Reciprocal plant-mediated antagonism between a legume plant virus and soil rhizobia

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Abstract

1. Beneficial plant-associated soil microbes can promote plant tolerance to stress and promote nutrient uptake. Yet, the benefits of microbes for plant health can be altered by above-ground stressors like herbivores and pathogens. However, few studies have assessed reciprocal plant-mediated interactions between beneficial soil microbes and multiple above-ground stressors.

2. We assessed if soil rhizobia influenced complex interactions among pea host plants, a vector herbivore (aphids) and a plant virus (Pea enation mosaic virus, PEMV). We also examined how aphids and PEMV affected the function of soil rhizobia.

3. We show that plants attacked by PEMV produced fewer root nodules and had lower fresh root nodule biomass per g of fresh plant root biomass, and decreased expression of genes associated with nodulation, suggesting PEMV inhibited nitrogen fixation by rhizobia. However, soil rhizobia decreased aphid abundance and PEMV titre on host plants, such that rhizobia decreased the susceptibility of plants to herbivores and pathogens.

4. Assays of amino acids, and gene expression related to hormone signalling, show interactions among rhizobia, plants, aphids and PEMV were mediated by plant defence and nutrients. Viruliferous aphids induced salicylic acid in plants, and salicylic acid suppressed the function of rhizobia. Aphids feeding on plants grown in rhizobia-inoculated soil also obtained fewer essential amino acids than those feeding on plants grown in un-inoculated sterilized soil.

5. Mutually antagonistic plant-mediated interactions between soil microbes and above-ground stressors affected plant susceptibility and herbivore nutrient uptake. This suggests ecological effects of soil microbes and above-ground stressors for plant health will likely vary based on multi-trophic plant-mediated interactions among herbivores, pathogens and soil microbes.

Keywords

antagonism, nodulation, pathosystem, pea aphid, PEMV, soil rhizobia
1 | INTRODUCTION

Soil harbours diverse microbial communities that can alter plant susceptibility to abiotic stressors such as drought and nutrient deficiency (Müller et al., 2016; Teng et al., 2015). Beneficial plant-associated soil microbes also mediate plant susceptibility to biotic stressors including pathogens and herbivores (Ballhorn et al., 2016; Jaber & Vidal, 2009; Santos et al., 2014). By affecting plant stress responses, soil microbes affect the resilience of entire plant communities and ecosystem processes such as biomass production, biological control, nutrient cycling and pollination (A’Bear et al., 2014; Bardgett & van der Putten, 2014). Above-ground plant antagonists such as herbivores and pathogens may also affect the abundance and function of soil microbes by affecting plant biomass and nutritional quality (Bardgett et al., 1998).

Soil rhizobia are ecologically important bacteria that colonize legume roots, forming root nodules that fix atmospheric nitrogen (Herridge et al., 2008; Smil, 1999). Rhizobia alter tolerance of legumes to pathogens by mediating programmed cell death, hormone signalling and physical defences (Dodds & Rathjen, 2010; Kumar et al., 2011). At the same time, abiotic and biotic stress above-ground may affect mutualisms between plants and rhizobia. For example, early nodule formation in legumes can be inhibited by pathogens (López et al., 2017; Rao et al., 1987), although herbivory can promote abundance and greater size of nodules (Heath & Lau, 2011; Simonsen & Stinchcombe, 2014). Recent syntheses suggest while herbivores and pathogens alter benefits of microbes for plants, the plant-mediated mechanisms underlying these interactions need further investigation (Friman et al., 2020; Pangesti et al., 2013).

Plant defence and nutrients are affected by soil microbes and above-ground antagonists. By altering plant traits, soil microbes may affect above-ground stressors and vice versa. For example, induction of salicylic acid, a hormone involved in plant defence, can inhibit nodule formation in rhizobia-infested legumes (Martínez-Abarca et al., 1998; Nagata & Suzuki, 2014). Enhanced rhizobia colonization, in contrast, has been observed in plants with reduced salicylic acid (Stacey et al., 2006; van Spouw et al., 2003). Consequently, phytohormone signalling may mediate interactions among rhizobia, plants and biotic stressors (Thaler et al., 2012). However, few studies have assessed hormones other than salicylic acid in mediating interactions among soil microbes and above-ground stressors (Rashid et al., 2017) while also looking at how rhizobia may affect nutrient uptake by herbivores (Dean et al., 2014; Johnson et al., 2017), which may interact to affect plant susceptibility to vector-borne pathogens. Such studies are needed given that the composition of soil microbial communities varies considerably across ecological contexts and may thus broadly mitigate the spread of disease.

