A plant-feeding nematode indirectly increases the fitness of an aphid.

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Keywords

aboveground-belowground interactions, Aphids, induced defence, Jasmonic acid (JA), salicylic acid (SA), Plant Parasitic Nematode

Abstract

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Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: No

A plant-feeding nematode indirectly increases the fitness of an aphid.

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Running title: Nematodes indirectly influence aphids.

Abstract

Plants suffer multiple, simultaneous assaults from above and below ground. In the laboratory, pests and/or pathogen attack are commonly studied on an individual basis. The molecular response of the plant to attack from multiple organisms and the interaction of different defence pathways is unclear. The inducible systemic responses of the potato (*Solanum tuberosum* L.) host plant were analysed to characterise the plant-mediated indirect interactions between a sedentary, endoparasitic nematode (*Globodera pallida*) and a phloem-sucking herbivore (*Myzus persicae*). The reproductive success of *M. persicae* was greater on potato plants pre-infected with *G. pallida* compared to control plants. Salicylic acid (SA) increased systemically in the leaves of potato plants following nematode and aphid infection singly with a corresponding increase in expression of SA-mediated marker genes. An increase in jasmonic acid (JA) associated with aphid infection was suppressed when plants were co-infected with nematodes. Our data suggests a positive, asymmetric interaction between a sedentary endoparasitic nematode and a sap-sucking insect. The systemic response of the potato plant following infection with *G. pallida* indirectly influences the performance of *M. persicae*. This work reveals additional secondary benefits of controlling individual crop pests.

Keywords:

Aboveground-belowground interactions; aphids; induced defences; jasmonic acid; plant parasitic nematodes; salicylic acid

Introduction

Plants are simultaneously attacked by a number of invading organisms, both above and below ground. Pests and pathogens sharing the same host can, despite their spatial separation, together elicit a response that is more complex than the additive response of those sole agents (van Dam & Heil, 2011). Infection of a host plant that carries a pre-existing pest or pathogen burden will influence the success of the secondary or primary infection, depending on a range of factors including the species under investigation, the sequence of pest arrival, the severity of the infestation (Erb *et al.*, 2011; Johnson *et al.*, 2012; Huang, *et al*, 2016; Papadopoulou and van Dam, 2017), and the changes in primary and secondary metabolites in the shared plant tissues (Bezemer *et al.*, 2003; Wardle *et al.*, 2004; Schoonhoven *et al*, 2005; van Geem *et al*, 2016). Given this context dependency, it is unsurprising that both positive and negative effects of below-ground organisms on those above-ground have been reported. For example, a positive indirect influence by generalist root herbivores resulted in an increased abundance of a tephritid (Diptera: Tephritidae) seed predator and two of its dominant parasitoids (Hymenoptera: Chalcidoidea) on the marsh thistle (Masters *et al.*, 2001), whereas negative indirect effects of wireworms below ground led to a reduced performance and fecundity of the beet armyworm, a major foliage feeding pest of cotton (Bezemer *et al.*, 2003).

Host-mediated interactions between plant-feeding organisms are particularly significant in agricultural systems: many economically important crops are attacked simultaneously by aboveground insect pests, such as aphids, and by belowground pathogens, such as plant parasitic nematodes. Aphids, the largest group of phloem feeders, use their stylet-like mouthparts to feed on photoassimilates found in the phloem sieve elements (Pollard, 1972). Aphids also transmit

viruses, which can adversely affect the fitness of the host plant (Dixon, 1998). Primarily, their importance is as vectors of virus diseases but due to their ability to reproduce rapidly (Foster *et al.,* 2000), high populations can also result in substantial reductions in yield (Kolbe, 1970). Cyst nematodes are a group of highly evolved sedentary endoparasites and are pathogens of temperate, subtropical and tropical plant species. Following root penetration, cyst nematode second-stage juveniles migrate intracellularly towards the vascular cylinder where each chooses an initial syncytial cell from which it will form a highly metabolically active feeding site (Lilley *et al*., 2005). Large scale gene expression profiling has identified genes that are differentially regulated by cyst nematode infection following a compatible interaction (Alkharouf *et al.*, 2006; Ithal *et al.*, 2007, Szakasits *et al.*, 2009) and many genes related to metabolic pathways including phytohormone regulation are up-regulated in the host plant (Uehara *et al.*, 2010). Salicylic acid (SA)-dependent signalling seems to be crucial for resistance against biotrophic pathogens (Glazebrook, 2005; Loake & Grant, 2007) and cyst nematodes have been reported to activate a strong salicylic acid-mediated defence response in shoots of *Arabidopsis thaliana* from five days post inoculation (Wubben *et al.*, 2008).

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 influences 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 & Müller, 2012).

