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Responses of pea plants to multiple antagonists are mediated by order of attack and phytohormone crosstalk

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Abstract

Plants are often attacked by multiple antagonists and traits of the attacking organisms and their order of arrival onto hosts may affect plant defences. However, few studies have assessed how multiple antagonists, and varying attack order, affect plant defence or nutrition. To address this, we assessed defensive and nutritional responses of *Pisum sativum* plants after attack by a vector herbivore (*Acrythosiphon pisum*), a nonvector herbivore (*Sitona lineatus*), and a pathogen (*Pea enation mosaic virus*, PEMV). We show viruliferous *A. pisum* induced several antipathogen plant defence signals, but these defences were inhibited by *S. lineatus* feeding on peas infected with PEMV. In contrast, *S. lineatus* feeding induced antiherbivore defence signals, and these plant defences were enhanced by PEMV. *Sitona lineatus* also increased abundance of plant amino acids, but only when they attacked after viruliferous *A. pisum*. Our results suggest that diverse communities of biotic antagonists alter defence and nutritional traits of plants through complex pathways that depend on the identity of attackers and their order of arrival onto hosts. Moreover, we show interactions among a group of biotic stressors can vary along a spectrum from antagonism to enhancement/synergism based on the identity and order of attackers, and these interactions are mediated by a multitude of phytohormone pathways.

KEYWORDS
disease ecology, plant defensive chemistry, plant nutrients, plant-insect-pathogen interactions, species interactions

1 | INTRODUCTION

Plants have defences to counter attacks from herbivores and pathogens (Miller et al., 2017; Pandey et al., 2015). Many studies have tested plant responses to single antagonists, but plants can be challenged by many stressors concurrently, and plant defences can depend on the order in which antagonists arrive on plants (Nejat & Mantri, 2017; Thaler et al., 2012). For example, herbivory often induces the jasmonic acid pathway, and induction of this pathway may impede a plant's ability to activate the salicylic acid pathway that controls pathogen defence (Koornneef & Pieterse, 2008; Thaler et al., 2012). Prior herbivory thus can interfere with plant defence against pathogens, but herbivores often have low impacts on pathogen defence if they arrive after pathogens (Lin et al., 2019; Okada et al., 2015). Certain organisms also can induce more robust plant defence through "priming", where they activate defences that are active against subsequent attackers (Mauch-Mani et al., 2017; Ramírez-Carrasco et al., 2017).

While there has been considerable research on the jasmonic and salicylic acid pathways, to understand complexities of plant defence there is a need to assess how antagonists mediate other pathways (Lacerda et al., 2014; Suzuki, 2016). Hormones associated with plant defence include salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA). SA activates defence against pathogens, JA and ET are induced against herbivores, while ABA promotes biotic and abiotic stress tolerance. However, the induction of any pathway

can affect resource allocation in plant hosts and the ability of the plant to mount defences against a subsequent attacker, a phenomenon called "phytohormone cross-talk" (Aerts et al., 2021; Yang et al., 2019). Yet, few studies have examined cross-talk in a broad ecological context with a subsequent analysis of multiple signalling pathways.

In addition to altering plant defence, biotic stressors can alter host plant quality by altering levels of amino acids such as proline, tyrosine, valine, histidine, and alanine (Mauck et al., 2012; Patton et al., 2020; Wang et al., 2012). Altered levels of amino acids can in turn affect performance of both pathogens and herbivores (Ángeles-López et al., 2016). For example, *Tomato yellow leaf curl virus* alters amino acid levels in phloem of tomato plants, which in turn alters the amino acid composition of whitefly (*Bemisia tabaci*) honeydew and whitefly fitness (Guo et al., 2019). However, few studies have correlated effects of multiple biotic stressors on both plant chemical signalling and nutrition (Petek et al., 2014; Su et al., 2016). Because plants are often challenged by multiple pathogens and herbivores, which impose selection pressures and various adaptive modifications, it is critical to more broadly examine how multiple organisms affect both plant defence and nutritional properties in a food web context (Thaler et al., 2012).

To better understand plant responses to multiple stressors, studies are needed that assess the ecological costs and benefits of plant defence and nutrition and how they are affected by order of arrival of stressors and food web complexity. We addressed this by assessing responses of *Pisum sativum* plants to attack from a piercing-sucking vector herbivore, the pea aphid (*Acrythosiphon pisum*), a chewing non-vector herbivore, the pea leaf weevil (*Sitona lineatus*), and an aphid-borne pathogen, *Pea-enation mosaic virus* (PEMV). These organisms co-occur in ecosystems of eastern Washington and northern Idaho, USA, and interactions between them can affect plant signalling pathways and nutrition. First generation *S. lineatus* adults typically attack plants before arrival of *A. pisum* and PEMV, but second generation *S. lineatus* typically attack plants after *A. pisum* and PEMV have arrived. However, it is unknown if responses of *P. sativum* differ based on the number of stressors and their order of attack. Moreover, the molecular mechanisms that mediate interactions among these stressors are largely unknown (Bera et al., 2020; Chisholm et al., 2019). Here, we varied the diversity, identity, and order of attack among this community of biotic antagonists and assessed resulting changes in gene expression and phytohormones related to plant defence and nutrition. Our study revealed how plant chemical and nutritional responses to diverse stressors can mediate complex species interactions within a ecologically and economically-relevant pathosystem.