Here, we addressed these key knowledge gaps by examining reciprocal interactions among a legume crop plant (pea Pisum sativum), soil rhizobia (Rhizobia leguminosarum biovar. viciae), a vector herbivore (pea aphid Acyrthosiphon pisum) and a vector-borne pathogen (Pepa enation mosaic virus, PEMV). In the Palouse region of eastern Washington and northern Idaho, these organisms co-occur on wild and cultivated legumes. However, it is unknown whether rhizobia decrease plant susceptibility to aphids and/or PEMV, or whether aphids and/or PEMV affect mutualisms between rhizobia and legume plants. We hypothesized that both soil rhizobia and above-ground antagonists would affect plant hormonal signalling and nutrient uptake, such that soil rhizobia could alter plant susceptibility to aphids and/or PEMV, or vice versa, through plant-mediated mechanisms. In controlled experiments, we examined reciprocal interactions among these organisms and characterized the underlying mechanisms. Our results show that rhizobia can interact with above-ground stressors through multiple plant-mediated pathways.

2 | MATERIALS AND METHODS

2.1 | Study system

In the Palouse region of eastern Washington and northern Idaho, USA, A. pisum and PEMV attack P. sativum hosts, which are widely cultivated as a nitrogen-fixing crop (Clement et al., 2010). Infection of P. sativum with PEMV, which is transmitted by A. pisum, stunts plant growth and reduces yield (Clement et al., 2010). The symptoms of P. sativum plants infected with PEMV include small yellowish spots on leaves which gradually turn white, crinkled and twisted leaf textures, presence of cracks and blisters in the adaxial side of leaves, stem deformation, as well as twisted and small pods. In peas, R. leguminosarum biovar. viciae, is a common root colonizing, nodule inducing, bacteria (Mutch & Young, 2004).

All A. pisum used in experiments were reared in greenhouses (16:8 hr light:dark; 22:17°C light:dark) on P. sativum (cv. Banner). We reared both viruliferous and non-viruliferous A. pisum colonies. Both were started in 2012 from a field-collected population of viruliferous A. pisum from the Palouse. To create the non-viruliferous colony, we transferred 50 viruliferous A. pisum adults to petri-dishes with filter paper to reproduce for 3 days. All A. pisum nymphs produced were non-viruliferous, as PEMV is not maternally transmitted and the nymphs never fed on PEMV-infected plants. Thereafter, these individuals were reared on uninfected P. sativum plants; the viruliferous A. pisum colony was reared on PEMV-infected plants. Colonies of viruliferous and non-viruliferous A. pisum were maintained in separate greenhouses (Chisholm et al., 2018).

2.2 | Effects of soil microbes and nitrogen on PEMV

We tested effects of rhizobia and fertilizer on susceptibility of peas to A. pisum and PEMV. Soil from the Palouse Conservation Farm (Pullman, WA) was exposed to four treatments: (a) control; (b) autoclaved to remove microbes; (c) autoclaved with rhizobia; and (d) control plus fertilizer; six replicates of each. Soil was autoclaved to remove microbes by placing it in 61 × 91 cm bags in an autoclave at 7 psi and 111°C overnight. In rhizobia treatments autoclaved soil was inoculated with pea-specific rhizobia (R. leguminosarum biovar.
viciae) by mixing N-charge®, a peat-based inoculant, with P. sativum seeds using a manufacturer’s protocol (Verdasian Life Sciences). The rhizobium inoculum concentration was 2 × 10⁵ colony forming units per g of soil. The fertilizer treatment was included as fertilizer can suppress nodule formation, and this treatment thus served as a form of ‘nitrogen control’. For this, ammonium nitrate was added to soil at 90 kg/ha (~5 mM N), a rate that suppresses nodulation in P. sativum (Harper & Gibson, 1984).

Experiments were conducted in greenhouses (16:8 hr light:dark, 23°C light, 17°C dark) with plants grown in potting mix (Sunshine® LC1) before transplantation into treated soil after 2 weeks. Plants were put in 1-L pots with soil exposed to one treatment and standardized to 75% moisture. Plants were not provided supplemental nutrients other than fertilizer in select treatments as prior experiments show potting mix provides suitable nutrients (Chisholm et al., 2018). Plants were put in 0.6 × 0.6 × 0.6 m cages and 1 d later exposed to one of two A. pism treatments: (a) PEMV: 10 5-day-old viruliferous A. pism for 48 hr or (b) sham-inoculation: Ten 5-day-old non-viruliferous A. pism for 48 hr. After 48 hr aphids were removed by aspiration; the removal of all individuals was confirmed by 72 hr of observations. To assess PEMV titre, half the plants in each treatment were harvested at an early time point (4 days post inoculation, dpi) and half at a late time point (10 dpi). To harvest plants, plant tissue was cut at the soil and plant fresh shoot biomass was measured. Then, we carefully washed soil off plant roots and measured plant root biomass. The experiment included six randomly assigned replicates of each treatment in a 4 × 2 factorial design (4 soil treatments × 2 aphid treatments × 2 time points × 6 replicates = 96 experimental units).