The mechanism underpinning this asymmetric interaction may be changes to plant biomass, although changes in primary and secondary metabolites appear to be more important at least in some cases. 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 (Vandegehuchtee *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.*, 2008; 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* (Hoffmann *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).

Phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) 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. Research aimed at developing stress-tolerant crops is therefore increasingly focussing on crosstalk between phytohormones (Miller *et al*, 2010; Denancé *et al*, 2013; Kissoudis *et al*, 2014). 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 (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 and ET, 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).

In this study we examined plant-mediated interactions between the plant parasitic nematode, *Globodera pallida* and the generalist aphid *Myzus persicae* Sulzer (Hemiptera:Aphididae) in the potato crop (*Solanum tuberosum* cv. Désirée). The potato cyst nematode *G. pallida* is an important pathogen of potato crops that can cause reported yield losses in excess of 50% (Trudgill, 1986) and the species is estimated to be present in 64% of potato-growing fields in England and Wales (Minnis, 2002). *M. persicae* feeds on a large variety of plants belonging to different families and worldwide is the most important insect pest of potato (Radcliffe, 1982). Although there is an increasing number of studies on nematode-aphid interactions in the model species *Arabidopsis thaliana* (Kutyniok *et al,* 2012, Kutyniok *et al*, 2014), the plant-mediated mechanisms responsible for such effects at both the biochemical and molecular level remain unexplored in crop plants. Using a combination of molecular and biochemical techniques, we test the hypothesis that systemic changes in endogenous phytohormones and the expression of associated genes can indirectly influence these plant-mediated interactions between organisms feeding above and below ground. We examine the induced systemic defence response of potato plants following nematode infection and how these responses impact on aphid-induced SA production which is required for systemic acquired resistance (SAR), leading to the expression of *PR*-genes. We also describe levels of endogenous JA and the expression of a gene involved in

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jasmonate signalling. Finally, we show the impact of *G. pallida* pre-infection of potato plants on *M. persicae* abundance.

Materials and Methods

Aphids and nematodes

Nymphs of the peach-potato aphid (*Myzus persicae*) were obtained from the James Hutton Institute, Invergowrie, Dundee, Scotland. The aphids were asexual clones of a wild population isolated in Scotland (Kasprowicz *et al.,* 2008). Aphid colonies were maintained on potato plants (*S. tuberosum* L. cv. Désireé) inside a mesh cage in a containment glasshouse at 20-22 °C under a 16 h/8 h light/dark cycle. Only apterous (wingless) aphids were used and transferred to experimental plants using a fine paintbrush.

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Cysts of *G. pallida* were extracted from infected soil stocks using the Fenwick can method (Fenwick, 1940). Infective second-stage juveniles (J2s) were hatched from the cysts following treatment with 1% sodium hypochlorite aqueous solution (Huengens *et al*., 1996). J2 nematodes were stored in autoclaved tap water at 10°C and their viability was checked prior to use by observation using a stereobinocular microscope.

Pest and pathogen infection and sample collection

Potato tuber cuttings (*S. tuberosum* L. cv. Désireé) were planted in 18 cm pots containing pesticide-free compost. Growth took place in a glasshouse at 20-22 °C under a 16 h/8 h light/dark cycle for a period of three weeks. For potato plants infected with nematodes only, ten thousand J2 nematodes suspended in six millilitres of autoclaved tap water were introduced into the compost

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around the roots of each potato plant. Uninfected potato plants used as a control were mock-inoculated with autoclaved tap water. At 14 days post inoculation (dpi), a fully expanded terminal leaf from the top of each plant was excised using fine tweezers, divided into three samples for RNA, SA and JA extractions and immediately snap frozen in liquid nitrogen. Five-week old potato plants were used for infection with aphids alone so ensuring each set of experimental plants were the same age. Twenty apterous aphids of various life-stages were transferred to the second fully expanded leaf with a fine paintbrush and confined to the abaxial surface of the leaf in a 2.5 cm diameter clip-cage. Aphid-free clip-cages were used in control experiments. After 48 hours, aphids were carefully removed and the leaf was excised and sampled as previously described. Co-infected potato plants were initially inoculated with ten thousand J2 nematodes, then 14 days later 20 apterous aphids were applied to either infected or control plants for 48 hours as previously described. Co-infected samples were collected 48 hours post infection (hpi) with aphids.