2 | MATERIALS AND METHODS

2.1 | Study system

The Palouse region of eastern Washington and northern Idaho, USA is home to many legumes including *P. sativum* (Black et al., 1998). In *P. sativum* fields, *S. lineatus*, a chewing herbivore, co-occurs with *A. pisum*, a phloem-feeding herbivore that can transmit pathogens such as PEMV, a pathogen that is obligately transmitted by aphids in a persistent manner (Chisholm et al., 2019).

Sitona lineatus adults overwinter outside of *P. sativum* fields and migrate into fields in late spring before *A. pisum* arrive (Cárcamo et al., 2018). After *S. lineatus* eggs hatch, larvae burrow into the soil to feed and pupate before emerging as adults in the summer (Cárcamo et al., 2018); these second-generation adults often occur on plants under attack from *A. pisum* and PEMV (Chisholm et al., 2019). Thus, *S. lineatus* can attack individual plants in the field both before or after *A. pisum* and PEMV, and while some plants are attacked by all three stressors, others are attacked by zero, one, or two. Understanding how the diversity and complexity of interactions among these species affects plant defence and nutrition is thus critical.

To address these questions, we conducted greenhouse assays to assess interactions between *S. lineatus*, *A. pisum*, and PEMV on spring pea (cv. Banner), and molecular mechanisms affecting these interactions. First-generation adult *S. lineatus* for experiments were collected from commercial *P. sativum* fields, or wild patches of *Vicia villosa*, immediately prior to experiments. Colonies of infectious *A. pisum* with PEMV, and uninfectious *A. pisum*, were started from Palouse field-collected individuals (Chisholm et al., 2019) and were maintained on *P. sativum* plants in a greenhouse (21–24°C during day cycle, 16–18°C during dark cycle, 16:8 h light:dark). The presence of PEMV was confirmed by sequencing coat protein (CP) gene (Data S1) using PEMV-CP specific primers (Table S3) designed from the evolutionary conserved regions of CP from multiple isolates of PEMV and confirming the sequence via Basic Local Alignment Search Tool (BLAST) search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2 | Experimental design

We conducted a 3 × 2 greenhouse (21–24°C day, 16–18°C night, 16:8 h day:night) study that varied *S. lineatus*, *A. pisum*, and PEMV (Figure 1). There were three *S. lineatus* arrival order treatments: (i) control: no *S. lineatus* adults prior to *A. pisum*, (ii) aphid first: *A. pisum* fed for 48 h prior to adding *S. lineatus*, and (iii) weevil first: two adult *S. lineatus* fed for 48 h before *A. pisum* were added. Pathogen treatments were: (i) uninfected: ten 5-day old uninfectious *A. pisum* adults that fed on plants for 48 h, and (ii) infected: ten 5-day old viruliferous *A. pisum* adults that fed for 48 h. For treatments with *S. lineatus* first, they were hand removed prior to adding *A. pisum*; for treatments with *A. pisum* first, they were removed by aspirator prior to adding *S. lineatus*. While we might expect differences in responses across our attack order treatments due to the difference in plant age (48 h), the goal of this experiment was to assess how the order of arrival of multiple antagonists affected plant defence and nutritional status. Thus, it was not possible to have all the treatments done on plants of exactly the same age.

Treatments were conducted on individual *P. sativum* plants in mesh "bug dorms" (0.6 × 0.6 × 0.6 m), with six replicates randomly assigned to treatments in a factorial design (3 *S. lineatus* arrival order

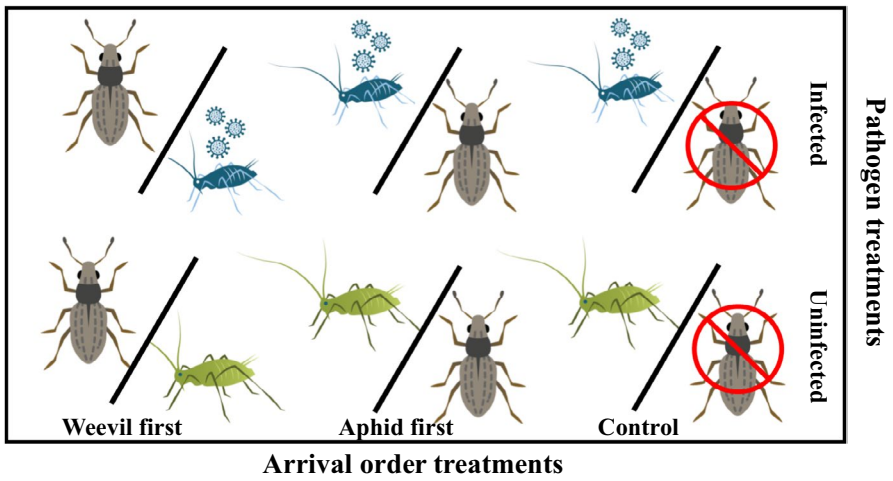

FIGURE 1 Schematic representation of 2 × 3 factorial design. Green-coloured aphids indicate sham (noninfective) *A. pisum*, while blue-coloured aphids indicate viruliferous *A. pisum*. Slashes indicate order of *S. lineatus* treatments (*S. lineatus* first, *A. pisum* first, or no *S. lineatus*)