For measuring relative PEMV-1 titre (per 100 mg plant biomass), after weighing, plant shoot tissue samples were wrapped in aluminium foil, frozen in liquid N₂ and snapped chilled in dry ice before storing in −80°C and ground into fine powder in liquid N₂ using mortar and pestles. Homogenized tissue (100 mg) was used for total RNA extraction using Promega SV total RNA isolation kits (Promega) and cDNA from 1 µg of total RNA using Bio-Rad iScript cDNA synthesis kits. PEMV-1 specific primers (Table S1) were used in qRT-PCR reactions (10 µl) containing 3 µl of ddH₂O, 5 µl of iTaq Univer SYBR Green Supermix, 1 µl of diluted primer mix (forward and reverse [concentration 10 µM]) and 1 µl of diluted (1:25) cDNA template. The qRT-PCR program included an initial denaturation for 3 min at 95°C followed by 40 cycles of denaturation at 95°C for 15 s, annealing for 30 s at 60°C and extension for 30 s at 72°C. For melting curve analysis, a dissociation step cycle was added (55°C for 10 s, and then 0.5°C for 10 s until 95°C). The relative viral titre of PEMV-1 (per 100 mg plant biomass) at two different time points (4 dpi and 10 dpi) were then calculated using the delta–delta Ct method, (2−ΔΔCt) with β-tubulin as a housekeeping gene (Kozera & Rapacz, 2013; Livak & Schmittgen, 2001). We measured PEMV-1 titre as our metric of PEMV transmission, rather than visual symptoms, given that visual symptoms of PEMV cannot be reliably quantified (Chisholm et al., 2018). However, all plants with positive PEMV titre showed visual viral symptoms (leaf yellowing, curling, etc).

### 2.3 Aphid abundance in response to soil treatment

We next conducted an assay to assess whether soil treatments, aphid type (sham or viruliferous) and their interaction affected A. pism abundance, with the same soil treatments: (a) control; (b) autoclaved; (c) autoclaved with rhizobia added after; and (d) control plus fertilizer. We had five replicates per soil treatment, for each aphid type, where each replicate had a single pea source plant grown in an individual pot, along with 10 recipient plants. Seeds were sown in 9-cm² pots in treated soil, and pots were placed in 0.6 × 0.6 × 1.0 m cages. 20 adult ‘sham’ or viruliferous A. pism were added to individual 2-week-old plants, and after 24 hr for establishment, we placed two rows of five recipient P. sativum plants directly adjacent to the source plant in the same cage; each of these plants was grown in an individual pot containing soil with the same treatment as the source plant. We used this design to allow aphid populations to have sufficient plant resources so they would not reach a carrying capacity, while allowing them to move naturally among plants, allowing us to more precisely measure absolute population growth rates. We counted maternal and nymph A. pism on all plants daily for 7 days. For analyses, we included the last 6 days since the first day only recorded establishing A. pism density. After 7 days, harvested plants were cut at the soil surface, and plant shoot fresh biomass was measured. All pea plants from each treatment were checked for the presence of PEMV infection using PCR, and 100% of plants exposed to viruliferous aphids had a positive PEMV titre.

### 2.4 Effects of A. pism and PEMV on soil rhizobia

We next assessed how stress from A. pism and PEMV affected nodulation and nodule biomass, key metrics of rhizobia function. This experiment had three treatments: (a) control – no A. pism; (b) sham-inoculation: plants infested with non-viruliferous A. pism; (c) PEMV: plants infested with viruliferous A. pism. In this experiment, P. sativum plants were grown in Sunshine Mix LC1 potting mix (Sun Gro Horticulture) that did not contain rhizobia and were exposed to one of two rhizobia treatments: (a) control – no rhizobia or (b) rhizobia: inoculated with rhizobia as described previously. We had 20 replicates for each combination of treatments (3 A. pism × 2 soil). In treatments with A. pism, ten 5-day-old A. pism were released on each plant (either all viruliferous or all sham) for 24 hr, after which they were removed. Prior studies have shown that a 24-hr exposure is enough for plants to respond to aphids and PEMV (Chisholm et al., 2018). After A. pism were removed, P. sativum plants were grown for 10 days to assess root development. Representative pea plants from each treatment were checked for the presence of PEMV infection through PCR using PEMV-1 specific primers, and 100% of the plants exposed to viruliferous aphids were infected; 0% of plants exposed to non-viruliferous aphids were infected.