RNA extraction, cDNA synthesis & qRT-PCR for the analysis of *PR*-gene expression

Total RNA was prepared from frozen leaf tissue of control and infected potato plants using the RNeasy® Plant Mini Kit (Qiagen, Inc., Valencia, CA, USA). First-strand cDNA was synthesised from 1000 ng RNA using SuperScript II reverse transcriptase (Invitrogen, Carlesbad, CA) and Oligo$(dT)_{17}$ primer (500 µg/ml) following the manufacturer's instructions. Quantitative reverse transcriptase (qRT)-PCR was carried out on the resulting cDNA using Brilliant III Ultra-Fast SYBR® Green Master Mix and a Mx3005P (v. 4.10) instrument (Agilent Technologies, La Jolla, CA). Genes for expression analysis were selected according to their previously recorded involvement in biotic stress responses (Kombrink *et al.*, 1988; Matton and Brisson, 1989;

Fidantsef *et al.*, 1999; Reiss and Horstmann, 2001; Wang *et al.*, 2005) (see results section for further details). Potato *ELONGATION FACTOR 1-α* was used to normalise the results (Nicot *et al.*, 2005). Sequences of primers used for amplification of each gene are detailed in Supporting Information Table S1. Sequences for the chosen genes were found on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) and primers were designed using the online Primer 3 software (http://primer3.ut.ee/). Controls for qRT-PCR included reactions containing no template. All primer pairs had an amplification efficiency of 93-101% and R^2 correlation coefficients for standard curves ranged between 0.94 and 0.99. qRT-PCR was performed on five biological replicates for control and infected samples and each reaction was carried out in triplicate. Ct values were determined using the MxPro software. Relative expression between control and infected samples was determined using the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001).

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Extraction and quantification of salicylic acid

Salicylic acid (SA) extraction was performed on leaf tissue that had been treated with aphids and nematodes both singly and in combination 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 a GeneVac (EZ-2 series). The remaining phase was subjected to acid hydrolysis using 8M HCl and incubated at 80°C for one hour to quantify sugar-conjugated (or stored) SA. The sugar-conjugated (or stored) SA 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. The pooled stored SA extract was re-suspended in 600 µl of water and acetonitrile (95:5) and quantified by high-pressure liquid chromatography (HPLC). Analysis was performed using a Supelcosil™ 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. SA was determined and quantified by comparing peaks of recovered SA using calibration standards. Total SA was calculated as the amount of free SA in plant samples to the amount of sugar-conjugated (or stored) SA in plant samples. The efficiency of SA recovery was calculated by using a deuterium-labelled internal standard of SA-d_6. Twelve biological replicates were used for each condition analysed.

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Jasmonic Acid Quantification

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 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 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).

Aphid Abundance

To test the effect of *G. pallida* infection on aphids, ten apterous adults were placed in a 2.5 cm diameter clip cage on a fully expanded, terminal leaf second from the top of a potato plant pre-infected with 10,000 J2 nematodes 14 days previously or mock-inoculated with water. After 24 hours all aphids except for five nymphs were removed. The five nymphs were allowed to develop and the number of aphids inside the clip-cage were counted for 8 days to determine the abundance of aphids on nematode-infested plants and non-infected control plants. Five biological replicates for each condition were used in the experiment.

Data Analysis

The effects of the treatments on gene expression and the levels of endogenous phytohormones JA and SA were determined using a Mann-Whitney U test. A Mann-Whitney U test was also carried out to compare the abundance of aphids on nematode infected plants against non-infected control plants.

Results

Infection of potato plants with *Globodera pallida* or *Myzus persicae* elicits a SA-mediated systemic defence pathway in the leaves.

There was a significant increase in endogenous SA in the leaves of potato plants 14 days after infection with *G. pallida*. The level of free SA was significantly greater in nematode-infected plants compared to non-infected control plants (mean ± standard error), 571.33 ± 70.09 ng/g FW for infected plants and 231.20 ± 27.21 ng/g FW for control plants (Mann-Whitney U = 497.5, P = 0.001, sig ≤ .05, 2-tailed) (Fig. 1A). The presence of nematodes also significantly increased total levels of SA in leaves of potato plants, (4541.42 ± 268.2 ng/g FW for nematode-infected plants and 2132.77 ± 758.57 ng/g FW for control plants, $P \leq 0.01$) (Fig. 1A). These results suggest an activation of the systemic acquired resistance (SAR) pathway in the leaves of potato plants, which is mediated by salicylic acid (Gaffney *et al.*, 1993).

An elevated level of the endogenous phytohormone SA is known to lead to the expression of pathogen-related (*PR*) genes, some of which are commonly used molecular markers of SAR (Bowling *et al.*, 1994; Cao *et al.*, 1994; Uknes *et al.*, 1993). We therefore measured the expression of *PR-1*, *PR-2* and *PR-5*, all of which are co-ordinately regulated by SA (Cao *et al.,* 1994), in nematode-infected plants 14 dpi. Transcripts of all three *PR*-genes were detected in leaf tissue from both infected and non-infected potato plants. However only the expression of *PR-5* was significantly induced in nematode infected plants (Mann-Whitney U = 1.000, P = 0.027) (Fig. 1C). Transcripts of *PR-5*, which encodes a thaumatin-like protein, were approximately three-fold higher in nematode-infested plants relative to control plants (Fig. 1C).

