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Soil rhizobia promote plant yield by increasing tolerance to pests and pathogens under field conditions

Paul J. Chisholm [a], Akaisha Charlton [a], Riley M. Anderson [a], Liesl Oeller [a], John P. Reganold [b], David W. Crowder [a,*]

[a] Department of Entomology, Washington State University, Pullman, WA 99164, USA
[b] Department of Crop and Soil Sciences, Washington State University, Pullman, WA 99164, USA

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ABSTRACT

Nitrogen-fixing, root-colonizing rhizobia are abundant soil microbes that form mutualisms with legumes. Rhizobia provide direct benefits to hosts by fixing nitrogen and promoting nutrient acquisition. However, whether rhizobia indirectly alter plant yield by affecting insect pests and insect-borne pathogens is less well understood, with conflicting results from existing lab studies. Here we used a field experiment to test whether effects of rhizobia on plants extended beyond nitrogen provisioning to include greater tolerance to aphids and pathogens. Specifically, we manipulated field soil with four treatments: (i) untreated, (ii) sterilized, (iii) sterilized with nitrogen fertilizer, and (iv) sterilized with rhizobia; we then assessed the direct effects on plant yields as well as tolerance to pea aphids (*Acyrthosiphon pisum*) and an aphid-borne pathogen, *pea enation mosaic virus* (PEMV). Peas grown in soil inoculated with rhizobia had fewer aphids and lower PEMV incidence, which had strong positive effects on plant yield. Structural equation models further showed that rhizobia inoculation outperformed synthetic nitrogen fertilization in improving pea tolerance to aphids and PEMV, and rhizobia in turn had greater benefits on yields than fertilizer. In contrast, sterilization of soil increased aphid abundance and PEMV incidence compared to rhizobia-inoculated treatments and decreased pea yields. Our results show that mutualistic soil microbes can exert strong effects on aboveground pathosystems by directly promoting plant growth and altering the tolerance of plants to insects and pathogens.

1. Introduction

Soil contains diverse microbe communities that mediate many aspects of plant growth (Van Der Heijden et al., 1998; Wall et al., 2010). Soil rhizobia, for example, are important microbes that fix atmospheric nitrogen into mineralizable forms accessible to plants (Avis et al., 2008; Kiers and Denison, 2008). Soil rhizobia also increase plant tolerance to abiotic stressors such as salt, drought, and heavy metal contamination (Weyens et al., 2009; Yang et al., 2009). Nitrogen provided by rhizobia also promotes synthesis of secondary metabolites with activity against pests (A'Bear et al., 2014). Yet, increased free amino acid availability in plants grown with rhizobia can increase herbivore fitness (Wilson and Stinner, 1984; Basu et al., 2021). Few studies have tested whether rhizobia affect pests aboveground, with conflicting results, with some showing reduced pest abundance and others the reverse (Brunner et al., 2015; Katayama et al., 2011).

Despite their role in mediating plant development and tolerance to pests, effects of rhizobia in mediating multi-trophic interactions aboveground are poorly understood (A'Bear et al., 2014; Bustos-Segura et al., 2024; Pineda et al., 2010). This is a key knowledge gap given the complex direct and indirect pathways by which rhizobia may affect plant tolerance to pathogens and also plant growth. For example, many insects that attack legumes are vectors of pathogens, and plants grown with rhizobia may have nutritional benefits such as increased amino acid availability that increase herbivore abundance (Wilson and Stinner, 1984; Basu et al., 2021). Yet, plants grown with rhizobia also often have greater levels of chemical defenses as well as physical defenses such as callose that limit pest feeding and virus transmission (Pineda et al., 2010; Basu et al., 2021). An experimental approach under realistic field settings that examines the role of rhizobia on pests, virus transmission, and plant growth would aid in unraveling direct and trait-mediated indirect pathways by which soil microbes affect insects, pathogens, and plant yield.

While benefits of rhizobia on plants are complex, it is often unclear

* Corresponding author.
E-mail address: dcrowder@wsu.edu (D.W. Crowder).

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whether they largely stem from increased nitrogen-fixation or whether the effects extend beyond what can be obtained with synthetic fertilization. Plants require resources to maintain mutualisms and will often not associate with rhizobia if synthetic nitrogen is available (Avis et al., 2008; Kiers and Denison, 2008). However, chemical by-products of rhizobia metabolism can induce tolerance to pests in plants, even in those that do not form nodules, suggesting benefits beyond nitrogen fixation (Reitz et al., 2002; Whitaker et al., 2014). More broadly, separating and quantifying the direct and indirect effects of rhizobia and fertilizer on plant growth and tolerance to stressors may improve our understanding of the mechanisms underlying legume-rhizobia symbiosis.

