary endoparasitic infection, albeit with root knot nematodes. A reduced expression of the salicylic acid biosynthesis gene, *OsICS1* in rice was found in above-ground rice tissue following infection with the root knot nematode, *Meloidogyne graminicola* (Kyndt *et al.*, 2012) and also a reduction in the expression of *PR*-genes was found in the leaves of *Arabidopsis* following infection with *M. incognita* (Hamamouch *et al.*, 2011). Although there has been a suppression of salicylic acid-mediated defences in response to root knot nematodes, conflicting

results to our study were found in the shoots of *Arabidopsis* following infection with the sugarbeet cyst nematode *H. schachtii* (Hamamouch *et al.*, 2011). At 5 dpi there was no significant difference in the expression of *PR*-1, *PR*-2 or *PR*-5 following infection with the nematode, however at 7 dpi there was a significant increase in the expression of all three genes suggesting a salicylic acid-mediated SAR defence response was present following nematode infection. In a separate study there was no significant difference found in the transcript levels of *PR*-1 of *Arabidopsis* plants 8 dpi with *H. schachtii* compared to non-infected control plants (Wubben *et al.*, 2008). Jasmonic acid defensive genes were also analysed at 7 dpi with *G. pallida* and although there was a down-regulation trend observed in the expression of *PR*-3 and *JAZ*-1, there was no significant difference relative to non-infected control plants. An up-regulation in the expression of *PR*-3 was reported in the leaves of *Arabidopsis* 5 dpi and 9 dpi with *H. schachtii* suggesting that jasmonic acid-dependent SAR may be altered in the plant however, there was no up-regulation of *PR*-4, which is also mediated by jasmonic acid (Hamamouch *et al.*, 2011). These conflicting results in addition to the results found in this study with *G. pallida* infection of potato plants suggests that the jasmonic acid signalling pathway is differentially expressed depending on the host plant and nematode infecting the plant. Jasmonic acid has been shown to be up-regulated in roots of plants infected with cyst nematodes (Ithal *et al.*, 2007), however more studies on defence gene expression and the quantification of endogenous hormones are needed that include systemic tissues of host plants.

Approximately 14 dpi of the host plant with nematodes, the syncytium has been established and growth of the nematode continues as it feeds and moults into its next life stages. In this study, there was a significant increase in the expression of *PR*-5 found in nematode infected plants compared to non-infected control plants. Although, there was no significant up-regulation in the expression of *PR*-1 and *PR*-2 observed, it is hypothesised that infection with *G.* pallida for 14 days activates the salicylic acid-mediated defence pathway in the leaves of potato plants due to an observation that free, stored and total endogenous salicylic acid levels were significantly increased at this time-point. This again, is in contrast to what was

observed in two studies using *Arabidopsis* as the host plant. In both studies, there was no significant difference found in the expression of *PR*-1 at 13 dpi and 14 dpi, respectively, with *H. schachtii* (Wubben *et al.*, 2008, Hamamouch *et al.*, 2011) or in the expression of *PR*-2 and *PR*-5 following infection with *H. schachtii* for 14 days (Hamamouch *et al.*, 2011). This lack of salicylic acid signalling in these studies is surprising given the fact that syncytia formed by cyst nematodes continue to grow with the developing nematode for 3-4 weeks (Hamamouch *et al.*, 2011). Interestingly, the functions of *PR*-5 can include osmotic regulation (Zhu *et al.*, 1995), which may occur during syncytium growth (Jones, 1981), therefore lending an explanation to the up-regulation in the expression of *PR*-5 observed in this study with *G. pallida.* The endogenous levels of jasmonic acid were also measured in the leaves of potato plants following infection with *G. pallida*, however there was no significant difference found between endogenous levels in nematode infected plants and control plants. In addition, there was no significant difference in the expression of *PR*-3 and *JAZ*-1 observed in nematode infected plants. Similar results were observed in the leaves of *Arabidopsis* following infection with *H. schachtii* at 14 dpi where no change in the expression of *PR*-3 was observed in nematode infected plants, although there was a significant reduction in the expression of *PR*-4 in infected plants (Hamamouch *et al.*, 2011). Taken together, the results found in this study in potato, alongside results observed during the study using *Arabidopsis* (Hamamouch *et al.*, 2011), suggests that jasmonic acid signalling is not initiated in the leaves of certain plants following cyst nematode infection.

4.4.1.2. Resistant potato cultivar (cv. Maris Piper)

A hypersensitive response has been reported in resistant cultivars of plants when nematodes fail to set up a syncytium as they become surrounded by necrotising cells (Bleve-Zacheo *et al.*, 1998). However, this has only been reported for root-knot nematodes. Instead a hypersensitive-like response has been described when potato cyst nematodes invade a resistant cultivar, whereby a syncytium is initiated but slowly deteriorates or abnormal development of the feeding site occurs (Grymaszewska and Golinowski, 1998, Holtmann *et al.*, 2000). Neither *G. rostochiensis* nor *G. pallida* up-regulated the salicylic acid defence pathway in the

leaves of a susceptible potato cultivar 3 dpi with the pathogen. In contrast, in response to infection by *G. rostochiensis* at the same time-point there was a rapid accumulation of free salicylic acid in the leaves of the resistant cultivar. Salicylic acid has been reported to play a role in programmed cell death by the hypersensitive response (Alvarez, 2000), therefore a sharp rise in salicylic acid in the leaves of a potato cultivar resistant to *G. rostochiensis* may indicate a systemic signalling caused by a hypersensitive-like response is occurring in the plant at the site of infection. *Globodera rostochiensis* did not alter levels of salicylic acid in the susceptible potato cultivar following 7 days of infection. This is in contrast to a down-regulation of salicylic acid, which was observed in the same potato cultivar but with infection with *G. pallida.* This suggests that the two potato cyst nematodes elicit a different signalling pattern of the salicylic acid pathway in a susceptible potato cultivar. Salicylic acid-mediated defences were not altered in the resistant cultivar 7 dpi with *G. rostochiensis* suggesting that the development of the nematode had been restricted following the sharp rise in salicylic acid in the leaves of plants following 3 days of infection. These observed results are supported by published studies that report the degeneration of the syncytium within 3 days in plants expressing H_1 resistance, therefore leading to cessation in the development of the nematode (Rice *et al.*, 1985, Hoopes *et al.*, 1978).

4.4.2. Molecular defence responses in the leaves of different potato cultivars to aphid infection

4.4.2.1. Solanum tuberosum (cv. Désirée)

Many plant defence signalling pathways are up-regulated in response to aphid feeding (De Vos et al., 2005, Kusnierczyk et al., 2008, Broekgaarden et al., 2011). How different phytohormones are elicited in the plant following aphid attack, whether by molecules directly produced by the aphids or by products of aphid bacterial endosymbionts, is largely undescribed (Urbanska *et al.*, 1998, Miles, 1999, Forslund *et al.*, 2000).

It should be noted that although potato plants were only infected with aphids for a period of 48 hours the time-points are annotated as 3, 7, 14 and 21 dpi to correspond with the time-points used for nematode infection. This was to ensure

that all experimental groups being compared (nematode only infection or aphid only infection) were the same age for phytohormone and gene expression analysis. The salicylic acid pathway was up-regulated in leaves of potato plants 3dpi locally with aphids. Our results corroborate other studies as an increase in the expression of *PR*-1 and *PR*-2 has already been reported in *Arabidopsis* at 48 hours (Moran and Thompson, 2001) and transcript levels of *PR*-1 reached maximum levels of 750-fold over the control in potato following 48 hours of aphid infestation (Kerchev *et al.*, 2012). However, in our study the levels of stored salicylic acid were not different between aphid infected plants and control plants. Most of the salicylic acid that is produced *in planta* is converted to a storage form called salicylic acid *O*-*β*-glucoside (SAG) by a salicylic acid glucosyltransferase (SAGT), which is inducible by pathogens (Dean and Mills, 2004, Dean *et al.*, 2005). SAG is actively transported from the cytosol into the vacuole where it may function as an inactive storage form that can be converted back into free salicylic acid (Dean and Mills, 2004). No significant change in stored salicylic acid between aphid infected plants and non-infected control plants may suggest that stored salicylic acid has already been converted into the free salicylic acid needed in order to activate downstream salicylic acid defence genes. It may be assumed that as free salicylic increases that stored salicylic acid may decrease; however there are not enough studies in the literature to support this hypothesis.

There was indication that aphids activated the salicylic acid pathway 7 dpi as there was an increase in defence genes, however it is assumed that these defence genes were up-regulated independently of salicylic acid as endogenous levels of salicylic acid were not different between aphid infected plants and control plants. *PR*-1, *PR*-2 and *PR*-5 are commonly used molecular markers for the activation of salicylic acid-mediated SAR, however *PR*-gene activation does not always coincide with an increase in levels of salicylic acid (Vallélian-Bindschedler *et al.*, 1998). Furthermore, the set of *PR*-genes activated upon pathogen attack doesn't always match the defence genes activated by exogenous application of salicylic acid or its functional analogues (Schweizer *et al.*, 1997, Pieterse and van Loon, 1999). It may also be construed that there was no elicitation of endogenous levels of salicylic acid if

aphids stopped feeding due to the plant being one week older than those plants used in aphid infections for 3 days. It has been reported that many herbivores prefer to attack young and vigourous plants more than old and mature plants (Price, 1991), however this is unlikely the case in our study due to the activation of *PR*-genes indicating that the aphids began feeding and caused a defence response in the plant.

The expression of *PR*-2, which encodes a β-1,3-glucanase was not up-regulated 14 dpi with aphids and this was also the case 21 dpi with aphids. This is in contrast to previous aphid studies as the expression of *PR*-2 has been shown to be highly up-regulated in aphid-infested leaves in *Arabidopsis* (Moran and Thompson, 2001, De Vos *et al.*, 2005, De Vos and Jander, 2009) and has also been shown to be up-regulated in response to attack by other phloem-sucking insects such as the whitefly *Bemicia tabacci* (Kempema *et al.*, 2007). *PR*-2 has also been shown to be up-regulated in a potato species, *Solanum stoloniferum* following probing of *M. persicae* and *M. euphorbidae*, suggesting it is up-regulated in potatoes by aphids (Alvarez *et al.*, 2013). The significant increase in the expression of *PR*-2 in plants 3 dpi and 7 dpi with *M. persicae* but not at 14 dpi or 21 dpi may suggest that up-regulation of certain defence genes may be dependent on the age of the plant. The expression of salicylic acid-mediated genes may also be dependent on the age of the host plant being affected as the expression of *PR*-1, although still significantly up-regulated relative to the control, did not increase as high in older plants compared to younger plants following aphid infection.