Five-week old potato plants infected with aphids were analysed for endogenous SA and the expression of SA-mediated defence genes. There was a significant increase in free (686 ± 76 ng/g FW, $P \leq 0.001$), stored (7010 ± 547 ng/g FW, $P \leq 0.001$) and total (8046 ± 555 ng/g FW, $P \leq 0.001$) SA in the leaves of potato plants infected with aphids compared to control plants (Free: 276 ± 32 ng/g FW; Stored: 3581 ± 392 ng/g FW; Total: 4055 ± 396 ng/g FW) (Fig. 2A). The expression of SA-mediated genes *PR*-1 ($P \leq 0.001$) and *PR*-5 ($P \leq 0.001$) was also significantly elevated. There was no significant increase in *PR*-2 expression (Fig. 2C).

Infection with *Myzus persicae* but not *Globodera pallida* elicits a JA-mediated systemic defence pathway in the leaves of potato plants.

In addition to SA-mediated effects, it is well established that jasmonic acid (JA) has an important role in the plant defence pathway. Hence we also measured endogenous levels of JA as well as transcript levels of *JAZ-1*, which is a nuclear-localised protein involved in jasmonate signalling in addition to *PR-3*. There was a significant increase in endogenous jasmonic acid in the leaves of plants infected with aphids (729 ± 22 ng/g FW) compared to control plants (356 ± 88 ng/g FW) ($P \leq 0.025$) (Fig. 2B). In addition there was a significant increase in transcript levels of *PR*-3 ($P \leq 0.001$) and *JAZ*-1 ($P \leq 0.001$) (Fig. 2C). However, there was no significant increase in endogenous levels of the phytohormone JA in nematode-infected plants 14 dpi (Mann-Whitney U = 66.000, $P = 0.76$, sig ≤ .05, 2-tailed) (Fig. 1B) or in the expression of genes involved in the signalling of JA, *PR*-3 ($P \leq 0.11$) or *JAZ*-1 ($P \leq 0.286$) (Fig. 1C) suggesting that nematode infection does not elicit a systemic JA defence response in the leaves of potato plants.

Co-infection with both *G. pallida* and *M. persicae* elicits an additive SA defence but a reduction in the JA defence signalling pathway in the leaves of potato plants.

The SA-mediated defence pathway was investigated in the leaves of potato plants that had been infected with both *G. pallida* and *M. persicae*. There was a significant increase in the levels of stored (9943 ± 1522 ng) and total SA (10750 ± 1557 ng) in the leaves of dual infected plants compared to the controls (Stored: 4665 ± 906 ng; Total: 5409 ± 930 ng; $P \leq 0.012$) (Fig. 3A). There was no significant difference in the levels of free SA in the leaves of plants that were co-infected (691 ± 45 ng) compared to the controls (743 ± 146 ng) (Fig. 3A). There was no significant increase in transcript levels of SA-mediated defence genes (Fig. 3A). The significant increase in the levels of stored SA indicates that the SA-mediated defence pathway is up-regulated in the leaves of potato plants; however it has not been converted into free SA.

There was no significant changes in the levels of endogenous JA in plants that had been co-infected with both pests (372 ± 73 ng) compared to the controls (392 ± 64, $P \leq 0.855$) (Fig. 3B). Similarly, when the expression of genes involved in the JA signalling pathway were analysed, there was no significant differences between the leaves co-infected plants and control plants (Fig. 3C). Due to a significant increase in endogenous levels of JA and the expression of SA-mediated defences in the leaves of plants infected with aphids only, the reduction of JA in co-infected plants may indicate an antagonistic suppression of JA by the additive increase in SA caused by both nematode and aphid infection together.

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The peach-potato aphid, *Myzus persicae* has a higher abundance on potato plants pre-infected with *Globodera pallida*

There was a significant increase in the abundance of aphids reared on potato plants pre-infected with nematodes for 14 days compared with aphids reared on non-infected control plants (Mann-Whitney U = 3.000, $P = 0.011$, sig ≤ .05, 2-tailed) (Fig. 4).

Discussion

Our results show how the molecular and biochemical response of the potato plant to attack by a below-ground pathogen, in this case plant-parasitic nematodes, can indirectly influence herbivore populations above ground through systemic changes in endogenous phytohormones and expression of associated genes.