Here we conducted a field experiment to test effects of rhizobia and synthetic fertilizer on pea yield, aphids, and *pea enation mosaic virus* (PEMV), an aphid-borne pathogen (Basu et al., 2021; Chisholm et al., 2019). These organisms co-occur in the inland Northwest US, where peas are widely grown in rotation with wheat. We hypothesized rhizobia would affect aboveground interactions, and benefits to peas will extend beyond nitrogen provisioning. Testing this requires experiments to avoid confounding variables, with settings reflecting typical field conditions, all of which are limited in the literature. Our study tested four predictions: (i) rhizobia will increase plant tolerance to aphids and PEMV, (ii) rhizobia will increase aphid and PEMV tolerance more than nitrogen fertilizer, (iii) rhizobia will increase plant yields more than nitrogen fertilizer, and (iv) direct effects of rhizobia on plant yield will exceed indirect effects on yield that are mediated through impacts on aphids and PEMV. Alternatively, if maintenance of rhizobial mutualisms is energetically costly for plants (Wendlandt et al., 2025), we may observe higher aphid abundance and PEMV incidence on plants with rhizobia compared to controls or fertilized treatments.

2. Materials and methods

2.1. Field experiment

The field experiment was conducted in 2019 at the Washington State University Spillman Farm in Pullman, WA. The site had Palouse silt loam soil (fine-silty, mixed, superactive, mesic Pachic Ultic Haploxeroll) and a history of pea and wheat rotation. No fertilizer was added after winter wheat harvest in 2018, so soils were nitrogen poor. To initially assess if pea-specific rhizobia were present, we collected 8 soil samples using a post-hole digger from 8 plots measuring 1-m^2. For each plot, subsamples were homogenized and divided in thirds, with one sample from each plot subjected to each of three treatments: (i) control, (ii) sterilized, or (iii) sterilized with added rhizobia. Sterilized treatments involved placing soil in 61 × 91 cm polypropylene autoclave bags in a steam autoclave at 7 psi/111 °C for 8 h. For the sterilized with added rhizobia treatment, seeds were inoculated with pea-specific *Rhizobia* (*R. leguminosarum* biovar. *viciae*) using N-Dure, a peat-based inoculant (Verdesian Life Sciences, Cary, NC) via a slurry method (Deaker et al., 2004). After treatment, soil was mixed at a 1:1 ratio with sand and put in 2.6-L pots. Two pea seeds were planted in each pot, and thinned to one after emergence. After emergence, plants were grown for an additional 50 d in greenhouses (16:8 h light:dark; 21–24 °C during light; 16–18 °C during dark), which allowed them to complete full development. After this period plants were removed, soil adhering to the root system was washed off, and nodules were counted. This trial revealed three major factors associated with the experiment: (i) plants grown in sterilized soil without rhizobia did not produce nodules (mean = 0, SD = 0), (ii) plants grown in sterilized soil with rhizobia added had the most nodules (mean = 28.20, SD = 10.40), and (iii) rhizobia were present in field soil, but plants in untreated soil had fewer nodules than treatments where seeds were rhizobia-inoculated (mean = 11.60, SD = 6.23) (Post-hoc, $t_{21} = -3.32$; $P < 0.01$) (Fig. S1).

After our preliminary trial showed the treatments were effective, our field experiment was conducted using a randomized block design with 8 blocks. Each block had 4 replicates, with one of each of four treatments: (i) untreated field soil, (ii) sterilized field soil, (iii) sterilized field soil with added nitrogen fertilizer and (iv) sterilized field soil with added rhizobia. The experimental area was established by digging 32 holes in a 4 × 8 grid with 1-m spacing (Fig. 1). A plastic 68-L tub (0.6 × 0.3 × 0.4) was inserted in each hole to allow for adding back treated soil in a contained arena (Fig. 1); each tub had 8, 1-cm^2 holes in the bottom to allow drainage, a setup up that allows peas to perform well (Basu et al., 2021). Autoclaved and rhizobia treatments were performed as described earlier. Fertilizer treatments received ammonium nitrate fertilizer at 90 kg N ha^{-1}, a standard commercial rate. Because fertilizer can suppress legume-rhizobia symbiosis (Harper and Gibson, 1984), fertilized plots indicated plants that obtain nitrogen mostly from fertilizer rather than rhizobia. Treated soil was added back to tubs 48 h later, with each container having the same amount. 60-cm^3 mesh cages were erected over tubs to create enclosed mesocosms.

Peas (*Pisum sativum* cv Banner) were initially planted on 22 April 2019 in a greenhouse (16:8 h light:dark; 21–24°C during light; 16–18°C during dark). Peas were grown from seed in Sunshine Mix LC1 media (Sun Gro, Agawam, MA); prior work shows the media does not have rhizobia and plants do not nodulate. Plants were watered regularly from the tap and transplanted to field mesocosms on 13 May, a typical planting date. Twenty-five plants were transplanted to each mesocosm ≈ 10 cm apart in a 5 × 5 grid (Fig. S2), a commercial farm density. Because steam from autoclaving adds moisture to soil, water was added to non-sterilized soil until soil moisture across plots was equal (measured using soil moisture sensors). All plants were watered after transplanting to aid establishment, but after received no additional water for the duration of the experiment, as peas are rainfed in the area. Monthly rainfall for May, June, July, and August 2019 was 31, 23, 2.3, and 16 mm, respectively. Monthly average high temperatures for May, June, July, and August were 20.6, 24.1, 27.7, and 29.5 °C respectively, and monthly average low temperatures for May, June, July, and August were 6.44, 9.11, 9.78, and 10.9 °C, respectively.