treatments ×2 *A. pisum* treatments). After insects were removed, plants were allowed to develop for 7 days before we harvested tissue to assess viral titre, gene expression, and nutrients. Tissue samples from the whole aboveground portion of plants were collected and flash frozen in liquid nitrogen and stored in a −80°C freezer for processing. Virus testing confirmed that 100% of plants in the PEMV treatments became infected over the course of the experiment.

2.3 | Analysis of plant defence and biosynthetic genes

Plant tissue was processed using liquid nitrogen in sterilized mortars and pestles. Powdered tissue (50–100 mg) was used for RNA extraction with Promega SV total RNA isolation kits (Promega). The quantity and quality of RNA was estimated on a NanoDrop1000 and agarose gel electrophoresis, respectively, and 1 μg of total RNA from each sample was used for cDNA synthesis (Bio-Rad iScript cDNA Synthesis kits). Gene specific primers (Table S3) for qRT-PCR were designed using the IDT Primer Quest Tool. Each qRT-PCR reaction (10 μl) was set up with 3 μl of ddH2O, 5 μl of iTaq Univer SYBR Green Supermix, 1 μl of specific primer mix (forward and reverse [concentration 10 μM]), and 1 μl of diluted (1: 25) cDNA template. Reactions were set up in triplicates for each sample and ran on a CFX96 qRT-PCR machine. The programme included an initial denaturation of 3 min at 95°C, followed by 40 denaturation cycles for 15 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C. For melting curve analysis, a dissociation step cycle (55°C for 10 s and then 0.5°C for 10 s until 95°C) was added. The comparative $2^{-\Delta\Delta Ct}$ method (Kozera & Rapacz, 2013; Livak & Schmittgen, 2001) was used to calculate the relative expression level of each gene, with β-tubulin as an endogenous control.

We assessed expression of seven genes associated with defence in peas. Gene sequences were obtained using accession numbers or using Pea Marker Database (Kulaeva et al., 2017) and blast searching through the reference genome (Kreplak et al., 2019). Four genes were associated with hormone biosynthesis: (i) *Isochorismate synthase1* (*ICS1*) (salicylic acid), (ii) *Lipoxygenase 2* (*LOX2*) (jasmonic acid), (iii) *Aldehyde oxidase 3* (*AO3*) (abscisic acid), and (iv) *Gibberellin 2-oxidase* (*GA2ox*) (gibberellic acid). *ICS1* converts chorismate to isochorismate, a precursor of salicylic acid (Seguel et al., 2018), while *LOX2* converts α-linolenic acid (18:3), a precursor to jasmonic acid, to an intermediate product 13S-hydroperoxy-(9Z,11E,15)-octadecatrienoic acid (13-HPOT) (Wasternack & Hause, 2013; Yan et al., 2013). *AO3* catalyses abscisic acid biosynthesis by oxidizing abscisic aldehyde, and *GA2OX* catalyses bioactive giberrelic acids or their immediate precursors to inactive forms (He et al., 2019; Serova et al., 2019; Zdunek-Zastocka & Sobczak, 2013).

Three additional genes were associated with defence responses that occur downstream from hormone induction. Pathogenesis-related protein 1 (PR1) affects systemic acquired resistance-mediated defence signalling and occurs downstream in the salicylic acid pathway (Fondevilla et al., 2011; Miranda et al., 2017). The second gene transcript was an antimicrobial defensin peptide (*disease resistance response* gene, *DRR230*), which can provide resistance in peas against pathogens (Lacerda et al., 2014; Selim et al., 2017). The third defence response transcript assessed was *lectin (PsLectin)*. Plant lectins are a group of carbohydrate binding proteins, and *lectin* genes can be induced by salicylic acid, jasmonic acid, and herbivores to stimulate phytoalexin and pistatin production in peas (Armijo et al., 2013; Fondevilla et al., 2011; Macedo et al., 2015).