4.4.2.2. Solanum tuberosum (cv. Maris Piper) resistant to the potato cyst nematode, Globodera rostochiensis

There have been genes described that confer resistance to aphids and highly effective plant resistance to specific aphids has been linked to single resistant (*R)* gene loci or alleles in a variety of plants (Züst and Agrawal, 2016). The *Vat* locus is linked to resistance against the melon aphid, *Aphis gossyphi* in melon (Dogimont *et al.*, 2014). Another gene, which belongs to the same receptor family (Smith and Clement, 2012), *Mi-1.2*, conveys resistance against the phloem-sucking insects and nematodes in tomato (Kaloshian, 2004). However, *Mi-1.2* only conveys resistance

to the potato aphid, *Macrosiphum eurphorbidae* and not to the generalist species, *Myzus persicae* (Goggin *et al.*, 2001).

Aphid infection induced both the salicylic acid and jasmonic acid-mediated defence pathway in the resistant cultivar as well as in the susceptible cultivar, however it was noted that 3 dpi with aphids that the expression of each *PR*-gene was up-regulated to a much higher level in resistant cultivars compared to the susceptible cultivar. This may suggest that although this potato cultivar is only resistant to infection with *G. rostochiensis*, it may be more sensitive to aphid infection than the susceptible cultivar. There was significant down-regulation of free salicylic acid in the leaves of the resistant potato cultivar 7 dpi with aphids suggesting that aphids may be able to suppress defence mechanisms. In recent years, evidence has been published to suggest that insects, including aphids, produce effectors that modulate plant defences (Wu and Baldwin, 2010, Bos *et al.*, 2010). However, because the aphids in this experiment were present on the leaves of the resistant potato plant for 48 hours, the same amount of time as on the resistant potato plants 3 dpi with aphids, it seems unlikely that any defences are being suppressed.

More than 200 species of aphids have the ability to transmit phytopathogenic viruses, and aphids are the most common vector of plant viruses (Ng and Perry, 2004). The indirect damage that these viruses cause the plant far exceeds that of the direct damage inflicted by the aphid feeding and this is the case for the majority of aphid species including *M.* persicae (Goggin, 2007). Throughout all experiments 'clean' aphids (aphids that did not harbour a phytopathogenic virus) were used to analyse the molecular response of different potato cultivars (both susceptible and resistant to plant parasitic nematodes), therefore it can be construed that all molecular results observed during the aphid experiments were due to the direct damage of feeding aphid. Aphids are known to elicit defence responses in the plant, which are traditionally associated with a pathogen defence response rather than that of an insect response (Kaloshian and Walling, 2005, Goggin, 2007), and viral disease symptoms have been reported to strongly influence the attractiveness of the plants, feeding behaviour and reproduction (Moran and Thompson, 2001). Research scope remains to include aphids vectoring viruses in experimental design

to elucidate the underpinning molecular mechanisms of the plant response to aphid feeding and viral transmission in potato to assess if aphid performance and survival would be enhanced even further with nematodes present on the plant. Alternatively, it would be interesting to assess any possible molecular interactions between plant-parasitic nematodes and the transmitted viruses and the resulting effect on the fecundity of the aphids.

4.4.3. Molecular defence responses in the leaves of resistant and susceptible potato cultivars to combined nematode and aphid infection

4.4.3.1. Susceptible potato cultivar (cv. Désirée)

Induced plant defences, which are regulated by different phytohormones, are increasingly being recognised as important in plant-mediated interactions between different pests and pathogens. These defences are induced differently depending on not only the type but also on the sequence and intensity of the attack from the stressor (Awmack and Leather, 2002, Howe and Jander, 2008, Stam *et al.*, 2014, Thaler *et al.*, 2012, Lazebnik *et al.*, 2014). Changes in levels of phytohormones and in the expression of defence genes in response to pathogen attack whether singly or in combination can affect the whole community; therefore studying dual stresses in host plants can yield important insights into the mechanisms underpinning ecological interactions.

Co-infection of the potato with both *G. pallida* and *M. persicae* had an impact on the levels of endogenous phytohormones and expression of related defence genes when compared to plants that had been infected with each pest singly. Salicylic acid mediated defences were up-regulated in co-infected plants at both time-points, however this differed to when potato plants were infected with nematodes only, where either there was no change or there was a suppression in salicylic acid-mediated defences between infected and control plants. It may be postulated that the up-regulation of endogenous salicylic acid and expression of related genes in co-infected plants may be due to the presence of aphids and not the presence of nematodes. However, at 7 dpi with aphids only the levels of endogenous salicylic acid were not significantly different between infected plants and control plants but

levels were up-regulated in co-infected plants, suggesting that it is a combination of both pests together that intensifies the salicylic acid pathway in potato plants, rather than just the presence of aphids alone because in both experiments aphids were present for the same 48 hour period.

Levels of endogenous stored and total salicylic acid significantly increased in the leaves of potato plants that were co-infected with both pests for 14 days while levels of jasmonic acid levels did not change. It could be construed that antagonistic crosstalk between the salicylic acid and jasmonic acid pathways is occurring at this time-point because, although the presence of nematodes singly did not up-regulate jasmonic acid, the presence of aphids singly did. Therefore it could be postulated that an up-regulation of jasmonic acid would still be present in the leaves of co-infected plants as the aphids were present for the same time period of 48 hours in each experiment. There is a lot of literature to suggest that phytohormones do not act independently of one another. The interaction between salicylic acid and jasmonic acid is complex with the main interaction between these two pathways being mutual antagonism (Kunkel and Brooks, 2002). Salicylic acid has been shown to have an inhibitory effect on jasmonic acid in tomato (Doherty *et al.*, 1988, Pena-Cortés *et al.*, 1993) and in *Arabidopsis* (Gupta *et al.*, 2000, Clarke *et al.*, 2000). Both *eds4* and *pad4* mutants, which are impaired in accumulating salicylic acid, show an increased response to inducers of JA-dependent gene expression (Gupta *et al.*, 2000). It has been reported that salicylic acid inducing biotrophic pathogens often suppress jasmonic acid-dependent defences (Spoel *et al.*, 2007). The total salicylic acid that was accumulated in plants that were co-infected with both pests was greater than the sum of total salicylic acid that was accumulated in the leaves of plants that been infected with each pest singly. Therefore it is postulated that the additive effect on total salicylic acid content by co-infection of the plant is enough to suppress the amount of jasmonic acid produced in the plant by the presence of aphids for 48 hours. It may be argued that there was no crosstalk due to the lack of free salicylic present in the leaves of co-infected plants; however it is unknown whether the stored form of salicylic acid can induce disease resistance or cause

reduced levels of other phytohormones, therefore antagonistic crosstalk between the salicylic acid and jasmonic acid pathways is a possibility.

4.4.3.2. Resistant potato cultivar (cv. Maris Piper)

To date only one resistant gene, *Mi-1.2*, has been described that confers resistance to nematodes and aphids (Rossi *et al.*, 1998). This gene was introduced into and used in cultivated tomato, *Lycopersicon esculentum* (Smith, 1944) to attain resistance against organisms from two different phyla. However this gene only confers resistance to root knot nematodes (*Meloidogyne* spp.) and the potato aphid (*Macrosiphon euphorbidae*). The gene confers resistance to all isolates of *M. euphorbidae*, however no resistance was observed for different isolates of the green peach aphid, *Myzus persicae* (Goggin *et al.*, 2001). In addition, when another Solanaceous species, eggplant (*S. melongena*), was transformed with *Mi-1.2* it retained its resistance against root knot nematode (*M. javinica*); however it lost its resistance to the potato aphid (Goggin *et al.*, 2006). To date, potato has not been transformed successfully with this gene, therefore studies in dual resistance to both organisms would be an interesting addition to plant-mediated interactions between nematodes and aphids in resistant plants.

A susceptible cultivar co-infected with both *G. rostochiensis* and *M. persicae* showed an activation of the salicylic acid and jasmonic acid pathways 3 dpi. These results are similar to those that were observed in the leaves of the same susceptible potato cultivar when infected with a different potato cyst nematode, *G. pallida,* with the exception of the jasmonic acid signalling pathway where no changes were observed. This indicates that although both cyst nematodes belong to the same genus, there are differences in the pathways that they up-regulate. It was observed that there was a more rapid accumulation of endogenous salicylic in leaves of resistant cultivars compared to susceptible cultivars, suggesting that there was a stronger defence response to both pests when present on the resistant cultivar. When compared to resistant plants infected with only nematodes, the accumulation of stored salicylic acid was more rapid in resistant plants co-infected with both pests, suggesting that the presence of aphids in addition to nematodes causes the plant to synthesise salicylic acid at a faster rate. Salicylic acid- and

jasmonic acid-mediated defence responses were up-regulated in the susceptible cultivar 7 dpi with both *G. rostochiensis* and *M. persicae.* It was observed that the levels of endogenous salicylic acid were much higher in susceptible plants infected with both pests compared to susceptible plants infected with either *G. rostochiensis* singly or *M. persicae* singly, suggesting that defences responses are activated more strongly in potato plants suffering dual biotic stress compared to plants suffering stress by just a single pest or pathogen. The salicylic acid-mediated defence pathway was activated to higher levels in the leaves of the resistant cultivar when co-infected with *G. rostochiensis* and *M. persicae* compared to plants infected with *G. rostochiensis* singly. This increase in endogenous salicylic acid in co-infected resistant potato plants suggests that it is due to the presence of aphids as when resistant potato cultivars were infected with only *G. rostochiensis* for 7 days there was no up-regulation of any plant defences due to a possible breakdown of the permanent feeding site following 3 days of infection, therefore rendering it impossible for the nematode to develop and complete its life cycle (Hoopes *et al.*, 1978, Rice *et al.*, 1985).