Plant Responses to Cyst Nematode and Aphid Infection Singly and in Combination

Previous studies have revealed that defence signalling pathways are involved in compatible interactions of plants with cyst nematodes (*Heterodera* and *Globodera* spp.) (Ithal *et al*., 2007; Jammes *et al.,* 2005; Wubben *et al.*, 2008). Similarly, it is well known that 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). Our analysis has shown that expression of *PR-5*, a molecular marker commonly used to indicate activation of systemic acquired resistance (SAR) (Unkes *et al.,* 1992; Bowling *et al.,* 1994), was significantly increased in leaves of potato plants following infection with *G. pallida* for 14 days and also in the leaves of five-week old plants infected with *M. persicae* for 48 hours. This correlates with the significant increase in free and total SA in leaves of potato plants: the accumulation of the phytohormone SA is required for

the activation of SAR in distal tissues of the infected plant (Gaffney *et al.*, 1993). Taken together these results indicate activation of an SAR-induced potato defence pathway following parasitism by *G. pallida* and infection with *M. persicae* singly. There was no significant increase in the expression *PR*-1 or *PR*-2 in the leaves of nematode-infected potato plants at the time-point examined. Expression of the orthologous genes was reported to increase in the leaves of *Arabidopsis thaliana* in response to cyst nematode infection, however this increase was transient and varied considerably between investigations (Wubben *et al.*, 2008; Hamamouch *et al.*, 2011). The length of time post-infection, together with the initial nematode burden, may be critical in determining if *PR*-gene induction is observed. It is well documented that there is mutual antagonism between SA and JA signalling pathways (Pieterse *et al.*, 2012), therefore the phytohormone JA and the expression levels of the JA-dependent associated genes *PR-3* and *JAZ-1*, a nuclear-localised protein involved in jasmonate signalling (Thines *et al.*, 2007) were quantified. No significant differences were found between nematode-infected plants and control plants in either the amount of JA or the expression of *PR-3* and *JAZ*-1, suggesting that infection with the potato cyst nematode does not alter the jasmonic acid signalling pathway in the potato plant at 14 dpi. Alternatively, this could indicate antagonistic cross-talk between the SA and jasmonic acid pathways following infection with *G. pallida*, as both endogenous SA and the expression of *PR-5* was significantly up-regulated. In contrast, it was found that aphid infection induced the JA signalling pathway in the leaves of potato plants as both JA and the expression of *PR*-3 and *JAZ*-1 were significantly up-regulated compared to control plants.

Co-infection of the potato with both *G. pallida* and *M. persicae* had a different and unique impact on the levels of endogenous phytohormones and expression of defence-related genes compared to

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plants that had been infected with each pest singly. An additive effect on SA was observed in co-infected plants, an effect that may be assumed when two pests are applied to a plant. However, a reduced JA effect was noted in dual infected plants even though JA was present in the leaves of plants infected with aphids in isolation. There is literature to suggest that phytohormones do not act independently of one another. The interaction between SA and JA is complex with the main interaction between these two pathways being mutual antagonistic (Kunkel and Brooks, 2002). SA 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). Therefore, a lack of JA in the leaves of co-infected plants could be construed as antagonistic crosstalk because although infection with plant-parasitic nematodes did not elicit the JA defence pathway in potato plants, infection with aphids alone did.

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Herbivore Responses to Plant Parasitic Nematode Infection

Plant-mediated interactions between plant parasitic nematodes and aerial pests studied to date have been variable: susceptibility to shoot pathogens and resistance to phloem feeders have been reported with the outcome depending on the parasitic strategy of the nematode involved in the interaction (Biere and Goverse, 2016). To the best of our knowledge there have been no studies of plant-mediated interactions between the potato cyst nematode and specialised above-ground pests or pathogens of potato, however there have been reports of interactions between *G. rostochiensis* and below-ground pathogens such as the soil-borne fungus of potato, *Rhizoctonia solani* (Back *et al*., 2006). 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*, 2013). 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). Our observation that *G. pallida*, a sedentary endoparasitic nematode, indirectly and positively influences the abundance of *M. persicae* highlights how aphids may be more damaging to the potato crop in areas where *G. pallia* is present compared to such areas where there is no infection, however this requires further investigation. Our study is in contrast to these previous studies and to our knowledge is the first to report the combined molecular and biochemical response of the potato to nematode infection.

Comment [GH[11]]: Change

Systemic plant resistance to insect herbivores is mediated by the SA and JA wound signalling pathways and the, usually antagonistic, crosstalk between them (Pieterse *et al*., 2012; Stam *et al*., 2014). In addition to their role in regulating resistance to biotrophic pathogens, SA-mediated defensive pathways are known to be induced by phloem-feeding insects, and there have also been reports suggesting that SA itself is an effective chemical defence against phloem-sucking herbivory animals (Kaloshian and Walling, 2005; Donovan *et al.*, 2013). As expected, we found induction of the SA pathway in response to nematodes, but any adverse effects of this on the aphids are likely to be negated by the benefits of SA-mediated reductions of the JA-mediated pathway responsible for plant resistance to herbivores (Lazebnik et al., 2014). Indeed, aphids are believed to circumvent the plant's immune system by eliciting the SA signalling pathway in order to antagonise and suppress the JA one, which is important in mediating resistance to phloem

Comment [GH[12]]: Change

feeders (Zhu-Salzman *et al.,* 2004; Ellis *et al.,* 2002). Thus, our observation of more aphids present on nematode infested plants could reflect circumvention of the SA-mediated defence pathway of the potato plant by *M. persicae*. Our analysis of the JA-mediated defence pathway in the potato plant showed no up-regulation of endogenous JA or expression of *PR-3* or *JAZ-1* in leaves of potato plants infected with nematodes when compared to control plants. Aphids could benefit from the situation in which the hormone has not been elicited or even suppressed.