2.4 | Measurement of phytohormones

Plant tissue samples were assessed for three phytohormones: jasmonic acid, salicylic acid, and abscisic acid following procedures of Patton et al. (2020). Briefly, tissue samples were flash frozen in liquid nitrogen before being lyophilized and weighed. Hormones were extracted in isopropanol:H_2O:HCL_{1MOL} (2:1:0.005) with 100 μl of internal standard solution (1,000 pg of each). Samples were evaporated to dryness, resuspended in 100 μl of MeOH, filtered, and 10 μl of each sample was injected into an Agilent Technologies 6420 triple quad liquid chromatography-tandem mass spectrometry instrument (Agilent). A Zorbax Extend-C18 column 3.0 × 150 mm (Agilent) was used with 0.1% formic acid in water (A) and 0.1% (v/v) formic

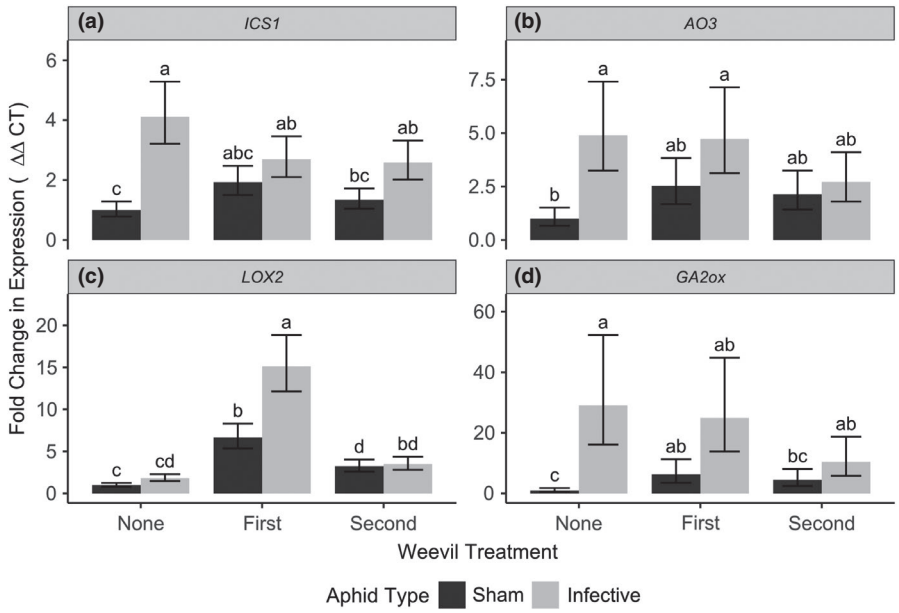

FIGURE 2 Relative transcript accumulation of plant hormone biosynthesis genes associated with four hormonal signalling pathways. (a) *ICS1* (salicylic acid), (b) *LOX2* (jasmonic acid), (c) *AOX3* (abscisic acid), and (d) *GA2ox* (gibberellic acid) following attack with various combinations of *S. lineatus*, *A. pisum*, and PEMV. Within each panel, bars separated by a different letter were significantly different based on MANOVA (Tukey's HSD, $\alpha = 0.05$). Bar height and error bars indicate marginal mean and standard error of the regression coefficient for each respective treatment

acid in acetonitrile (B) at a flow rate of 600 ml/min. The gradient used was 0–1 min, 20% B; 1–10 min, linear gradient to 100% B; 10–13 min, 100% A. Retention times were: jasmonic acid (D5) standard (5.740 min), jasmonic acid (5.744 min), salicylic acid D4 standard (4.677 min), salicylic acid (4.720 min), abscisic acid (D6) standard (4.855 min), and abscisic acid (4.882 min).

2.5 | Analysis of plant nutritional components

For amino acid analysis, leaf tissue was lyophilized, weighed, and extracted with 20 mM of HCL (Patton et al., 2020). Derivation was done using AccQTag reagents following the manufacturer's instructions (Waters), and derivatised samples (10 µl) were then injected. Ground tissue was extracted with 100 µl of 20 mM HCl, centrifuged, and the supernatant was saved. Amino acids were derivatized using AccQ-Fluor reagent kits (Waters), with L-Norleucine as an internal standard. Then, 10 µl from each sample was injected with an Agilent 1260 Infinity pump with a Nova-Pak C18 column and fluorescence detector, and Agilent Chemstation software for data recording. Amino acid derivatives were detected with an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Peak areas were compared to a standard curve made from a serial dilution of amino acid standards (Sigma-Aldrich) injected into a Agilent 1260 Infinity HPLC (Agilent) with a Nova-Pak C18 column (Casteel et al., 2014). Solvent A, AccQ-Tag Eluent A, was premixed from Waters; Solvent B was acetonitrile:water (60:40). The gradient used was 0–0.01 min, 100% A; 0.01–0.5 min, linear gradient to 3% B; 0.5–12 min, linear gradient to 5% B; 12–15 min, linear gradient to 8% B; 15–45 min, 35% B; 45–49 min, linear gradient to 35% B; 50–60 min, 100% B. The flow rate was 1.0 ml/min. Amino acid derivatives were measured with an Agilent fluorescence detector with an excitation wavelength of 250 nm and an emission wavelength of 395 nm. For concentration calculations, standard curves were generated for each amino acid using dilutions of the standard.