Summary

- The potato cyst nematode, *Globodera pallida* suppresses the salicylic acid pathway in susceptible potato plants (*Solanum tuberosum* cv. Désirée) following 7 days of infection.

- Susceptible potato plants infected with only nematodes up-regulate the salicylic acid pathway and not the jasmonic acid pathway 14 days post inoculation, possibly due to antagonistic crosstalk between the salicylic acid and jasmonic acid signalling pathways.

- The green peach potato aphid, *Myzus persicae* up-regulates both the salicylic acid and jasmonic acid defence pathways in potato plants (*S. tuberosum* cv. Désirée).

- Salicylic acid and jasmonic acid mediated defence pathways are activated in potato plants (*S. tuberosum* cv. Désirée) infected with both *M. persicae* and *G. pallida* at earlier time-points (3 dpi and 7 dpi) rather than later time-points (14 dpi and 21 dpi).

- Salicylic acid defence signalling is activated in the leaves of potato plants resistant (cv. Maris Piper) to the golden potato cyst nematode, *G. rostochiensis* at 3 dpi but not 7 dpi suggesting a breakdown of the syncytium rendering it impossible for the nematode to develop and complete reproduction.

- Aphid infection activates the salicylic acid and jasmonic acid-mediated pathways in potato plants resistant to *G. rostochiensis*.

- Dual biotic stress in potato plants resistant to *G. rostochiensis* leads to a more rapid and intense defence response compared to that observed in potato plants susceptible to nematode infection at an early time-point (3 dpi).

Chapter 5

Characterising the complete metabolic profile of a susceptible potato cultivar (*Solanum tuberosum* cv. Désirée) infected with *Globodera pallida* and *Myzus persicae* alone and in combination

5.1. Introduction

Metabolomics is an approach which allows all metabolites in a biological sample to be analysed comprehensively. It is often used in conjunction with genomics, transcriptomics and proteomics in order to gain a deeper understanding of biological processes (Sumner *et al.*, 2003, Saito *et al.*, 2008). Metabolites are diverse and differ greatly in their physical and chemical properties therefore multiple instruments based on different analytical properties are required for a non-targeted metabolomics approach, for example, liquid chromatography coupled with mass spectrometry (LC-MS) (Sawada *et al.*, 2009). Mass spectrometry allows the detection of mass-to-charge species that pin-point the molecular masses and fragment patterns of compounds depending on which ionisation technique is used. For example, electrospray ionisation (ESI) used in LC-MS is a soft ionisation method (Whitehouse *et al.*, 1989), provides highly sensitive information on the molecular masses of compounds and causes less fragmentation of said compounds unlike electron ionisation used in gas chromatography, which gives rise to fragment ions derived by cleavage of a compound (Sawada *et al.*, 2009). Identities to compounds can then be assigned by combining accurate mass and fragmentation patterns from the biological sample and comparing this to the accurate mass and fragmentation patterns of an authentic analytical standard.

Metabolomics has emerged as a valuable technology as it has allowed for the generation of extensive metabolic fingerprints of plants following pest and

pathogen attack (Kaplan *et al.*, 2004, Pushpa *et al.*, 2014, Sato *et al.*, 2013, Hofmann *et al.*, 2010). A comprehensive non-targeted metabolomic profile should include primary metabolites such as carbohydrates, amino acids, vitamins and organic acids as well as a range of secondary metabolite compound classes including phenylpropanoids, terpenoids, alkaloids and glucosinolates with this list varying according to the species under study (Sumner *et al.*, 2003). Primary metabolites are involved in plant metabolism and cell growth whereas many secondary metabolites found in plants have a role in defence against herbivores, pests and pathogens (Bennett and Wallsgrove, 1994). Metabolomic studies have been carried out in nematode-infected plants to complement gene expression studies where altered levels of starch, maltose and protein levels of glucose-6-phosphate isomerase and isoflavone reductase were reported (Wieczorek *et al.*, 2008, Afzal *et al.*, 2009). More recently, metabolic profiling of *Arabidopsis* roots parasitised by *Heterodera schachtii* showed increased levels of amino acids and phosphorylated metabolites in the syncytia as well as showing an accumulation of 1-kestose, raffinose and α,α-trehalose in systemic roots (Hofmann *et al.*, 2010). Studies analysing the metabolome of plants infected with aphids have also been carried out (Sato *et al.*, 2013, Hodge *et al.*, 2013). Alkaloids and flavonoids as well as concentrations of metabolites involved in the tricarboxcylic acid (TCA) cycle were altered in soybean infected with the foxglove aphid, *Aulacorthum solani* (Sato *et al.*, 2013). In a separate study, trehalose was up-regulated in systemic tissues of *Arabidopsis* and dependent on aphid density (Hodge *et al.*, 2013).

Presently, there are no studies in the literature that investigate the metabolome of a plant that has been infected with a pest and a pathogen simultaneously. Potato plants (cv. Désirée) susceptible to infection with the potato cyst nematode were infected with *Globodera pallida* and *Myzus persicae* both singly and in combination. A non-targeted metabolomics approach was used to elucidate the impacts of dual biotic stresses on the metabolome of potato plants.

5.2 Aims

1. To investigate metabolic changes in the leaves of potato plants infected with the potato cyst nematodes, *Globodera pallida*

2. To investigate metabolic changes in the leaves of potato plants infected with the peach-potato aphid, *Myzus persicae*

3. To investigate metabolic changes in the leaves of potato plants infected with both *G. pallida* and *M. persicae* simultaneously.

5.3 Materials and methods

5.3.1. Infection of Solanum tuberosum (cv. Désirée) with the *Globodera pallida* and *Myzus persicae*

Potato plants were infected with the potato cyst nematode, *G. pallida* and the peach potato aphids, *Myzus persicae* (see chapter 2).

5.3.2. Non-targeted metabolite analysis using LC-HRMS

Leaf samples were collected at 14 days post inoculation with nematodes and aphids singly as well as in combination. Samples were immediately placed on dry ice and lyophilised for 24 hours. Dried material was ground into a fine powder using a mini bead beater (Glen Mills, New Jersey). Five milligrams of tissue was extracted using 1.5 ml methanol and water (1:1). The samples were disrupted using a disruptor genie (Scientific Industries, NY, USA) for 30 minutes and centrifuged in a microcentrifuge at 14, 500 rpm for 10 minutes. Supernatants were diluted four fold with methanol:water (1:1) and analysed in a random order by liquid chromatography-high resolution mass spectrometry (LC-HRMS). LC analysis was performed on an Accela 600 High Speed LC system (Thermo Fisher Scientific Waltham, Massachusetts, USA). Extracts were separated on an ACE AQ (Hichrom, UK), 150 mm x 3 mm, 300 Å. Mobile phases were 0.1% formic acid in water (mobile phase A, MPA) and 0.1% formic acid in acetonitrile (mobile phase B, MBA). The gradient applied was 100% MPA for 5 minutes before increasing to 100% MPB over 15 minutes. This was held for 10 minutes before reverting back to 100% MPA and held for 2 minutes. Injection volume was 10 µl, flow rate was 0.4 ml/min and column temperature was 25°C.

For profiling, the MS used was a Thermo Exactive high resolution-accurate mass-mass spectrometer (Thermo Fisher Scientific Waltham, Massachusetts, USA) set at 50,000 resolution FWHM at 200 m/z, with an acquisition speed of 2Hz. Sample extracts were analysed separately for both positive and negative ionisation mode. For quality control purposes a pooled extract of each sample was prepared and analysed every 6 injections. All data was analysed using Progenesis QI® software (Newcastle Upon Tyne, UK).

MS^3spectra were detected on an Orbitrap Velos Pro hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in positive mode and MS/MS analysis was performed using a collision induced dissociation energy of 35eV. The Xcalibur Version 2.2 Software was used for data evaluation. Mass Frontier 7.0 Spectral Interpretation Software (Thermo-Scientific) was used for theoretical fragmentation prediction and generation of mechanistic pathways.

5.3.3. Data Analysis

For non-targeted metabolite analysis, data was analysed using Progenesis QI® software (Principal Components Analysis, (PCA), ANOVAs and mean fold change between groups) to assess the difference in abundance of metabolites between PCN infected plants and non-infected control plants. Compounds of interest were discovered by filtering on $p \leq 0.01$, fold-change ≥ 2 and percentage coefficient of variance between replicates <25%. A q-value (an FDR-corrected p-value) cut off of 0.05 was used. Tentative identifications were assigned to compounds of interest using an in-house *m/z* database of plant metabolites and the Metlin: Metabolite MS Database (Scripps Centre for Metabolomics, California, USA).

5.4 Results

After initial processing of the MS data sets, 7078 and 4249 signals were detected in positive electrospray ionisation (+ESI) and negative electrospray ionisation (-ESI) modes, respectively. Analysis of these data sets revealed that the mean coefficient of variance of the peak areas in the pooled quality control (QC) was between 6% and 16% for either ESI mode, indicating little drift in MS response during the analyses. Only tentative identities based on acquired accurate masses were assigned to metabolites of interest due to a lack of matching information between online databases and the information obtained from theoretical fragmentation.

5.4.1. Metabolic profile of the leaves of potato plants infected with the potato cyst nematode, *Globodera pallida* for 14 days.

Filtering of data from +ESI and - ESI mode revealed 81 and 54 metabolites of interest, respectively. These metabolites showed a significant response in the leaves ($P \leq 0.01$) with a fold change of ≥ 2 to the presence of parasitic nematode infection in the roots. The metabolites of interest in either electrospray ionisation mode were then subjected to a 5% false discovery rate (FDR) correction. This reduced the metabolites of interest found in +ESI and – ESI mode to 4 and 1, respectively (Table 5.1). Of the 4 metabolites of interest revealed in +ESI mode, 3 had a higher abundance in the leaves of non-infected control plants compared to nematode-infected plants suggesting that perhaps nematodes have the ability to suppress certain metabolites systemically in potato plants. Only tentative identifications based on acquired accurate mass could be assigned to the masses of interest found in +ESI mode (Table 5.1). A tentative identification could be assigned to the one mass of interest found in – ESI mode (Table 5.1). No metabolic pathways could be assigned to masses of true significant difference in either electrospray ionisation mode.