Conclusion

Our biochemical and molecular data reveal the potential mechanisms underpinning a positive asymmetric interaction between a sedentary endoparasitic nematode and a sap-sucking insect. The SA pathway and PR defence gene expression is altered in the potato plant following infection with *G. pallida* and these changes indirectly influence the performance of the peach potato aphid *M. persicae.* Our study highlights how multiple stresses elicit a unique molecular and biochemical response compared to singly stressed plants. It also demonstrates the importance of analysing hormonal crosstalk when seeking to understand plant defensive responses to co-incident attack by pests and pathogens.

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667

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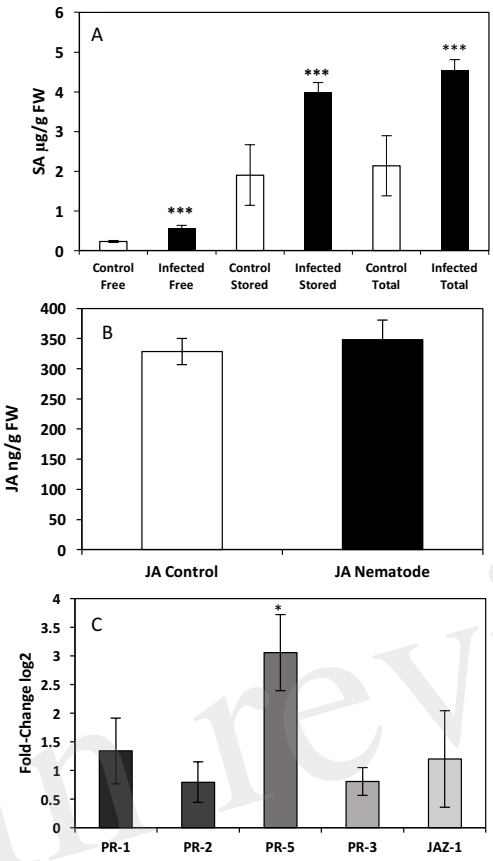

Figure 1. Quantification of endogenous salicylic acid and jasmonic acid and analysis of *PR*-gene expression by qRT-PCR in the leaves of potato plants (*Solanum tuberosum* cv. Désirée) infected with the potato cyst nematode, *Globodera pallida.* A. Levels of endogenous salicylic acid in leaves of potato 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 14 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)).

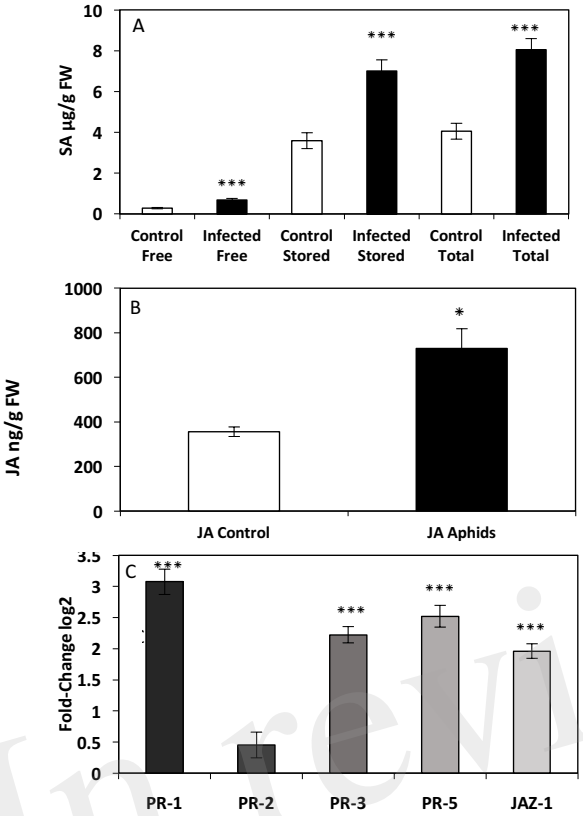

Figure 2. Quantification of endogenous salicylic acid and jasmonic acid and analysis of *PR*-gene expression by qRT-PCR in the leaves of potato plants (*Solanum tuberosum* cv. Désirée) infected with the peach-potato aphid, *Myzus persicae*. A. Levels of endogenous salicylic acid in leaves of potato plants infected with *M. persicae* 48 hours post inoculation (hpi). **B.** Levels of endogenous jasmonic acid in leaves of potato plants infected with *M. persicae* 48 hpi. **C.** Expression levels of *PR*-genes in the leaves of potato plants infected with *M. persicae* 48 hpi. 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)).