2.6 | Data analysis

To evaluate effects of our treatments on host-plant defences and host-plant quality, we ran a series of multivariate models using R ver. 3.5.2 (R Core Team, 2018). First, gene expression was evaluated with *ICS1*, *LOX2*, *GA2ox*, *AO3*, *PR1*, *DRR230*, and *PsLectin* as the responses, with MANOVA to assess treatment effects on relative gene expression ($2^{-\Delta\Delta Ct}$) based on cycle threshold values for each observed gene transcript. Estimated marginal mean of Ct values, and standard error of the mean, were generated using the emmeans package in R (Lenth, 2016). The methodology for $2^{-\Delta\Delta Ct}$ followed modified recommendations from Rao et al., (2013) and Kozera and Rapacz (2013), using housekeeping gene *β-tubulin* to normalize expression and a sham aphid (noninfective pea aphid and no weevil addition) treatment as a control.

Hormone levels were evaluated using MANOVA, with salicylic acid, jasmonic acid, and abscisic acid as responses (three variables, log-transformed to meet normality assumptions). Total amino acid content was evaluated with a generalized linear model (GLM) with total concentration among all amino acids as the response. All models assessed treatment effects, using *S. lineatus* addition, *A. pisum* infection status, and their interaction as predictors. Finally, changes in the amino acid profile were evaluated using nonmetric multidimensional scaling (NMDS). This analysis tests if the 14 amino acids aggregate into group based on treatments. NMDS were performed using the vegan package (Oksanen et al., 2019) using the Bray-Curtis index following Ceulemans et al., (2017).

3 | RESULTS

3.1 | Effects of multiple antagonists and attack order on plant gene transcripts

Transcription of plant genes associated with biosynthesis of salicylic acid (*ICS1*), jasmonic acid (*LOX2*), abscisic acid (*AO3*), and gibberellic acid (*GA2ox*) were induced by PEMV when *S. lineatus* was not present (Figure 2a–d, Table S1, *Pillai* = 0.942, $p = .002$). However, there was no induction of any of these biosynthesis genes in response to PEMV when *S. lineatus* attacked first, indicating herbivory by *S. lineatus* inhibited subsequent plant defence against PEMV (Table S1, A × W interaction, *Pillai* = 1.509, $p = .021$, Figure 2). In both the presence and absence of PEMV, subsequent feeding by *S. lineatus* induced transcription of *LOX2*, but *S. lineatus* did not directly modify the expression of *ICS1*, *AO3* or *GA2ox* (Figure 2b–d, Table S1). When viruliferous *A. pisum* attacked following *S. lineatus*, there was greater induction of *LOX2* compared to when *S. lineatus* attacked alone (Table S1, A × W interaction, *Pillai* = 1.509, $p = .021$, Figure 2). In contrast to the antagonism exerted by *S. lineatus* on *P. sativum* responses to PEMV, where plant responses to *S. lineatus* inhibited subsequent defences against PEMV, this LOX2 response represents enhancement of plant defence when PEMV infection followed attack by *S. lineatus*.

All three defence response transcripts (*PR1*, *DDR230*, *PsLectin*) were induced by PEMV when *S. lineatus* was not present (Figure 3); similarly, each transcript was induced by *S. lineatus* when PEMV was not present (Figure 3, Table S1; A × W interaction, $F = 2.64$, $p = .111$). When *S. lineatus* attacked second, the expression level of *PR1* and lectin did not change compared to when weevils were absent. The effects of PEMV on the transcripts was modified by the presence of *S. lineatus* and attack order. While *DDR230* was induced by PEMV (Table S1, $F = 47.181$, $p < .001$), this effect diminished when *S. lineatus* was present after PEMV (Figure 3b). Similarly, the effects of PEMV on *PR1* were inhibited when *S. lineatus* attacked second (Figure 3), whereas the induction of lectin by PEMV was not altered by *S. lineatus* in either order (Figure 3).

3.2 | Effects of multiple antagonists and attack order on phytohormones

We observed variation in phytohormones in response to *A. pisum* (Table S2, *Pillai* = 0.95, $p < .001$) and *S. lineatus* (Table S2, *Pillai* = 1.195, $p < .001$). Viruliferous *A. pisum* induced salicylic acid (Table S2, $F = 254.2$, $p < .001$), but this was attenuated when *S. lineatus* attacked after PEMV (Figure 4a, Tukey HSD). PEMV did not affect jasmonic acid (Table S2, $F = 0.97$, $p = .34$), but the attack order of *S. lineatus* did (Table S2, $F = 5.30$, $p = .018$). Both *S. lineatus* (Table S2, $F = 4.10$, $p = .037$) and infectious *A. pisum* induced abscisic acid (Table S2, $F = 9.96$, $p = .006$), contingent on the attack order (Table S2, A × W, $F = 4.32$, $p = .032$, Figure 4, Tukey's HSD). Jasmonic acid levels were suppressed by *S. lineatus* when attacking prior to noninfectious sham *A. pisum*, but not on plants already attacked by PEMV (Figure 4b, Tukey's HSD).

3.3 | Effects of multiple antagonists and attack order on plant nutrients

Feeding by *S. lineatus* increased the total amino acid levels (GLM, $\chi^2 = 9.19$, $p = .01$, Figure 5), but attack by viruliferous *A. pisum* did not (GLM, $\chi^2 = 0.044$, $p = .83$), and there was no significant interaction between aphid and weevil treatments (GLM, A × W interaction, $\chi^2 = 0.24$, $p = .63$). Nonmetric multidimensional scaling (NMDS) analysis of amino acid composition also showed that changes to amino acid availability was most different among treatments for alanine, arginine, lysine, and glycine (ordination plot, Figure S1).