Following the experiment period, plants were cut at the soil surface, and plant fresh shoot biomass was measured. Then, plant
roots were carefully uprooted and the roots were washed with tap water to clean up attached soil particles. The total number of nodules were visually counted before they were excised and collected in separate plastic trays using a sharp blade. Nodule fresh weights were taken immediately after collection, and plant root biomass (without nodules) was measured. Nodules were dried for 5 days at 37°C for dry weight measurements. None of the plants in the control soil produced nodules, indicating that the soil was free of rhizobia.

2.5 Analyses of transcripts related to phytohormone signalling

To assess if phytohormones mediated interactions among soil rhizobia, A. pism, and PEMV, we conducted a 2 × 3 factorial experiment that varied rhizobia (present or absent) and A. pism (none, sham or viruliferous) and assessed the relative expression of two plant genes, PR1 (PATHOGENESIS-RELATED PROTEIN 1) and LOX2 (LIPEROXYGENASE 2). PR1 acts downstream of the salicylic acid pathway and is induced by pathogens (Johnson et al., 2003; Zhu et al., 2018). In contrast, LOX2 is involved upstream of the jasmonic acid pathway and induced by chewing insects (Turner et al., 2002; Zhu et al., 2018). The expression of these genes reflects the salicylic acid and jasmonic acid signalling pathways, which can mediate responses of plants to biotic stress (Kouzai et al., 2016; Lemarié et al., 2015). Expression of these genes also mirror production of salicylic acid and jasmonic acid (Chisholm et al., 2018). Pea plants were grown in potting mix (Sunshine® LC1) in 1-L pots, and rhizobia treatments were inoculated as previously described. For A. pism treatments (sham or viruliferous), we added ten 5-day-old A. pism to plants when they were 2 weeks old in 0.6 × 0.6 × 0.6 m mesh cages. After 48 hr, A. pism were removed by aspiration and plants were grown for 7 days before harvesting. While transcript abundances can vary over time, we waited 7 days to harvest plants as this is the amount of time for PEMV to be detectable and to allow for comparison to other studies that used this time period for analysis (Bera et al., 2020; Chisholm et al., 2018). Each of the treatments [2 soils (rhizobia or control) × 3 A. pism (viruliferous, sham and none)] had four biological replicates.

After A. pism treatments were complete, above-ground plant tissue was harvested and prepared for RNA extraction as described earlier (see 'Effects of soil microbes and nitrogen PEMV'). After processing, primers specific to phytohormone genes, PR1 and LOX2 (Table S1) were used in qRT-PCR reactions (10 μl) following procedures described earlier (see 'Effects of soil microbes and nitrogen on A. pism and PEMV'). Relative transcript abundances of PR1 and LOX2 were then calculated using the delta–delta Ct method, (2–ΔΔCt) with Pstj-tubulin as a housekeeping gene (Kozera & Rapacz, 2013; Livak & Schmittgen, 2001). PEMV infection in the infected plants were confirmed through PCR using PEMV-1 specific primers, and 100% of plants exposed to viruliferous A. pism had a positive PEMV titre.

2.6 Effects of A. pism and PEMV on gene transcripts associated with nodule development

We grew plants for 4 weeks in rhizobia-inoculated Sunshine Mix LC1 potting mix (Sun Gro Horticulture) and then exposed them to three A. pism treatments: (a) control – no A. pism, (b) sham: infested with non-viruliferous A. pism, and (c) PEMV: infested with viruliferous A. pism. In A. pism treatments, ten 5-day-old A. pism were released on each plant (all viruliferous or sham) for 48 hr, after which they were removed. Plants were then grown for 7 days for nodule development. Following this period, plants were carefully uprooted and the roots were washed with tap water to clean up soil particles. From each plant, five nodules of similar size were excised using a sharp blade and collected in microfuge tubes, frozen in liquid N2, and snap chilled in dry ice before storing in −80°C. For analyses, samples were homogenized using a micro pestle using liquid N2. Homogenized tissue was used for total RNA extraction and cDNA synthesis as previously described (see ‘Effects of soil microbes and nitrogen on A. pism and PEMV’). Relative transcript abundances of Nodule Inception Protein gene (PsNIN) and Nodule Lectin (PsNLEC1) were determined using qRT-PCR reactions with gene specific primers (Table S1) and Pstj-tubulin as a housekeeping gene (Kozera & Rapacz, 2013; Livak & Schmittgen, 2001). Presence of infection in the PEMV treated plants were confirmed by using PEMV-1 specific primers, and 100% of plants exposed to viruliferous A. pism had a positive PEMV titre.