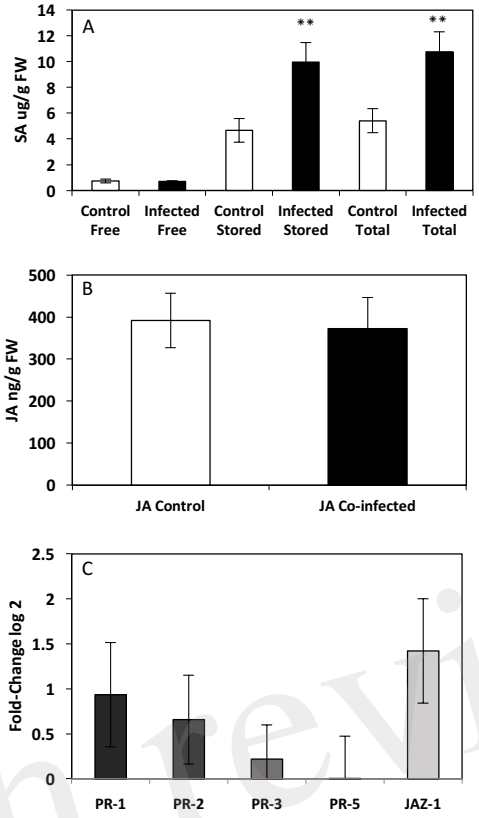

Figure 3. Quantification of endogenous salicylic acid and jasmonic acid and analysis of *PR*-gene expression by qRT-PCR in the leaves of potato plants (*Solanum tuberosum* cv. Désirée) infected with both the potato cyst nematode, *Globodera pallida* and the peach-potato aphid, *Myzus persicae.* A. Levels of endogenous salicylic acid in leaves of potato plants infected with *G. pallida* 14 dpi and *M. persicae* 48 hours post inoculation (hpi). **B.** Levels of endogenous jasmonic acid in leaves of potato plants infected with *G. pallida* 14 dpi and *M. persicae* 48 hpi. **C.** Expression levels of *PR*-genes in the leaves of potato plants infected with *G. pallida* 14 dpi and *M. persicae* 48 hpi. The presented data are the mean fold changes ± standard errors of biological replicates in qRT-PCR graphs. The *PR* transcript levels are relative to uninfected control tissue (baseline set at 0) from biological replicates (Mann-Whitney U, * $P < 0.05$, n = 5 (qPCR and JA analysis), n=12 (Endogenous SA)).

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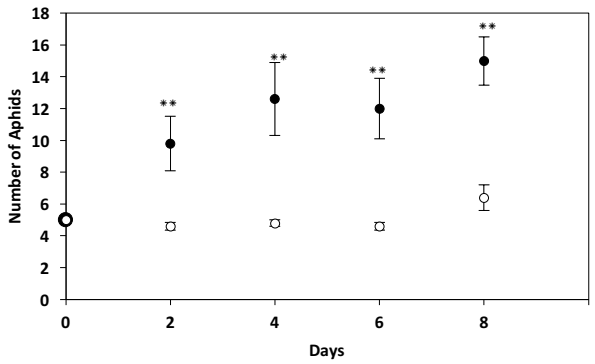

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705 Figure 4: No choice performance assays of *M. persicae* on potato plants pre-infected with 10,000 *G.*
706 *pallida* J2s for 14 days or non-infected control potato plants. Black dots represent aphids present on plant
707 pre-infected with nematodes. White dots represent aphids present on non-infected control plants. There
708 were more *M. persicae* present on nematode-infested plants from Day 2 to Day 8 compared to non-
709 infected control plants (n=5, ** = P<0.01).