Only 13 metabolites of interest were found in the leaves of potato plants infected with *G. pallida* for 21 days in +ESI mode. However, when the 5% FDR cut off was applied, only one metabolite of interest remained statistically significant (Table 5.2). This metabolite of interest was reduced 4–fold in comparison to the control

Table 5.1. Metabolite markers identified in leaves of *Solanum tuberosum* cv. Désirée 14 dpi with the potato cyst nematode *Globodera pallida*

Metabolite Number	Retention Time	*m/z* Observed Ion	Ion Species	Formula of Ion	*P*-value	Identity of Metabolite	Group
1	13.93	315.1341	M+NH_4	$C_{17}H_{15}NO_4$	0.0001	(S)-Annocherine Longifolonine	-
2	12.94	202.0890	M+NH_4	$C_9H_{12}O_2S$	0.0001	(+/-)-3-[(2-methyl-3-furyl)thio]-2-butane	-
3	13.82	178.1355	-	-	0.0001	-	-
4	13.26	366.2022	M+H	$C_{18}H_{27}N_3O_5$	0.0001	Amino Acid combination	-
5	14.49	607.2376	M+K-2H	$C_{25}H_{46}O_{14}$	0.0568	2-O-(beta-D-galactopyranosyl-(1->6)-beta-D-galactopyranosyl) 2S,3R-dihydroxytridecanoic acid	-

Table 5.2. Metabolite markers identified in leaves of *Solanum tuberosum* cv. Désirée 21 dpi with the potato cyst nematode, *Globodera pallida*

Metabolite Number	Retention Time	*m/z* Observed Ion	Ion Species	Formula of Ion	*P*-value	Identity of Metabolite	Group
1	16.01	165.1273	M+H	$C_{11}H_{16}O$	0.0001	Thymol methyl ester Santalone Trans-jasmone	Secondary metabolite production

plants suggesting that nematodes suppress this metabolite. Only tentative identifications could be assigned to this mass of interest. No metabolites of interest were found to be significantly different between nematode infected plants and control plants in – ESI mode.

5.4.2. Metabolic profile of the leaves of potato plants infected with the peach potato aphid, *Myzus persicae*.

Filtering of data from +ESI revealed 105 metabolites of interest that had changed in leaves of five week old potato plants that had been infected locally with aphids for 48 hours (equivalent to potato plants infected with *G*. *pallida* for 14 days). Of the 105 metabolites of interest revealed, 82 metabolites were more abundant in aphid-infected plants, whilst 23 were less abundant in aphid infected plants. Following application of a 5% FDR to the metabolites of interest found in +ESI, 17 were found to be statistically significant changes. Of these 17 metabolites of interest, 16 were found to be more abundant in plants that had aphids present compared to plants that had no aphids present. Of these, four could be grouped into flavonoids, three could be grouped as alkaloids and four could be grouped into phenylpropanoid biosynthesis or metabolism. One only metabolite of interest had a higher abundance in non-infected control plants compared to plants that had aphids present and this was tentatively identified as a coumarin, an effective metabolite against insects. Tentative identifications according to acquired accurate mass were initially assigned to all metabolites (Table 5.2).

Metabolomic data was also collected for potato infected with aphids on 6 week old plants (equivalent to potato plants infected with *G. pallida* for 21 days). Following filtering of acquired masses in +ESI mode, 10 metabolites of interest were observed and tentative identifications were assigned (Table 5.4). All of these metabolites were significantly more abundant in plants that aphids present compared to those that had no aphids present and six out of the ten metabolites of interest were found to be involved in the synthesis or metabolism of plant secondary metabolites.

5.4.3. Metabolic profile of the leaves of potato plants infected with the potato cyst nematode, *Globodera pallida* and the peach potato aphid, *Myzus persicae*.

Filtering of data from +ESI and –ESI mode initially revealed 31 and 22 metabolites of

Table 5.3. Metabolite markers identified in leaves of *Solanum tuberosum* cv. Desirée 14 dpi with the peach potato aphid, *Myzus persicae*

Metabolite Number	Retention Time	*m/z* Observed Ion	Ion Species	Formula of Ion	*P*-value	Identity of Metabolite	Group
1	13.57	352.1153		-	0.0001	No identity	-
2	14.71	306.1100	M+K	$C_{14}H_{21}NO_4$	0.0001	Diethofencarb	Pesticide
			M+Na	$C_{17}H_{17}NO_3$		Morphinone	Alkaloid
3	13.33	328.1178	$M+NH_4$	$C_{18}H_{14}O_5$	0.0001	6-Deoxyjacareubin	Flavanoid
				$C_{18}H_{14}O_5$		Hoslundal	Flavanoid
4	11.40	251.1389	-		0.0001	No identity	-
5	14.71	284.1279	M+H	$C_{17}H_{17}NO_3$	0.0001	Acrophylline	Flavanoid
			$M+NH_4$	$C_{17}H_{14}O_3$		7-Methoxy-2-methylisoflavone	Alkaloid
6	14.79	314.1385	$M+NH_4$		0.0001	Desmoflavone	Flavanoid
			$M+NH_4$	$C_{18}H_{16}O_4$		Dasytrichone	Flavanoid
				$C_{18}H_{16}O_4$		Acetylcaranine	Alkaloid
7	12.23	277.1656	M+H	$C_{14}H_{20}N_4O_3$	0.0001	p-Coumaroylagmatine	Amino acid metabolism
8	13.74	273.0866	$M+NH_4$	$C_9H_{10}ClN_5O_2$	0.0001	Imidacloprid	Pesticide
9	13.75	130.0650	M+H	C_9H_7N	0.0001`	Isoquinoline	Alkaloid
			M+H	C_9H_7N		Quinoline	Alkaloid
10	11.63	302.1383	$M+NH_4$	$C_{17}H_{16}O_4$	0.0001	Stercurensin	Flavanoid
			$M+NH_4$	$C_{17}H_{16}O_4$		Flavokawin B	Flavanoid
11	15.13	316.1897	$M+NH_4$	$C_{19}H_{22}O_3$	0.0001	Ostruthin	Coumarin
						Glepidotin C	Polyketides
12	12.47	195.0651	M+H	$C_{10}H_{10}O_4$	0.0001	Dimethyl phthalate	Insect Repellent
						5-hydroxyconiferaldehyde	Phenylpropanoid biosynthesis
						Ferulic Acid	Phenylpropanoid biosynthesis

13	11.84	365.1202	M+Na	$C_{16}H_{22}O_8$	0.0001	Coniferin	Phenylpropanoid biosynthesis
14	13.75	245.0922	-	-	0.0001	No identity	-
15	2.29	446.1867	-	-	0.0001	No identity	-
16	11.78	176.1068	-	-	0.0001	No identity	-
17	11.93	208.0966	M+H	$C_{11}H_{13}NO_3$	0.0001	N-Acetyl-L-phenylalanine	Phenylalanine metabolism

Table 5.4. Metabolite markers identified in leaves of *Solanum tuberosum* cv. Desirée 21 dpi with the peach potato aphid, *Myzus persicae*

Metabolite Number	Retention Time	*m/z* Observed Ion	Ion Species	Formula of Ion	*P*-value	Identity of Metabolite	Group
1	13.57	352.1153	-	-	0.0001	No identity	-
2	14.71	306.1100	M+K	$C_{14}H_{21}NO_4$	0.0001	Diethofencarb	Pesticide
			M+Na	$C_{17}H_{17}NO_3$		Morphinone	Alkaloid
3	13.55	312.1288	$M+NH_4$	$C_{18}H_{14}O_4$	0.0001	Pongamol	Flavanoid
						9-O-Methylneodunol	
						Glyzarin	
						Castillene A	
						Dehydrocycloguanandin	Polyketide
				$C_{18}H_{17}NO_4$		Actinodaphine	Alkaloid
4	6.67	121.0648	M+H	C_8H_8O	0.0001	Phenylacetyldehyde	Phenylalanine
						4-Hydroxystyrene	metabolism
							Phenylpropanoid
							biosynthesis
5	14.71	284.1279	$M+NH_4$	$C_{14}H_{14}O_3$	0.0001	Osthenol	Phenylpropanoid
						Kawain	biosynthesis
						Demethylsuberosin	Polyketides
						Dihydroresveratrol	Phenylpropanoid
						Demethylbatatasin IV	biosynthesis
							Polyketides
							Polyketides
6	13.05	298.1073	$M+NH_4$	$C_{17}H_{12}O_4$	0.0001	Neodunol	Flavanoid
7	14.79	272.1046	-	-	0.0001	No identity	-
8	2.47	132.1019	M+H	$C_6H_{13}NO_2$	0.0001	L-Leucine	Secondary
						L-isoleucine	metabolite
							synthesis

							Secondary metabolite
9	2.05	254.0189	-	.	0.0001	No identity	-
10	13.45	322.1049	-	.	0.0001	No identity	-

interest in leaves of potato plants infected when dual infected with both *G. pallida* and *M. persicae* for 14 days. Following application of a 5% FDR cut off point these metabolites were reduced to 6 and 4 in +ESI and –ESI mode respectively. Four of the 6 metabolites that were significantly different in +ESI mode were found to have a higher abundance in plants that had been co-infected with both pests compared to control plants and were found to be a range of flavonoids and a range of metabolites involved in the synthesis of phenylpropanoids. Two metabolites were found to have a lower abundance in the leaves of co-infected plants compared to control plants, one of which could have an identity assigned. All of the 4 metabolites found in –ESI mode had a higher abundance in plants that were co-infected with both pests compared to those that had no pests present, of which one could have an identity assigned.

There were 44 and 47 metabolites of interest in +ESI and –ESI mode respectively, found in the leaves of potato plants co-infected with both *G. pallida* and *M. persicae* for a period of 21 days. However, when the 5% FDR cut off point was applied these metabolites were reduced to 2 in +ESI mode and 3 in –ESI mode. All 5 of the metabolites found in +ESI and –ESI mode had a higher abundance in the leaves of co-infected plants compared to control plants and these were found to be a range of alkaloids, flavonoids and a range of metabolites involved in phenylpropanoid biosynthesis.