4 | DISCUSSION

Assessing plant-interactions among biotic stressors is critical for predicting how interactions may affect ecological and evolutionary processes. Plant phytochemical and nutritional responses to stressors are often attacker-specific (van Geem et al., 2016;

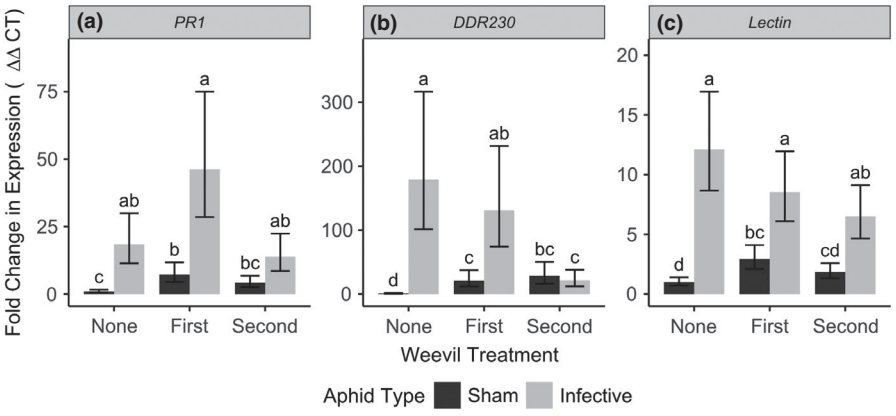

FIGURE 3 Relative transcript accumulation of plant defence response transcripts: (a) *PR1*, (b) *DDR230*, and (c) *PsLectin* following attack with various combinations of *S. lineatus*, *A. pisum*, and PEMV. Within each panel, bars separated by a different letter were significantly different based on MANOVA (Tukey's HSD, $\alpha = 0.05$). Bar height and error bars indicate marginal mean and standard error of the regression coefficient for each respective treatment

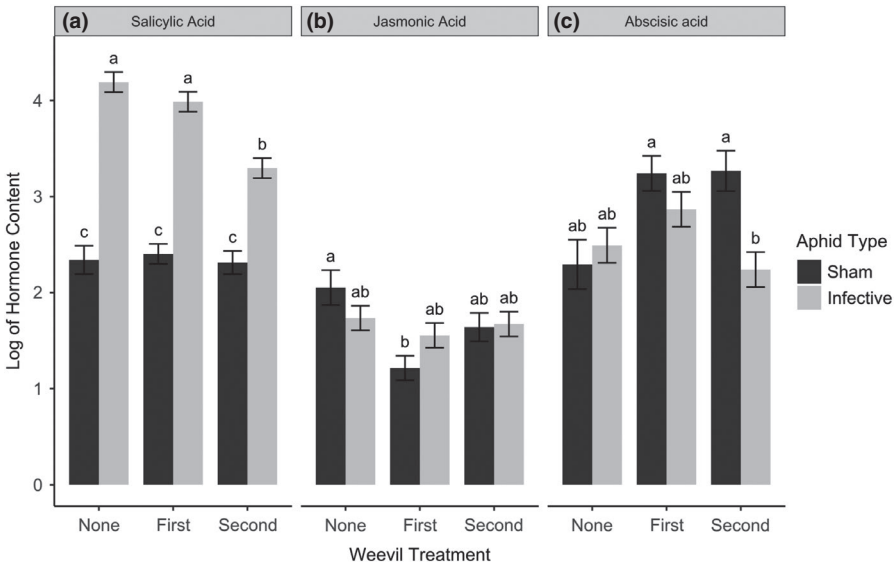

FIGURE 4 (a) Salicylic acid, (b) jasmonic acid, and (c) abscisic acid levels in *P. sativum* plants following attack with various combinations of *S. lineatus*, *A. pisum*, and PEMV. Within each panel, bars not connected by the same letter were significantly different (Tukey's HSD, $\alpha = 0.05$). Bar height and error bars indicate marginal mean and standard error of the regression coefficient for each respective treatment