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Figure 1.JPEG

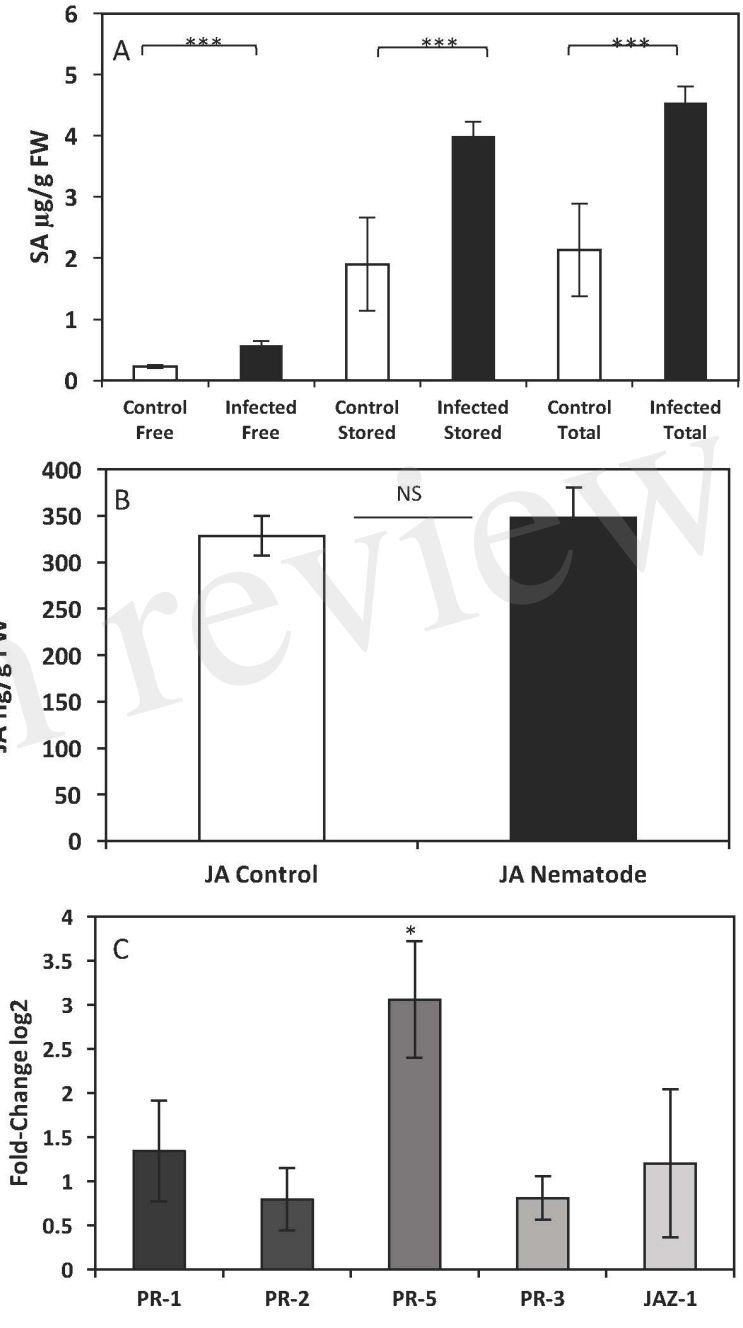

Figure 2.JPEG

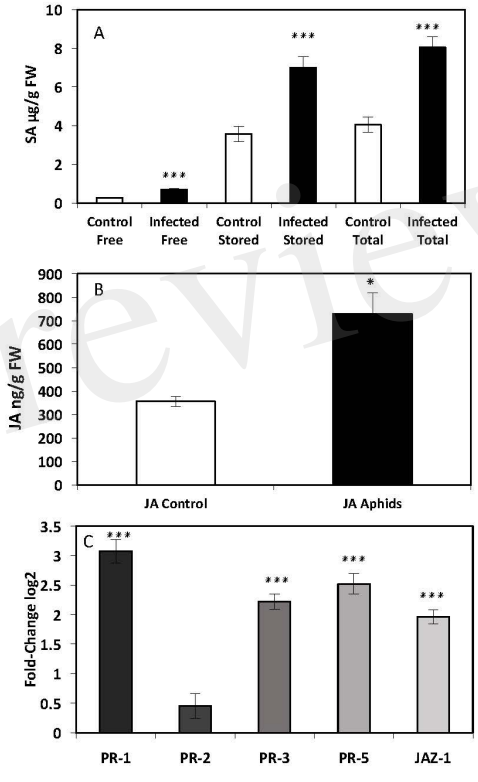

Figure 3.JPEG

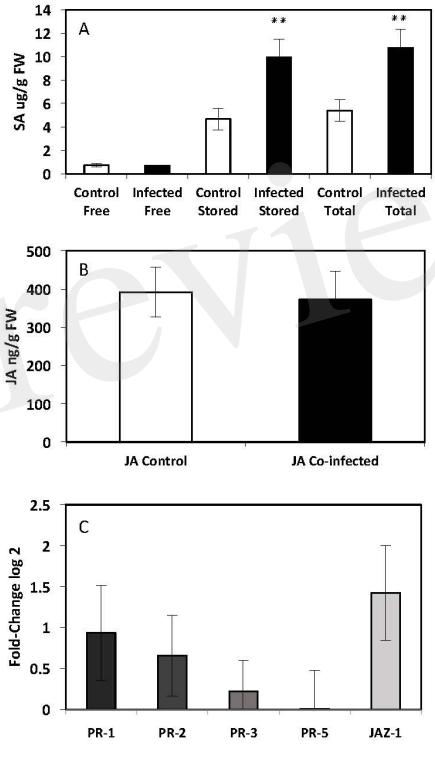

Figure 4.JPEG