Table 5.5. Metabolite markers identified in leaves of *Solanum tuberosum* cv. Desirée 14 dpi with the potato cyst nematode, *Globodera pallida* and the peach potato aphid, *Myzus persicae*.

Metabolite Number	Retention Time	*m/z* Observed Ion	Ion Species	Formula of Ion	*P*-value	Identity of Metabolite	Group
1	14.71	306.1100	M+K	$C_{14}H_{21}NO_4$	0.0001	Diethofencarb	Pesticide
			M+Na	$C_{17}H_{17}NO_3$		Morphinone	Alkaloid
2	13.05	298.1073	M+NH_4	$C_{17}H_{12}O_4$	0.0001	Neodunal	Flavanoid
3	14.71	284.1279	M+NH_4	$C_{14}H_{14}O_3$	0.0001	Osthenol	Phenylpropanoid
						Kawain	biosynthesis
						Demethylsuberosin	Polyketides
						Dihydroresveratrol	Phenylpropanoid
						Demethylbatatasin IV	biosynthesis
							Polyketides
							Polyketides
4	16.79	256.1097	M+H	$C_{13}H_{18}ClNO_2$	0.0001	Dimethachlor	Pesticides
5	16.79	315.1097	-	-	0.0001	No identification	-
6	13.57	352.1153	-	-	0.0001	No identification	-
7	12.97	326.1034	-	-	0.0001	No identification	-
8	13.53	298.1085	M-H_2O-H	$C_{17}H_{19}NO_5$	0.0001	Piplartine	Alkaloid
9	12.15	299.0773	M-H	$C_{13}H_{16}O_8$	0.0001	4-(β-D-Glucosyloxy)benzoate	Terpenoid
						Salicylic acid beta-D-glucoside	quinone
							biosynthesis
							Salicylic acid
							biosynthesis
10	12.43	153.0188	M-H_20-H	$C_7H_8O_5$	0.0001	5-dehydroshikimate	Shikimate
						3,4-dihydroxybenzoate	pathway

Table 5.6. Metabolite markers identified in leaves of *Solanum tuberosum* cv. Desirée 21 dpi with the potato cyst nematode, *Globodera pallida* and the peach potato aphid, *Myzus persicae*.

Metabolite Number	Retention Time	*m/z* Observed Ion	Ion Species	Formula of Ion	*P*-value	Identity of Metabolite	Group
1	2.03	350.9836	-	-	0.0001	No identity	-
2	13.33	328.117	$M+NH_4$	$C_{18}H_{14}O_5$	0.0001	6-Deoxyjacareubin	Flavanoid
				$C_{18}H_{14}O_5$		Hoslundal	Flavanoid
3	12.15	299.0773	M-H	$C_{12}H_{16}O_8$	0.0001	4-(β-D-Glucosyloxy)benzoate	Terpenoid quinone biosynthesis
						Salicylic acid beta-D-glucoside	Salicylic acid biosynthesis
4	12.78	319.0823	M-H	$C_{16}H_{16}O_7$	0.0001	4-Coumaroylshikimate	Phenylpropanoid biosynthesis
						Gliricoidol	Flavanoid
						Ourateacatechin	Flavanoid
						p-Coumaroylquinic acid	Phenylpropanoid Biosynthesis
5	13.99	282.1137	M-H	$C_{17}H_{17}NO_3$	0.0001	Morphinone	Isoquinoline alkaloid biosynthesis
						Acrophylline	Alkaloid

5.5. Discussion

Plants not only employ intricate chemical defences when attacked by pests and pathogens but they also alter metabolic pathways, in a process usually referred to as acclimation, in order to find a new state of homeostasis (Shulaev *et al.*, 2008). Metabolomic change is a major feature of genetic modification in plants experiencing abiotic and biotic stress as the metabolome reflects the cellular processes that control the biochemical phenotype of the cell, tissue or whole organism (Allwood *et al.*, 2008). Metabolomics has being carried out on a number of different plant species including, *Arabidopsis thaliana* (Kaplan *et al.*, 2004), tomato (Schauer *et al.*, 2005), potato (Roessner *et al.*, 2000), rice (Sato *et al.*, 2004), wheat (Hamzehzarghani *et al.*, 2005) and tobacco (Blount *et al.*, 2002). Plant defence responses include cell-wall modifications, and biochemical responses, which are of high energetic cost, and are based on modifications of the metabolism of infected plants both locally and systemically (Bolton, 2009). In this study, a non-targeted approach was used to elucidate the metabolic profile of the potato plant suffering nematode or aphid stress singly or a combination of both nematode and aphid stress.

Initially, a large number of signals were detected in both positive electrospray ionisation (+ESI) and negative electrospray ionisation (-ESI) modes following filtering of mass (MS) spectrometry data sets of leaves of potato plants suffering nematode and aphid stress. However, the number of signals with a significant difference between infected plants and control plants was greatly reduced in both ionisation modes following the application of a 5% false discovery rate (FDR) cut off point suggesting that there were not many true significant difference in the leaves of potato plants suffering stress from nematodes or aphids alone or from both pests simultaneously. Unlike testing a single hypothesis where a $P > 0.05$ cut off point will mean there is a 5% chance of a false positive (i.e. a significant result is presented, yet in reality no difference exists); testing a large data set for significant differences using a 5% cut off point can result in a large number of false positives. For example, if there are 4000 signals in an experiment and a t-test to each compound is performed then we would expect to get 200 (i.e. 5%) false positives by

chance alone. This large number of false positives is the problem with multiple testing therefore, a false discovery rate (FDR) must be applied when analysing a large data set. A 5% FDR cut off point was chosen based on previous reports that included large data sets (Benjamini and Yekutieli, 2001, Hartley et al., 2015), however, based on the study in question, for example proteomics, more stringent FDR cut off points such as 1% can be used (Davidsen *et al*., 2014). Similarly, less stringent FDR cut off point such as 25% may be used (Laguna *et al.*, 2015) however, this cut off point could not be found in any plant science-related studies and ultimately will decrease the amount of false positives that are detected. In this study, the q-value, which is an adjusted *P*-value found using an optimised FDR approach, was used to find true significant differences in compounds that had changed between infected plants and non-infected control plants. The q-value of a test measures the proportion of false positives incurred when a particular test is called significant (Storey, 2002).

5.5.1. Metabolic changes in the leaves of potato plants suffering nematode stress

Several transcriptomics studies have been carried out to elucidate plant-nematode interactions (Hammes *et al.*, 2005, Jammes *et al.*, 2005, Ithal *et al.*, 2007), however it is only in recent years that metabolic studies have been carried out in order to complement these findings (Hofmann *et al.*, 2008, Hofmann *et al.*, 2010). As obligate parasites, nematodes are fully dependent on plant-derived nutrients, which are imported into their permanent feeding structures and re-arrangement of plant cells caused by nematode infection suggests increased metabolic activity linked to a high energy demand (Hofmann *et al.*, 2010). In potato plants infected with nematodes for 14 days, only five compounds could be assigned tentative identities based on acquired accurate masses. Four of these compounds had a lower abundance in nematode-infected plants compared to control plants suggesting that perhaps nematodes have the ability to suppress compounds systemically in the leaves of potato plants. However, none of these identities have been reported in the literature as playing any particular defensive role in plants. Similarly, in potato plants infected with nematodes for 21 days only one compound could be assigned a tentative identification, trans-jasmone, which may be involved

in the production of plant secondary metabolites. Few studies exist in the literature of metabolic changes in the leaves of host plants in response to nematode infection and even these studies have used a targeted approach to investigate only certain metabolites such as terpenoids, phenolics and nicotine (Van Dam *et al.*, 2005, Kaplan *et al.*, 2008a, Kaplan *et al.*, 2008b). Metabolomic studies in response to nematode infection have mainly focussed on local changes in the roots of the host plant (Hofmann *et al.*, 2008, Hofmann *et al.*, 2009, Wieczorek *et al.*, 2008), with the exception of one study, which compared metabolic changes locally in the roots at the site of syncytia formation in addition to systemic changes in the roots and also in the shoots of *Arabidopsis thaliana* (Hofmann *et al.*, 2010). Metabolite changes were evident in syncytia with amino acids and certain sugars such as raffinose being up-regulated in nematode-infected roots compared to control roots. Levels of erythritol, a metabolite that is involved in terpenoid biosynthesis, was shown to be higher in control roots (Hofmann *et al.*, 2010). Terpenoids have been shown to be important deterrents of several insects and pathogens and recently have been shown to have a dose-dependent nematicidal effect on the free-living nematode, *Caenorhabditis elegans* (Abdel-Rahman *et al.*, 2013), therefore a higher level of erythritol in control roots may suggest that perhaps nematodes can suppress terpenoids in the roots of plants. Systemic changes in plants infected with nematodes were reported to be higher in the shoots of the host plant compared to systemic roots of the host plant with levels of carbohydrates such as α,α'-trehalose, 1-kestose and raffinose increasing the most in systemic shoots of nematode infected plants. A systemic decrease of these carbohydrates in roots of the infected plant in addition to a local increase in the syncytia highlights how the feeding site of the nematode is a strong sink character and a systemic increase of these carbohydrates in the shoots of the host plant could indicate an osmotic stress response (Hofmann *et al.*, 2010).