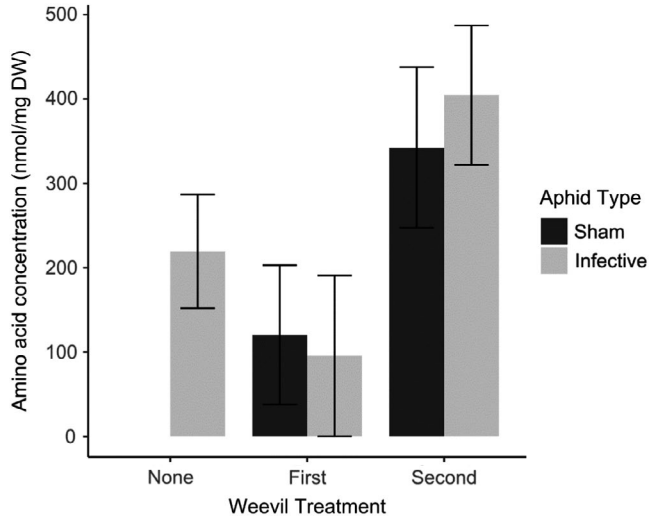

FIGURE 5 Nutritional analysis (total amino acid) in *P. sativum* following attack with various combinations of *S. lineatus*, *A. pisum*, and PEMV. *S. lineatus* increase total amino acid concentration in plants (GLM, $\chi^2 = 9.194$, $p = .01$). There was no "sham-none" treatment combination so that could not be estimated. Within each panel, bars not connected by the same letter were significantly different (Tukey's HSD, $\alpha = 0.05$). Bar height and error bars indicate marginal mean and standard error of the regression coefficient for each respective treatment

Shikano, 2017), and we show that plants responded differently to a piercing-sucking herbivore, a chewing herbivore, and a virus. Our results show that plant traits varied in response to the order of arrival of stressors. Moreover, we show that assessing multiple gene transcripts, phytohormones, and plant nutrients provides a more comprehensive perspective on mechanisms driving plant-insect-pathogen interactions than measurement of any response in isolation.

4.1 | Effects of PEMV infection on various pathogen-induced defence responses in peas

We found that PEMV caused broad defensive responses in *P. sativum* by inducing specific gene transcripts and phytohormones (Figures 2–4). Biotropic pathogens such as PEMV are known to activate salicylic acid signalling (Chisholm et al., 2018; Pan et al., 2021; Singh et al., 2018); this was reflected by increased expression of the *ICS1* biosynthesis gene, increased salicylic acid, and increased expression of the downstream defence transcript *PR1* when PEMV was present. However, effects of PEMV were not limited to salicylic acid signalling, as PEMV induced gene transcripts often associated with biosynthesis of abscisic acid (*AO3*) and giberrellic acid (*GA2ox*), while affecting defence genes that occur downstream from hormone induction (*PR1*, *DRR230*, *PsLectin*). Yet, PEMV alone did not induce gene transcripts associated with JA biosynthesis (*LOX2*), but PEMV infection after weevil herbivory induced *LOX2*. Similar results have been observed in response to fungal infection by *Mycosphaerella pinodes* and *Phoma koolunga*, which induce genes across multiple pathways (Fondevilla et al., 2011; Tran et al., 2018). However, increased expression of *LOX2* and *AO3* (Figure 2) were not reflected by increased jasmonic or abscisic acid (Figure 4), such that assessing phytohormones or gene transcripts in isolation may fail to reveal more complex pathways by which plants respond to pathogen effectors (Kazan & Lyons, 2014).

4.2 | Plant defence in peas was affected by the order of arrival of biotic stressors

While PEMV had broad effects on pea plants when infectious aphids attacked prior to *S. lineatus*, subsequent feeding by *S. lineatus* attenuated these responses for three biosynthesis gene transcripts

(Figure 2). Effects of PEMV on plant defence genes (*PR1*, *DDR230*, *PsLectin*) were also affected by *S. lineatus* and attack order. Overall, attack order had stronger effects on downstream plant defence responses than on hormone biosynthesis gene transcripts. For plants attacked first by either PEMV or *S. lineatus*, we also show stronger evidence for mutual antagonism in plant signalling responses at the gene transcript level rather than at phytohormone level (Figures 2–4). We show that weevils did not induce jasmonic acid even though they did induce *LOX2*. This may be because *LOX2* is upstream of JA biosynthesis and is rapidly induced following herbivory. Yet, biosynthesis of jasmonic acid also requires many intermediated steps. One intermediate, OPDA, is key for the production of jasmonic acid and can be regulated by various environmental and stress factors. Thus, when plants are exposed to multiple stressors, the level of jasmonic acid might induce differently than some of the JA-responsive genes at their transcriptional level.

Our results provide evidence that the order of arrival of biotic stressors on plants can play a crucial role in determining plants' response to these attackers. While mutual antagonism between *S. lineatus* and PEMV was common, for some genes these effects only occurred when *S. lineatus* attacked first, and for others when *S. lineatus* attacked second (Figures 2–4). Mutual antagonism has most often been studied as effects of a prior attacker affecting plants responses to a subsequent attacker, such as when a herbivore alters phytohormones in ways that attenuate performance of a pathogen (Erb et al., 2011; Huang et al., 2017; Kessler & Halitschke, 2007; Stam et al., 2014). However, our results suggest a second attacker may mitigate plant defensive responses against the first across multiple pathways in ways that might affect plant defence and pathogen transmission. For example, inhibition of pathogen defences by subsequent *S. lineatus* feeding should promote PEMV replication.