2.7 Effects of phytohormones on function of soil rhizobia in mediating PEMV infection

We assessed if phytohormones mediated the effects of rhizobia on providing tolerance to PEMV by treating leaves of 2-week-old P. sativum plants (grown in potting mix inoculated with rhizobia) with one of three treatments: (a) control – 0.01% tween solution (a surfactant), (b) jasmonic acid – 0.01% tween plus 0.45 mM methyl jasmonate, an active form of jasmonic acid (Adio et al., 2011) and (c) salicylic acid – 0.01% tween plus 1 mM methyl salicylate, an active form of salicylic acid (Ghazijahani et al., 2014). These concentrations reflect levels used in experiments with Arabidopsis spp., P. sativum, and other plants (Adio et al., 2011; Bera et al., 2020; Ghazijahani et al., 2014). Twelve replicates were used per treatment. Plants were individually placed in mesh cages (0.6 × 0.6 × 1 m), and after 24 hr, ten 5-day-old viruliferous A. pism were released on each plant for 24 hr, after which they were removed by aspiration. After 10 days, plants were uprooted, cleaned with tap water, and the number of nodules was counted. Representative plants from each treatment were confirmed for viral presence through PCR using PEMV-1 rep specific primers, and 100% of plants exposed to viruliferous A. pism had a positive PEMV titre.
2.8 | Analyses of amino acids

We also measured amino acid content of *A. pisum* from plants grown in each field-collected soil treatment. We used this method, rather than testing amino acids in phloem sap, because the most common technique to extract phloem sap (extrusion into ethylenediaminetetraacetic acid) can confound sugar and amino acid concentrations (Casteel et al., 2014). However, measuring amino acids directly from aphids can provide a fairly reliable estimate of nutrients available in phloem.

For the experiment, 20 age-standardized (5-day-old adults) *A. pisum* were collected from each replicate of the abundance experiment for each soil treatment into liquid N₂ and lyophilized (4 replicates each). Amino acids were extracted and analysed based on Casteel et al. (2014). Briefly, 10 mg of dried material was placed in a 2-ml micro-centrifuge tube with two 3-mm steel beads, and ground to powder using a Harbil 5G-HD paint shaker. Ground tissue was extracted with 100 μl of 20 mM HCl, centrifuged, and supernatant was saved. Amino acids were derivatized with AccQ-Fluor reagent kits with the manufacturer’s instructions (Waters), with L-Norleucine as a standard. From each sample, 10 μl was injected with an Agilent 1,260 Infinity pump system with a Nova-Pak C18 column and a fluorescence detector. Data were recorded using the Agilent Chemstation software. Amino acid derivatives were detected with an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Peak areas from samples were compared to a standard curve made from a serial dilution of amino acid standards (Sigma-Aldrich). Plants and aphids from all the soil treatments were tested for the presence of PEMV by using PEMV-1 specific primers, and 100% of plants exposed to viruliferous *A. pisum* had a positive PEMV titre.

2.9 | Data analysis

Analyses were conducted in R 3.6.1 (R Core Team, 2019). Using ANOVA, we assessed whether soil (control, sterilized, rhizobia, nitrogen) and aphid (sham or viruliferous) treatments affected plant shoot and root biomass in the experiment measuring PEMV titre. We then assessed if the four soil treatments affected shoot biomass in the experiment that assessed viruliferous aphid population growth. Finally, we assessed if three aphid treatments [none, sham, viruliferous] and soil treatment (rhizobia or not) affected plant root biomass in the experiment testing nodulation.

We analysed the fold change in viral titre using ANOVA to examine effects of soil and aphid treatments, and their interaction, on delta CT values for PEMV titre and relative transcript abundance for the *PR1*, *LOX2*, *PsNIN* and *PsNLEC1* gene transcripts. Parameter estimates and subsequent calculations for delta-delta CT (2^-ΔΔCT) were plotted on the log₁₀ scale. To evaluate the effect of soil treatments on *A. pisum* abundance, *A. pisum* counts per plant per day in each dorm was modelled as a Poisson-distributed response variable in a GLMM using soil treatment as a fixed effect and dorm number × duration as a random effect (GLMM, *lme*4 package, Bates et al., 2015). We used univariate models to assess whether soil treatments affected nodule weight and nodule count in rhizobia-treated soil. Nodule weight and nodule counts measurements were standardized based on plant root biomass. Finally, average amino acid content for 11 amino acids (serine, glycine, glutamine, histidine, homoserine, arginine, threonine, alanine, proline, tyrosine and lysine) was fitted to a generalized linear mixed model (GLMM, *lme*4 package, Bates et al., 2015), with soil treatment as a fixed effect and amino acid and replicate as random effects. For this analysis, amino acid concentration was log-transformed to meet normality assumptions. All post-hoc tests were calculated using the *emmeans* package (Lenth, 2016), while significance tests via analysis of deviance tables were generated using the *car* package (Fox & Weisberg, 2011).