5.5.2. Metabolic changes in the leaves of potato plants suffering aphid stress

Large-scale studies investigating plant responses to aphid feeding are also largely uncommon with most focussing on the transcriptome and proteome (Coppola *et al.*, 2013, Guan *et al.*, 2015, Tzin *et al.*, 2015). Aphids have the ability to trigger

inducible defences and in order to do this plant recognition of the pest followed by cell signalling and subsequent transcriptional and metabolic reprogramming leading to the production of toxic or deterrent compounds is required (Kusnierczyk *et al*., 2008). Only 17 metabolites were found to be significantly different between aphid infected plants and control plants, the majority of these metabolites showed a higher abundance in the leaves of plants that had aphids present. Only tentative identities could be assigned to metabolites that had a higher abundance in aphid infected plants, however the metabolites of interest could be grouped into alkaloids, flavonoids or were metabolites found to be involved in phenylpropanoid biosynthesis. Alkaloids, flavonoids and phenylpropanoids have all been reported to play a role in resistance to aphids (Züst and Agrawal, 2016, Liang *et al.*, 2015, Pickett *et al.*, 1992). Alkaloids are present in a large number of plants and are a diverse group of nitrogen-containing compounds belonging to the phenylpropanoid family (Herrmann, 1995) that have significant impacts on herbivorous insects (Roberts, 2013). However, the effects of alkaloids on aphids have been variable, with the pea aphid, *Acyrthosiphon pisum* being only mildly deterred by pyrrolizidine alkaloids, yet being strongly deterred by indolozidine and quinolizidine alkaloids (Dreyer *et al.*, 1985). In this study one metabolite that had a higher abundance in aphid-infected plants was tentatively identified as quinoline, an alkaloid, suggesting that the potato plant up-regulate alkaloids in response to aphid feeding. Solanaceae species have been reported to produce alkaloids (Blackman and Eastop, 2008), however resistance to aphids still depends on the specific alkaloid in question. Aphids were only deterred by aglycones of glycoalkaloids from potato when fed using an artificial diet, while in the same study, when fed glycoalkaloids, the aphids were stimulated to feed (Güntner *et al.*, 1997). Flavonoids are also derived from the phenylpropanoid pathway and play an important role in defence against herbivourous insects, such as aphids (Simmonds, 2003). Endogenous flavonoids were shown to accumulate in cowpea (*Vigna unguiculata*) in response to feeding by the cowpea aphid, *Aphis craccivora* and these flavonoids inhibited aphid reproduction rates in *in vitro* bioassays (Lattanzio *et al.*, 2000). Isoflavonoids, flavones, flavanols and anthocyanins have all been shown to be important in plant-insect interactions (Simmonds and Stevenson, 2001, Gould *et al.*, 2006, Lev-Yadun

and Gould, 2008). However, flavonoids are not only important for plant defence reactions but also as intracellular signalling molecules (Peer and Murphy, 2006). Therefore, the production of flavonoids may affect transcription, translation and enzyme activity in the host plant (Morkunas *et al.*, 2015). Seven of the metabolites of interest in this study that had a higher abundance in the leaves of aphid infected plants compared to control plants could be assigned putative roles as flavonoids or were found to be involved in phenylpropanoid biosynthesis. Whether these metabolites affect transcription, translation or enzyme activity in the leaves of potato plants in response to aphid feeding, however remains to be elucidated with further research.

5.5.3. Metabolic changes in the leaves of potato plants suffering nematode and aphid stress

Studies have been published on metabolic changes in response to nematode infection or to aphid infection in isolation and some of these studies have even reported the effects these systemic changes in metabolites caused by nematode infection may impact the fecundity of an above-ground pest (Kaplan *et al.*, 2009, Hol *et al.*, 2013). Nevertheless, currently in the literature there are no studies reporting metabolic changes in the host plant with two or more pests present. In this study, 3 metabolites present in the leaves of potato plants co-infected with both *G. pallida* and *M. persicae* for 14 days were also present in the leaves of plants infected with just aphids suggesting that these metabolites were up-regulated in the plant in response to aphid feeding and not up-regulated systemically in the leaves due to the presence of nematodes for 14 days. Four metabolites of interest that were unique to co-infected plants i.e. not present in plants infected with nematodes singly or aphids singly, were given putative roles as terpenoids or metabolites involved in the phenylpropanoid pathway, salicylic acid biosynthesis or the shikimate pathway. A metabolite with a putative role as a terpenoid was found to be more abundant in co-infected plants compared to control plants. The up-regulation of this terpenoid in co-infected plants is in contrast to tentatively identified terpenoid, which had a lower abundance in plants with just nematodes present, therefore perhaps the additional presence of aphids prevents nematodes from suppressing terpenoids or else the presence of the terpenoid in co-infected

plants was due to the presence of aphids on the plant. Two other metabolites of interest which appeared in plants that were co-infected with both pests either for 14 days or 21 days were tentatively identified as salicylic acid beta-D-glucoside and 5-dehydroshikimate. Salicylic acid beta-D-glucoside (SAG) is the dominant conjugate in plants (Lee *et al.*, 1995) and is actively transported from the cytosol into the vacuole where it may function as an inactive storage form that can be converted back into free salicylic acid (Dean and Mills, 2004). Salicylic acid, which is a benzoic acid is formed from the shikimic (or shikimate) pathway (Raskin, 1992) and the chemical compound 5-dehydroshikimic acid is related to shikimic acid (Banwell *et al.*, 2003) therefore it is likely that 5-dehydroshikimic acid is involved in the biosynthesis of salicylic acid. Levels of stored and total salicylic acid were significantly higher in potato plants that were co-infected with both *G. pallida* and *M. persicae* for a period of 14 days compared to non-infected control plants (see chapter 4), therefore the presence of the metabolites SAG and 5-dehydroshikimate in the same co-infected sample gives further confidence that more salicylic acid may be synthesised in the plant as a heightened defence response against two pests compared to when the plant is infected with only one pest. Nevertheless, these metabolites were only putatively identified, therefore more metabolomic research needs to be carried out in order to confirm tentative identities using authentic analytical standards.

It is clear from this study that aphids induce more metabolites in the leaves of potato plants compared to nematodes, however this may be due to the fact that the aphids were present locally on the leaf that was sampled rather than systemically such as the nematodes were. The literature reports that nematodes differentially induce primary metabolites both locally and systemically in plants such as *Arabidopsis* (Hofmann *et al.*, 2010), however, more research needs to be carried out to include systemic metabolic changes in secondary metabolites in the shoots of different plants in response to nematode infection. Further metabolic research needs to be applied to masses of interest in order to confirm their identities using authentic analytical standards. Following this, these compounds

may then be used as targets in engineering crops to effectively deal with the constraints of dual biotic stresses.

Summary

- Five metabolite markers had a higher abundance in plants infected with nematodes for 14 days compared to control plants. No biological roles could be assigned to these.

- Metabolite markers in the leaves of potato plants infected with aphids were putatively identified as a range of flavonoids, alkaloids and metabolites involved in phenylpropanoid biosynthesis

- Four metabolite markers up-regulated in the leaves of potato plants were found to be unique to co-infected plants, i.e. not present in the leaves of potato plants infected with nematodes singly or aphids singly.

- Metabolite markers unique to co-infected plants were tentatively identified as salicylic acid beta-D-glucoside and 5-dehydroshikimic acid, two compounds involved in salicylic acid biosynthesis.

Chapter 6

General Discussion

This study has elucidated plant-mediated interactions between the potato cyst nematode, *Globodera pallida* and the peach potato aphid, *Myzus persicae*, two important pests in the UK that infect the potato crop and often simultaneously. Glasshouse experiments were set up to investigate the biological interactions between the two pests. In addition, molecular and biochemical experiments were carried out to explore defence pathways in the host plant suffering nematode or aphid stress singly or a combination of both stresses together. These pathways were then analysed to interpret how they might play a role in determining consequence for pest success.

6.1. An increase in pests and pathogens with a changing climate

Crops are continually subjected to a variety of environmental pressures during development, which could lead to a reduction in the productivity of the plant. When plants grow in sub-optimal conditions their full genetic potential for growth and reproduction is compromised, a yield gap is observed and therefore the average yield obtained is much lower than the maximum potential yield for a particular crop (Lobell *et al.*, 2009, Atkinson *et al.*, 2015). This yield gap can be largely attributed to the potentially damaging physiological changes in the plant known as abiotic and biotic stresses (Boyer, 1982). Crop yield losses due to these stresses reduce food production in many parts of the world. For example, the average yields for tropical wheat has been estimated as less than 20% of the potential yield due to frequent nutrient, water, pest, and disease stresses (Bruinsma, 2003). Abiotic stresses alone are estimated to cause a 70% decrease in crop yield (Acquaah, 2009), while the estimated total global potential loss due to pests and pathogens varies from 50% in wheat to more than 80% in cotton production (Oerke, 2006) and with current climate change predictions, this decline in crop productivity will be exacerbated (Sree and Rajam, 2015). Forecasted climate changes are expected to include an increase in temperature, emission of green-

house gases and an increase in tropospheric ozone levels (Atkinson *et al.*, 2015). Temperature increases outside of the normal range for growth stages for crop plants will impact productivity and will lead to a shorter life cycle for the plant (Moriondo *et al.*, 2011). In addition, climate change will affect plant-biotic interactions. The positive commensalism interaction that was observed between nematodes and aphids in this study showcases how infection of a plant with one pathogen may indirectly affect the fecundity of a secondary arriving pest without climate change incorporated into the experimental model. It was deduced that climate change could affect the life cycle of pests and pathogens, whereby generations increase as well as the number of survivors of particular instar stages (Lonsdale and Gibbs, 1996, Collier *et al.*, 2008). Therefore, climate change could exacerbate even further the economic costs that these two pests cause the potato industry both in the UK and globally. Furthermore, the geographical distribution of pests and pathogens will be altered (Newton *et al.*, 2011) leaving crop plants vulnerable to a wide range of both abiotic and biotic environmental stresses, which act on the plant simultaneously.

Traditional methods of assessing molecular and biochemical changes in the plant in response to abiotic and biotic stressors have been based on applying a single stress in isolation or by exogenously applying phytohormones to model plant species such as *Arabidopsis* in a glasshouse setting (Moran and Thompson, 2001, Wubben *et al.*, 2008, Hamamouch *et al.*, 2011). These studies have enhanced our knowledge on signalling cascades and defence pathways mediated by phytohormones in stressed plants and engineered plants have been used to achieve tolerance to various stresses. However, tolerance attained from an engineered plant in a laboratory setting has been difficult to reproduce when the plant is placed in a field setting (Mittler, 2006, Atkinson and Urwin, 2012). This is because stresses in natural surroundings do not just encounter a single stress in isolation. They are subject to a variety of abiotic and biotic stresses simultaneously; therefore there is a need for studies to be carried out on crop plants suffering multiple stresses at the same time.