4.3 | Plant responses to biotic stressors stem from complex interactions among multiple pathways

Mutual antagonism in plant signalling pathways has most commonly been examined with regard to tradeoffs between jasmonic acid and salicylic acid signalling. Here, we show that PEMV infection induced several plant defences, including those associated with SA signalling, but these defences were inhibited when *S. lineatus* feed on peas already infected with PEMV. Conversely, *S. lineatus* feeding induced antiherbivore defence signals associated with JA signalling, but these defences were enhanced by PEMV. However, our results and other studies show these tradeoffs can affect other pathways. For example, jasmonic acid induction can limit production of abscisic acid in *Arabidopsis* following attack from *Fusarium oxysporum* (Anderson et al., 2004). Mutual antagonism between jasmonic acid and gibberellic acid, and jasmonic acid and abscisic acid, where induction of one hormone inhibits plant production of the other, have also been reported (Liu & Hou, 2018; Okada et al., 2015; Yang et al., 2013). For example, jasmonic acid facilitates defence over growth by repressing degradation of DELLA protein in rice and *Arabidopsis*, but elevated DELLA proteins interfere with the gibberellic acid pathway by binding to transcription factors associated with gibberellic acid signalling (Okada et al., 2015; Yang et al.,2012, 2013). However, antagonisms between salicylic acid and abscisic acid may actually lead to synergism between jasmonic acid and abscisic acid, a result seen following infection with *Pseudomonas syringae* in *Arabidopsis* (Fan et al., 2009). Overall, these results suggest that a broad examination of genes and hormones is needed to elucidate pathways underlying plant-insect-pathogen interactions in *P. sativum* and other plants.

Our results suggest interactions between PEMV and *S. lineatus* may also affect defence gene transcripts associated with a single signalling pathway. For example, the induction of *PR1*, a salicylic acid-responsive gene, was mitigated by *S. lineatus* attack after PEMV infection, as may be expected with cross-talk between jasmonic acid and salicylic acid. However, the expression of *ICS1*, another gene associated with salicylic acid biosynthesis, was not responsive to *S. lineatus*. This has been seen in other studies where *ICS1* was not induced by caterpillar feeding although other genes associated with salicylic acid were (Onkokesung et al., 2016). These results suggest that a plant's response to multiple stressors is unlikely to result from simple crosstalk but from interactions among multiple signalling pathways.

4.4 | Attack order and the diversity of attackers affected plant nutritional status

Plant pathogens can alter nutritional quality of their host plants in ways that affect vectors (Mauck et al., 2012; Wang et al., 2012; Patton et al., 2019). Similarly, nonvector herbivores may affect quantity and quality of plant nutrients (Ángeles-López et al., 2016). For example, Pepper golden mosaic virus (PGMV) increased levels of the amino acids proline, tyrosine, and valine in *Capsicum annuum* plants, but decreased levels of histidine and alanine. In this system, feeding by the greenhouse whitefly, *Trialeurodes vaporarioum*, reversed the levels of these amino acids (Ángeles-López et al., 2016). We show arrival of *S. lineatus* before PEMV infection suppressed total amino acid level, but enhanced amino acid levels were detected if *S. lineatus* damaged peas after PEMV infections. This suggests the intriguing possibility that antagonism between a pathogen and nonvector herbivore can occur at the level of amino acid production in plants.

Amino acids are main macronutrients and principal sources of nitrogen, necessary for herbivore growth and survival, and specific amino acid metabolic pathways are associated with distinct plant defence pathways (Schultz et al., 2013; Zeier, 2013). For example, amino acid profiles in peas may have changed following weevil herbivory if plants used nitrogen to produce antiherbivore defence compounds. Insect herbivory also sometimes induces various amino acid degrading enzymes such as proteases inhibitors and polyphenol oxidases (Kant et al., 2015). If weevils appear before viruliferous *A. pisum* in peas, reduced levels of total amino acids may thus be due to increased production of antiherbivore defence compounds or activation of wound-induced amino acid degrading enzymes. However,

prior infection with PEMV suppressed these enzymes, possibly because infected pea plants were primed with antipathogen defences. Thus, alteration of amino acid profiles following specific interactions may predict overall plant nutritional status (Kant et al., 2015; Schultz et al., 2013).

5 | CONCLUSION

Our study suggests complex plant-mediated interactions between a vector-borne plant pathogen and a nonvector herbivore can vary antagonism to enhancement, and manifest as changes in plant gene transcripts, phytohormones, and nutrients. Assessing the order of attack is necessary to understand the complexity and mechanisms of such interactions. Moreover, our study suggests more in-depth characterization of defence pathways is needed to avoid missing complexities of plant responses (Ángeles-López el al., 2016; Bedini et al., 2018; Shi et al., 2019). Characterizing the pathways by which plants respond to single and multiple stressors, with varying attack order, can in turn shed light on both the mechanisms and phenotypes that shape food web interactions among plants, herbivores, and pathogens.

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AUTHOR CONTRIBUTIONS

Saumik Basu and David W. Crowder conceived the ideas and designed the methodology; Saumik Basu, Robert E. Clark, Sayanta Bera and Clare L. Casteel collected the data; Robert E. Clark, Saumik Basu, Clare L. Casteel and David W. Crowder analysed the data; all authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data are publicly available from Figshare: https://doi.org/10.6084/m9.figshare.14227097.v1

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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