3 | RESULTS

3.1 | Treatment effects on plant shoot and root biomass

Soil treatment (control, sterilized, rhizobia, nitrogen) and aphid type (sham, viruliferous) did not affect shoot or root biomass for 4-week-old pea plants in PEMV titre experiments at 4 dpi (shoot biomass - soil: *F*₃,₄₀ = 0.88, *p* = 0.46, aphid: *F*₁,₄₀ = 0.73, *p* = 0.40, soil × aphid: *F*₂,₄₀ = 0.10, *p* = 0.96; root biomass - soil: *F*₃,₄₀ = 1.08, *p* = 0.37, aphid: *F*₁,₄₀ = 0.23, *p* = 0.64, soil × aphid: *F*₂,₄₀ = 0.85, *p* = 0.48) or 10 dpi (shoot biomass – soil: *F*₃,₄₀ = 0.043, *p* = 0.99, aphid: *F*₁,₄₀ = 0.52, *p* = 0.48, soil × aphid: *F*₂,₄₀ = 0.87, *p* = 0.47; root biomass - soil: *F*₃,₄₀ = 1.15, *p* = 0.34, aphid: *F*₁,₄₀ = 2.42, *p* = 0.13, soil × aphid: *F*₂,₄₀ = 1.43, *p* = 0.25). Soil treatments also did not affect shoot biomass in the experiment measuring effects on aphid population growth (*F*₃,₄₀ = 2.11, *p* = 0.14; Figure S1); the three aphid treatments (none, sham, viruliferous) also did not affect plant root biomass in the experiment testing nodulation (*F*₂,₄₀ = 2.17, *p* = 0.12).

3.2 | Soil treatment effects on viral titre and vector abundance

PEMV titre was affected by soil treatment at both 4 (GLMM, *χ*² = 43.45, *p* < 0.001) and 10 dpi (GLMM, *χ*² = 47.41, *p* < 0.001). At both dpi, the lowest PEMV titre occurred for plants treated with rhizobia (Figure 1A,B). At 4 dpi, sterilized soil had intermediate PEMV titre between rhizobia and control treatments (Figure 1A), but this was not observed at 10 dpi (Figure 1B). In the experiment assessing soil treatments on *A. pisum* abundance, the number of *A. pisum* on recipient host plants was significantly lower when plants were in soil inoculated with rhizobia compared to the control treatment (GLMM, *χ*² = 8.88, *p* = 0.030, Figure 1C). These results were consistent for both aphid types (sham or viruliferous; GLMM, *χ*² = 1.35, *p* = 0.25, Figure 1C), and there was no interaction between aphid type and soil treatment on abundance (GLMM, *χ*² = 2.47, *p* = 0.48).
3.3 Effects of A. pisum and PEMV on the function of rhizobia

PEMV infection decreased the number of root nodules per g of plant root biomass (GLMM, $\chi^2 = 68.7, p < 0.001$), nodule dry mass per g of plant root biomass (GLMM, $\chi^2 = 63.9, p < 0.001$) and nodule fresh biomass per g of plant root biomass (GLMM, $\chi^2 = 77.5, p < 0.0001$) compared to both sham-inoculated and control plants (Figure 2). These reductions in nodule size and biomass, and lateral root formation, could be observed visually on washed *P. sativum* roots (Figure S2).

3.4 Effects of stressors on plant gene expression across soil backgrounds

Differences in stress treatments, and soil background, affected the relative expression of both *PR1* (ANOVA, $F_{7,24} = 15.71, p < 0.001$) and *LOX2* (ANOVA, $F_{7,24} = 2.73, p = 0.03$; Figure 3). Treatments with viruliferous aphids had higher *PR1* expression in plants with or without rhizobia, compared to the control + sham aphid, rhizobia + no aphid and rhizobia + sham aphid treatments (Figure 3). Conversely, there was a significant reduction in *LOX2* expression in PEMV-infected plants compared to the sham treatment with non-viruliferous *A. pisum*. Rhizobia treated plants, regardless of PEMV infection, had intermediate levels of *LOX2* expression (Figure 3).

3.5 Effects of A. pisum and PEMV on gene transcripts associated with nodule development

We observed significant differences in the expression gene transcripts for *PSNLEC1* ($F_{3,11} = 27.6, p < 0.001$) and *PSNIN* ($F_{3,11} = 7.43, p = 0.007$). The lowest expression levels for either gene transcript occurred with plants exposed to PEMV (Figure 4, Tukey HSD). Sham aphids alone (no PEMV) did not significantly reduce expression of
PSNLEC1 or PSNIN compared to plants with no A. pism (Figure 4, Tukey HSD). Reduced nodulation in PEMV plants could be visibly observed from plant roots in this experiment (Figure S3).