In recent years, studies have emerged reporting the effect that climate change will impose on plant-pest interactions (Newman, 2004, Ziska and Bunce, 2007, Gregory *et al.*, 2009). It has been reported that increasing levels of CO_2 is likely to increase winged forms of the cereal aphid (*Rhopalosiphum padi*) by 10% therefore potentially leading to a greater spread and incidence of barley yellow dwarf virus for which the cereal aphid is a vector (Newman, 2004). Plant parasitic nematodes have also shown a neutral or positive response to elevated levels of CO_2, whereby some species have the potential to build up rapidly and therefore interfere with the plant's response to abiotic stress (Yeates *et al.*, 2003, Somasekhar and Prasad, 2012). Overwintering mortality of some aphids was reduced due to increasing temperatures, resulting in flight phenologies occurring a month earlier (Zhou *et al.*, 1995) and this is already the case in Scotland, with aphids of different genotypes being detected in suction traps several weeks earlier than previously reported (Malloch *et al.*, 2006). In addition, aphids are expected to be particularly responsive to climate change due to their ability to develop at low temperatures and the fact they have a short generation time (Sutherst *et al.*, 2007). Plant defence pathways are also predicted to be altered as a result of increasing CO_2 levels through manipulation of phytohormone pathways. Increased CO_2 levels were shown to cause suppression of jasmonic acid and ethylene levels, which resulted in a reduction in the levels of cysteine protease inhibitors, which then rendered soybean more susceptible to coleopterans (Zavala *et al.*, 2008). Expression of salicylic acid in soybean was found to be elevated in soybean in response to increased levels of CO_2 (Casteel *et al.*, 2012). This may be of benefit to the plant as salicylic acid has been reported to an effective defence mechanism against a number of pests and pathogens (Yu *et al.*, 1997, Vleeshouwers *et al.*, 2000, Wubben *et al.*, 2008). However, it may also render the plant more susceptible to attack by secondary arriving pests. Our study shows that the salicylic acid and jasmonic acid signalling pathways are different in the leaves of potato when plants are dual stressed with both aphids and nematodes compared to either pest singly. Dual stressing the potato with both pests simultaneously increased the total levels of endogenous salicylic acid, more than what was elicited by each pest singly. This additive salicylic acid effect may suppress the levels of jasmonic acid that the plant

elicits in the leaves in response to the presence of aphids. This could be of benefit to the aphids and explain a higher number of aphids on nematode-infected plants as both the peach potato aphid (*M. persicae*) the greenbug aphid (*Schizaphis graminum*) have the ability to circumvent the plant's immune system by eliciting the salicylic acid signalling pathway in order to antagonise and suppress the jasmonic acid pathway, which is important in mediating resistance to phloem feeders (Zhu-Salzman *et al.*, 2004, Ellis *et al.*, 2002).

The need for plants to produce an appropriate response to multiple stress conditions is also true for abiotic stresses. For example, plants often open their stomata in order to cool their leaves in response to heat stress, however in drought stress this would be disadvantageous as more water would be lost from the plant (Rizhsky *et al.*, 2004). In addition, uptake of salt or heavy metals could be enhanced due to increased transpiration due to said heat stress, thus intensifying damage to the plant under stress (Mittler and Blumwald, 2010). These studies mentioned highlight that research into the plant defence response to multiple attacks simultaneously is crucial as the plant response to various stressors is not just additive to the individual responses elicited (Rizhsky *et al.*, 2004, Atkinson *et al.*, 2013, Prasch and Sonnewald, 2013, Rasmussen *et al.*, 2013, Suzuki *et al.*, 2014). The particular stress response elicited by the combination of stresses should be treated as a new state of stress response in which the plant must adjust or adapt to (Mittler, 2006).

6.2. Cross-talk between molecular pathways for development of new resistant crops

Gene expression data has been used from studies on plants suffering abiotic or biotic stresses in isolation in order to identify combinatorial stress responsive genes (Luo *et al.*, 2005, Tippmann *et al.*, 2006, Narsai *et al.*, 2013, Shaik and Ramakrishna, 2013, Sharma *et al.*, 2013). However, these studies can be misleading as more recent evidence has shown that plants responds to multiple stressors by eliciting a specific tailored response and these responses cannot be understood by directly generalising results obtained from stress studies suffered in isolation (Atkinson and Urwin, 2012, Atkinson *et al.*, 2013, Bostock *et al.*, 2014, Kissoudis *et al.*, 2014,

Prasch and Sonnewald, 2013, Rasmussen *et al.*, 2013, Rivero *et al.*, 2014, Suzuki *et al.*, 2014). Plant hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) are or are at least partly shared by both abiotic and biotic stress signalling, indicating the likelihood of crosstalk and convergence of mechanisms in these molecular pathways therefore, research aimed at developing stress-tolerant crops is increasingly focussing on crosstalk between phytohormones (Miller *et al.*, 2010, Denancé *et al.*, 2013, Kissoudis *et al.*, 2014). Phytohormones are a diverse group of signalling molecules and crosstalk between different molecular signals is a way in which plants can fine-tune their responses to stress by controlling gene expression (Pieterse *et al.*, 2012, Lazebnik *et al.*, 2014). Phytohormones can act either at their site of synthesis or systemically elsewhere in the plant following their transport (Peleg and Blumwald, 2011), thus attack from a pathogen at one position in a plant may indirectly affect a secondary arriving pest through plant mediated interactions. Complex interactions between SA, JA, ET and ABA, however, are influenced by the invading pest or pathogen and the timing of the infection (Ton *et al.*, 2009, Dicke *et al.*, 2009, Atkinson *et al.*, 2015). ABA has traditionally been involved in responses against abiotic stresses, while SA, JA and ET are involved in defence responses against biotic stresses however studies indicate that ABA may also play a role in biotic defence responses as studies have revealed that ABA and ET can act synergistically with JA-regulated responses, while these responses generally antagonise SA signalling and *vice versa* (Pieterse *et al.*, 2012, Caarls *et al.*, 2015). ABA has been reported to be a key hormone in regulating crosstalk between other molecular pathways as ABA directly impacts salicylic acid biosynthesis (Yasuda *et al.*, 2008, de Torres Zabala *et al.*, 2009). Manipulation of the ABA pathway in plants can enhance resistance to pathogens. For example, enhanced callose deposition is positively regulated by ABA and leads to resistance against the penetration of fungal pathogens (Cao *et al.*, 2011). In addition, drought stress leads to stomatal closure, which conserves water loss, and this stomatal closure as an added defence mechanism prevents entry of pathogens such a *Psuedomonas syringae* into leaves (Sawinski *et al.*, 2013). Conversely, ABA can also render a plant more susceptible to *P. syringae* as when *Arabidopsis* plants were exogenously sprayed with ABA, susceptibility to infection was observed (Thaler and Bostock,

2004, de Torres Zabala *et al.*, 2009). The discrepancies between these two reports were explained based on the infection timing of *P. syringae*. ABA is needed in order to prevent initial infection by the bacteria, nevertheless once inside the plant it has the ability to take control of ABA signalling in order to maintain a high water potential in the apoplast, which is required for *P. syringae* colony growth (Beattie, 2011).

The plant defence signalling pathway is extremely complex and there are more than just four phytohormones that control defence responses. These include auxins, gibberellins, cytokinins and the newer defence hormones such as brassinosteroids and strigolactones (Großkinsky *et al.*, 2016, Shigenaga and Argueso, 2016). Auxin, a plant hormone, which has diverse roles in plant growth and development, is increasingly being described as having an involvement in defence against abiotic and biotic stressors (Kazan and Manners, 2009, Salopek-Sondi *et al.*, 2015, Großkinsky *et al.*, 2016) and IAA signalling my influence plant defence responses to biotic stresses by modulating other hormonal pathways and defences (Erb *et al.*, 2012, Machado *et al.*, 2016). Auxins can repress defence-related processes to invest in the growth and development of the plant, and similarly their actions can be antagonised by SA or JA leading to the activation of defence at the expense of plant growth (Pieterse *et al.*, 2012). Auxin has been reported as being involved in both resistance and susceptibility to pathogens depending on whether they feed off of living cells or dying and decaying cells (Llorente *et al.*, 2008). Auxin has the ability to suppress the SA mediated pathway in the *Arabidopsis-P. syringae* interaction (Wang *et al.*, 2007, Iglesias *et al.*, 2011), however auxin works synergistically with JA signalling therefore affecting disease development in plants (Kazan and Manners, 2009, Kidd *et al.*, 2009, Qi *et al.*, 2012). In addition, auxin has also been implicated in both root knot and cyst nematode infection. *Meloidogyne javanica* (Lambert *et al.*, 1999)*, Heterodera schachtii* (Vanholme *et al.*, 2009) and *H. glycines* (Bekal *et al.*, 2003) secrete an effector, chorismate mutase, which has the ability to suppress SA signalling in plants (Jones *et al.*, 2003, Djamei *et al.*, 2011). Soybean hairy roots expressing the *M. javanica* chorismate mutase showed an altered root phenotype, however when auxin was exogenously applied to the plant the root phenotype was

rescued. This suggests that the *M. javanica* chorismate mutase has the ability to suppress auxin levels, but how this suppression of auxin levels may be of benefit to the nematode is still unknown (Doyle and Lambert, 2003).

Points of crossover between hormonal signalling pathways is crucial in developing broad-spectrum resistant crops as such a close association of ABA and other phytohormones such as auxins with defence signalling pathways may result in a change in defence responses. A slight change in environmental conditions may lead to an increase in ABA, which in turn could cause a repression of the SA, JA and ET defence pathways, therefore rendering the plant more susceptible to pest and pathogen attack (Atkinson *et al.*, 2015).

6.3. Metabolomics of plant biotic interactions

Plant metabolites represent genetic variance, epigenetic modifications as well as transcriptomic and proteomic compounds, therefore metabolomic studies are important for plant-biotic interactions (Tenenboim and Brotman, 2016). To date, metabolomics has only been used in few studies, which investigates the response of the plant to combined stress even though the use of metabolomics shows great potential in studying unique defence responses from combinatorial stresses. When tomato plants were stressed with a combination of both water and nematodes, distinct metabolites were observed in dual stressed plants compared to when the plants were stressed singly with just water or nematodes (Atkinson, 2011). Levels of carotenoids lycopene and β-carotene were lower in water stressed plants, while in dual stressed tomatoes these metabolites showed a different pattern. In addition, sugars were only observed to be higher in dual stressed plants compared to plants suffering a single stress in isolation (Atkinson *et al.*, 2011). Similarly, metabolomics studies revealed a unique response when *Arabidopsis* was artificially stressed with exogenous application of ABA and SA, whereby few metabolic changes were observed in plants that were treated with SA alone, however when dual stressed, the plants showed significant changes in sugars and amino acids (Okamoto *et al.*, 2009). Metabolomics has also been used to characterise concoctions of volatile organic compounds (VOCs) in plants challenged with both abiotic and biotic stressors. When *Arabidopsis* was stressed with just temperature alone, emissions of

6 different volatiles were observed, however when these plants encountered stress from the peach potato aphid, *Myzus persicae* in addition to the temperature stress 8 volatiles emissions were observed (Truong *et al.*, 2014). VOCs have been shown to be important alarm pheromones against herbivory, for example, the volatile sesquiterpene (E)-β-farnesene was found to deter *M. persicae* from colonising the wild potato, *Solanum berthaultii* (Gibson and Pickett, 1983, Avé *et al.*, 1987). Efforts have been made to utilise this chemical compound to repel aphids through genetic engineering and in the laboratory have proved successful. When the (E)-β-farnesene gene was transformed into *Brassica juncea,* direct repellence against aphid colonisation was observed in the transgenic plants compared to control plants (Verma *et al.*, 2015). Similarly, aphid repellence observations were made in the laboratory when wheat plants were transformed with a gene derived from the peppermint plant, which enabled the wheat to release the (E)-β-farnesene pheromone. These results, however failed to translate over into field trials as there was no statistical difference in the number of aphids infesting the genetically modified wheat and those infesting the control plants (Bruce *et al.*, 2015).

Although these metabolomics studies have been beneficial in order to further understand plant defences responses to abiotic and biotic stresses in isolation and in combination, only small groups of metabolites have been extensively researched, for example, alkaloids. In order to investigate the neglected compounds further, metabolomics should be combined and integrated with the results from other 'omics' data sets so that a comprehensive overview of potential cellular processes in a physiological context is obtained. Studies have investigated biosynthetic pathways of alkaloids and toxic compounds found in tomato and potato using genomic and transcriptomic tools and these results were supported by metabolomics (Itkin *et al.*, 2013), however to date in the literature an investigation into plant defence responses to combined abiotic or biotic stressors, which incorporates two or more 'omics' data sets has not been published.

The metabolomics study in this thesis focussed on the different compounds that changed in susceptible potato cultivars in response to nematode and aphid infection both singly and in combination. Although metabolite changes in

susceptible cultivars are useful, one might argue that it is the changes in response to nematode and aphid infection in resistant cultivars that are more important. There is a lack of understanding of physiological and secondary chemistry traits in relation to plant defence and resistance against different pests and pathogens, therefore a non-targeted metabolomics approach in potato cultivars resistant to nematodes and aphids both singly and in combination could reveal new defence pathways to explore in order to develop crops that are resistant against multiple stresses.

6.4. Root exudates – a new defence mechanism against nematodes

Currently, synthetic nematicides are the most commonly used procedure to protect against nematode infection. However, as with the majority of exogenously applied chemical compounds, there is a high cost and a risk of toxicity to the environment, therefore new methods of nematode control need to be discovered. Second-stage juveniles (J2) of the golden potato cyst nematode, *Globodera rostochiensis* and the white potato cyst nematode, *G. pallida* hatch from eggs when they are exposed to root diffusate from host plants (Perry, 1989, Devine *et al.*, 1996). To date, only nine nematode hatching factors have been identified from root exudates including the potato glycoalkaloids solanine and α-chaconine (Devine and Jones, 2000). Purification and identification of hatching factors has been difficult due to their high specific activity therefore leaving only trace amounts available for analysis (Devine *et al.*, 1996). Exudates represent a source of defence molecules that could be exploited in order to provide resistance against below-ground pathogens (Nelson, 1991). Studies have described the defence activity that seed exudates may have against microorganisms (Scarafoni *et al.*, 2013), however it is only recently that seed exudates have been investigated as having a potential defence role against plant parasitic nematodes (Rocha *et al.*, 2015). Reduced hatching of *M. incognita* J2s was observed from eggs that were incubated in seed exudates compared to those that were incubated in distilled water suggesting the secreted proteins from these exudates were directly involved in plant defence against soil pathogens. In addition, in the same study, when J2s were pre-treated with soybean exudates, a 90% reduction of gall numbers in tobacco plants was observed. A proteomic

approach on the exudates from soybean seeds indicated the presence of 63 exuded proteins, including *β*-1,3-glucanase, chitinase, lectin, a trypsin inhibitor and a lipoxygenase, all of which have previously been reported to be involved in plant defences against biotic stressors (Rocha *et al.*, 2015). This study and the research presented in chapter 3 that exudates collected from aphid-infected potato plants reduces the emergence of *Globodera pallida* J2s from cysts compared to control plants highlights the need for an attempt into elucidating the role that plant exudates may play in plant defence and subsequent nematode resistance.

6.5. Developing new resistant crops for multiple biotic stresses

Success of developing stress tolerant crops in order to feed an ever increasing global population has been at the hub of plant science research. Although stress resistance success has been achieved by researchers for both abiotic and biotic stressors (Rossi *et al.*, 1998, Fuller *et al.*, 2008, Adhikari, 2016, Ramírez and Maiti, 2016, Kersting *et al.*, 2016, Papolu *et al.*, 2016), these studies do not necessarily test the resistant crop against further abiotic and biotic stresses. In recent studies, it has been highlighted that plants experiencing unfavourable environmental conditions will have a higher susceptibility to herbivore and pathogen attack, whereby the behaviour of the insect will be affected due to the quality of the host plant (Gregory *et al.*, 2009, McKenzie *et al.*, 2013, Foyer *et al.*, 2016). The results in this thesis confirm that when the potato plant is attacked by one pathogen, this can render the plant more susceptible to a secondary arriving pest, potentially due to crosstalk between different defence signalling pathways. It was also highlighted in this research that stress defence responses are not simply additive when two biotic stressors are applied to the plant, but are different to the individual response. Each stress that is applied to a plant, whether abiotic or biotic influences all aspects of the plant including its morphology, the metabolome, the transcriptome and the proteome therefore allowing overlap of defence responses and subsequently cross-tolerance (Foyer *et al.*, 2016). Therefore, in order to develop crops that survive in conditions found in the field, resistant plants should be tested under combined stress treatments (Mittler and Blumwald, 2010, Atkinson and Urwin, 2012). A theory has been proposed suggesting that when plants are attacked by more than

one pathogen at a time, common and overlapping signalling pathways are elicited therefore enhancing general defences, which can prime the plant for further attack against a wider range of stresses (Mullineaux *et al.*, 2011, Bjornson *et al.*, 2016). It is suggested that plants control their basal defence response through a network of genes that are regulated by heat shock factors, redox and hydrogen peroxide signalling, which protects the plant from low levels of stress and allows them to function, grow and development up to a certain point. When the plants endures a level of stress, which passes this basal stress threshold, the plant invests more energy into eliciting a stronger defence response, which allows the plant to endure extreme stress conditions, however this leads to a cost for growth and productivity of the plant (Mullineaux *et al.*, 2011). More recent studies in resistance against abiotic and biotic stresses include plant priming (Pastor *et al.*, 2013, Baccelli and Mauch-Mani, 2015, Savvides *et al.*, 2016, Martinez-Medina *et al.*, 2016). Priming a plant against defence induces a physiological state, whereby the plant is conditioned or prepared for the activation of defences against abiotic and biotic stresses (Frost *et al.*, 2008). It is currently being employed in plant science research due to its broad spectrum defence against multiple pests and diseases (Conrath *et al.*, 2015); its robustness as a defence mechanism and the low fitness cost caused to the plant (Martinez-Medina *et al.*, 2016). Scientists are challenged every day in order to develop new stress resistant traits against a broad range of abiotic and biotic stressors in economically important crops. In order to generate new varieties, researchers will need to take advantage of the latest technologies in crop breeding, including expression of transgenes, marker assisted selection, QTL analysis and CRISPR (Clustered, Regularly Interspaced, Short Palindromic Repeats), the newest targeted genome modification system, which could be utilised to improve crop plants (Belhaj *et al.*, 2013, Chen and Gao, 2014, Long *et al.*, 2015). Yields for crops can vary greatly between farms and countries even though they may have good climatic conditions for growing a crop. For example in the USA, maize had an average yield of 10.0 t/ha in 2013, while Zimbabwe only had an average yield of 0.9 t/ha (FAO, 2015). Given the timescale of approximately 20 years from the demonstration of innovative solutions to seeds of resistant crops, it is imperative that molecular engineering such as methods mentioned above and conventional

selective breeding methods be combined to achieve higher yields that can endure increasingly intensive biotic stresses presented by a changing climate.

The above mentioned advances in plant molecular and biochemical biotechnology have enhanced the capabilities of research for discovery of new resistant genes and their functional characterisation, the study of tissue-specific promoters in addition to discovering novel efficient methods for plant genetic transformation. By using incorporating the results from this study, researchers can utilise many techniques to provide farmers with seeds that are resistant to multiple stresses, in this case plant parasitic nematodes and aphids. All of the chapters presented link into each other and by utilising the molecular and biochemical data in chapters 4 and 5, respectively, marker assisted breeding could be applied in the laboratory, whereby genetic markers are used to identify specific genes, which will ultimately provide farmers with seeds that are resistant to multiple stresses, in this case plant parasitic nematodes and aphids. The results from chapter 3 present results indicating that aphids may cause a defence response in the roots or directly affect the composition of potato and tomato root exudates, which have the ability to reduce the emergence of juveniles from their cysts. This data can provide scientists the opportunity to pinpoint a marker in root exudates and allow biotechnology companies to develop an environmental friendly nematicide, which can be sprayed on the foliar parts of the potato plant, therefore eliciting a defence response or change in composition of exudates below ground. The results presented in this thesis opens up exciting new opportunities for future research into sustainable agricultural.

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