3.6 | Effects of plant phytohormones on function of rhizobia

As we observed that PEMV affected gene expression associated with the salicylic and jasmonic signalling pathways, we tested whether exogenous application of these phytohormones affected the function of rhizobia. While exogenously sprayed methyl jasmonate had no effect on nodule formation compared to plants with no phytohormone treatment, plants treated with methyl salicylate had a significantly reduced number of nodules (GLM, $\chi^2 = 96.84, p < 0.001$, Figure 5).

3.7 | Soil rhizobia effects on amino acids obtained by A. pism from P. sativum

Soil treatment had a significant effect on the average concentration of amino acids for feeding A. pism (GLMM, $\chi^2 = 13.34, p = 0.003$). Compared to sterilized soil, amino acid content in A.
**Discussion**

Our results show strong antagonistic interactions between soil rhizobia and an aphid-borne plant virus. Plants grown with rhizobia had lower PEMV titre and aphid abundance even though soil treatments did not affect root or shoot biomass. This shows lower plant susceptibility to PEMV and aphids in soil with rhizobia was not due to greater growth but from alterations of chemical defences or nutrients. As we autoclaved soil before adding rhizobia, we show rhizobia are a keystone microbe that alters plant tolerance to biotic stressors by altering plant functional traits. Decreased aphid abundance and PEMV titre in rhizobia-treated plants may be due to reduced aphid feeding or nutrient uptake, although application of nitrogen fertilizer did not affect aphids or PEMV, suggesting rhizobia affect aphids and virus transmission through mechanisms other than alterations of nitrogen. As rhizobia communities vary widely across ecological contexts, ecological interactions between rhizobia and biotic stressors such as those seen here may drive co-evolution among plants, herbivores and microbes (e.g. van Cauwenberghe et al., 2021).

At the same time, plants infected with PEMV had fewer nodules and lower nodule biomass per g of plant root biomass. This likely reduces nitrogen fixation, although analyses of nitrogen isotypes are needed to confirm this. PEMV reduced expression of gene transcripts associated with nodulation, further suggesting nitrogen fixation was likely limited due to PEMV. However, we did not observe direct effects of sham A. pisum on nodule number or biomass, although this may reflect the short aphid feeding duration. Many genes are involved in nodule development in P. sativum, including PsSym35/PsNIN (Constantin et al., 2008) and Nodule lectin, PsNLc1 (Brewin & Kardailsky, 1997; Constantin et al., 2008; Kijne et al., 1997; van Rhijn et al., 2001). For example, in legume crops, PEMV outbreaks occur early in the growing season (Clement et al., 2010). In other studies, infection of legume crops with viruses has been shown to reduce nitrogen inputs to soil by interfering with legume-rhizobia symbiosis (Marchetto & Power, 2021). Our study shows PEMV may similarly reduce nitrogen provided by rhizobia to soil.

Our study provides more evidence that rhizobia can decrease susceptibility to herbivores and pathogens (Dutta et al., 2008; Gopalakrishnan et al., 2015; Mabrouk et al., 2018; Ranjbar Sistani et al., 2017). In contrast, pathogens like PEMV can inhibit plant mutualisms with microbes by suppressing symbiotic genes or interfering with nutrient transfer (Duffy et al., 2003; López et al., 2017). By analysing phytohormone transcripts, we show that interactions between rhizobia, A. pisum, and PEMV were mediated by alterations of hormone signalling. Plants infected with PEMV had greater expression of a SA-gene, and application of exogenous salicylic acid to infected plants decreased decreased nodule formation by rhizobia. This suggests salicylic acid mediates interactions between viruses and rhizobia (e.g. Martínez-Abarca et al., 1998; Nagata & Suzuki, 2014; van Sprensen et al., 2003). In contrast, enhanced rhizobia colonization occurs in Lotus japonicus, Medicago truncatula and P. sativum when production of salicylic acid is inhibited (Blilou et al., 1999; Stacey et al., 2006). While we did not observe effects of rhizobia on gene transcripts, in alfalfa Medicago sativa rhizobia can reduce salicylic acid accumulation in roots, showing rhizobia may inhibit phytohormone production in some cases or at different points in the pathway (Martínez-Abarca et al., 1998).

While we did not quantify root growth, our qualitative data suggest that rhizobia not only affected hormone signalling but also promoted lateral root formation in peas, although not enough to impact biomass. Rhizobia secrete signalling molecules that can facilitate lateral root formation in legumes (Bensmihen, 2015; Ferguson & Mathesius, 2014). However, effects of PEMV on reducing nodule number and size were unlikely due to reduced root growth but appeared to be mainly due to disruption of nodule formation. In contrast, Phaseolus vulgaris plants attacked by *Southern bean mosaic* virus have severe root malformations, atypical root hairs, and fewer root hairs (López et al., 2017). The ability of rhizobia and other microbes to colonize plant roots are strongly affected by root architecture, including abundance of root hairs (Gage, 2004), suggesting virus-induced changes in root growth may be an additional mechanism that inhibits rhizobia establishment and function. Besides promoting root growth, soil rhizobia enhance water uptake by plants and promote drought tolerance, while aphid feeding often has the opposite effect, reducing total plant water content (Katayama et al., 2014; Mabrouk et al., 2018).
Rhizobia also reduced the uptake of amino acids in A. pism compared to those feeding on plants grown in sterilized soil. This suggests rhizobia may have altered feeding behaviour of A. pism (Brunner et al., 2015) or affected plant nutrients; it also suggests that soil sterilization may have released a cause of nutrients that were taken up by plants. This may partially explain the reduced PEMV titre in plants grown in soil with rhizobia, as if A. pism are able to feed less effectively on plants grown with rhizobia, their ability to transmit PEMV could decrease. However, we acknowledge that autoclaving of field soil, while a common sterilization technique, can also drive changes in soil texture, compaction and chemistry; unfortunately, we cannot exclude the possibility that such changes in soil from autoclaving may have affected results.

As a persistently transmitted pathogen, PEMV requires sustained feeding bouts for transmission, and inhibition of aphid feeding interferes with transmission (Chisholm et al., 2018). Our analysis of amino acids from A. pism reflected aphid feeding behaviour without confounding effects associated with assessing phloem, which can poorly reflect aphid nutrient uptake (Jakobs et al., 2019; Karley et al., 2002; Mauck et al., 2014). Alternatively, soil rhizobia can exert indirect effects on insect herbivores by altering primary and secondary plant metabolites (Pineda et al., 2010) or by limiting nutrient uptake (Dean et al., 2014; Katayama et al., 2014) in ways that affect aphid fitness (Kempel et al., 2009). Enhanced root nodulation due to silicon addition, in contrast, elevated accumulation of essential foliar amino acids in Medicago sativa and increased uptake by A. pism (Johnson et al., 2017). Moreover, increased nitrogen fixation by rhizobia can increase nitrogen-based defences in legumes, which may inhibit herbivores (Thamer et al., 2011). These studies suggest that effects of rhizobia on plant nutrients and metabolites can alter aphid performance and feeding behaviour.

Our results suggest soil rhizobia improve plant health by modulating plant defence against above-ground pathogens and lower amino acids obtained by vector herbivores. Although rhizobia likely increase nitrogen levels in plants, legume-rhizobia symbiosis triggers synthesis of secondary metabolites including pyrrolizidine alkaloids and various flavonoid compounds. These toxic secondary metabolites may act as feeding deterrents and explain reduced nitrogen uptake by aphids on plants grown in rhizobia-inoculated soil (Irmer et al., 2015; War et al., 2012). On the other hand, vectors and pathogens can interfere strongly with legume-rhizobia symbiosis by inhibiting root nodule development and nitrogen fixation. From an evolutionary perspective, this suggests soil microbes like rhizobia, and plant pathogens like PEMV, could co-evolve due to plant-mediated selection pressure. For example, co-evolution might lead to evolution of viral genomes to promote greater transmissibility and virulence in areas where rhizobia are common, and selection for rhizobia to promote greater plant chemical defence and limited nutrients where viruses are common. Thus, assessing reciprocal interactions between rhizobia, herbivores and pathogens is crucial for understanding disease ecology and plant productivity in both natural and agricultural ecosystems across both short (in-season) and long (evolutionary) time-scales.

ACKNOWLEDGEMENTS
We thank the many Crowder laboratory undergraduates who helped conduct the experiments and E. Lysian for assistance with data analysis. This research was supported by USDA-NIFA Grants 2016-67011-24693, 2017-67013-26537 and Hatch project 1014754.

AUTHORS’ CONTRIBUTIONS
S.B., C.L.C., A.M.C. and D.W.C. conceived the ideas and designed the methodology; S.B., R.E.C., R.B., A.M.C. and C.L.C. collected the data; R.E.C., R.B., C.L.C. and D.W.C. analysed the data; all authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT
All data associated with the manuscript have been uploaded to figshare (Crowder & Basu, 2021; https://doi.org/10.6084/m9.figshare.9917756).

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