After plants had 4 weeks to establish (10 June), 25 PEMV-infectious *A. pisum* individuals were released in each cage onto a single plant using a paint brush. The colony of *A. pisum* used for these releases was maintained on peas in greenhouses and originated from a field-collected population. On 8 July, the terminal leaflet was clipped from a predefined 9 plants from each cage (Fig. S2) to be tested with PCR for PEMV using a published diagnostic procedure (Basu et al., 2021). Briefly, homogenized tissue (100 mg) was used for total RNA extraction using Promega SV total RNA isolation kits (Promega, Madison, WI), and cDNA from 1 µg of total RNA using Bio-Rad iScript cDNA synthesis kits (Bio-Rad, Hercules, CA). PEMV-1 specific primers were used in qRT-PCR reactions

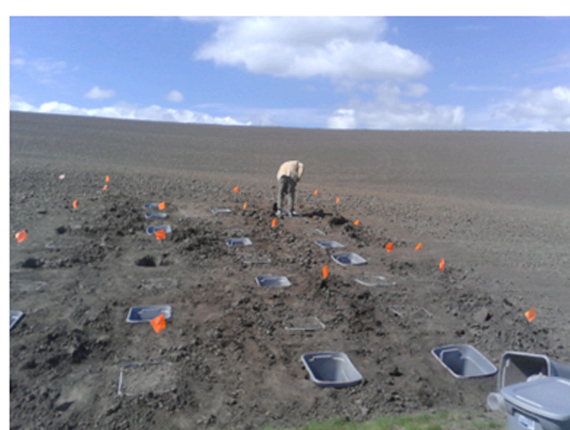

Fig. 1. Photo showing the process of putting tubs into holes on the 0.1-ha study site at Spillman Farm at Washington State University in Pullman, WA, and then filling with treated soil.

(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 Psβ-tubulin as a housekeeping gene (Basu et al., 2021).

To assess aphid population abundance, aphids were sampled at the height of the population size on 15 July using a DVAC suction sampler (Rincon-Vitova, Ventura, CA). Cages were lifted off each mesocosm, aphids were collected and then cages were replaced. The number of aphids was then counted. This time period reflects aphid population dynamics over the season; aphid populations were significantly reduced by 5 August as plants dried out, and final aphid counts would not have been informative of peak population size. Peas were harvested by hand on 12 August, hulled and dried in an oven for 5 d at 37°C before weighing. Plant roots were also carefully washed in tap water before the number of nodules present on each plant was counted.

A composite soil sample (0–40 cm) was taken from each mesocosm after the experiment and sent to Soiltest Consultants (Moses Lake, WA, USA) for nitrate (NO_3^-) and ammonium (NH_4^+) analyses; NO_3^- was tested with a chromotropic acid method and NH_4^+ with the salicylate method (Miller et al., 2013). Microbe respiration was measured using a Solvita 24-h CO_2 burst test (Haney et al., 2008). Briefly, the test applies water to dried soil to trigger a rapid release of CO_2 as microbes rapidly assimilating freshly wet soil organic matter. Quantity of CO^2 released in the 24-h "burst", by which time the reaction reaches a saturation point, is one measure of a soil's maximal biological activity (Haney et al., 2008). Each analysis was also performed on a subset of sterilized (n = 6) and control (n = 6) soil to assess effects of autoclaving on soil properties.

2.2. Data analysis

All analyses were conducted in R v. 4.2.3 (R Core Team, 2023). We modeled aphid abundance, PEMV prevalence (number of infected plants), and yield (dry mass), as a function of the four soil treatments using linear mixed models (LMM) and generalized linear mixed models (GLMMs) assuming a normally distributed random intercept of experimental block to account for the spatial design. Model diagnostics led to selection of negative binomial (GLMM), Poisson (GLMM), and Gaussian (LMM) error distributions for aphid abundance, PEMV prevalence, and yield, respectively. We conducted Šidák tests with a multiple comparison correction using the *emmeans* package to identify significant differences among the treatments.

As we hypothesized that changes in yield may be affected not only by soil treatment but also by aphid abundance or PEMV severity, we examined correlations between these responses. Pearson correlation analysis revealed strong correlations among yield, aphid abundance, and PEMV prevalence (see Results; Figs. 2 and 3), so that generalized linear models were unable to properly assess if yield differences were directly due to soil treatment or indirectly mediated through variation in aphid abundance or PEMV incidence across treatments (Clark et al., 2019). Consequently, we used local estimation structural equation models (piecewiseSEM package) (Lefcheck, 2016) to identify direct and indirect links between these variables. Structural equation modeling is a powerful tool that allows for the separation of direct and indirect effects that works well when analyses involve multiple response variables that may be tightly correlated (Lefcheck, 2016).

The function of structural equation models relies on the variance-covariance matrices of continuous predictor and response variables (Lefcheck, 2016). To incorporate categorical soil treatments, we used a coding matrix that approximated soil treatments as continuous

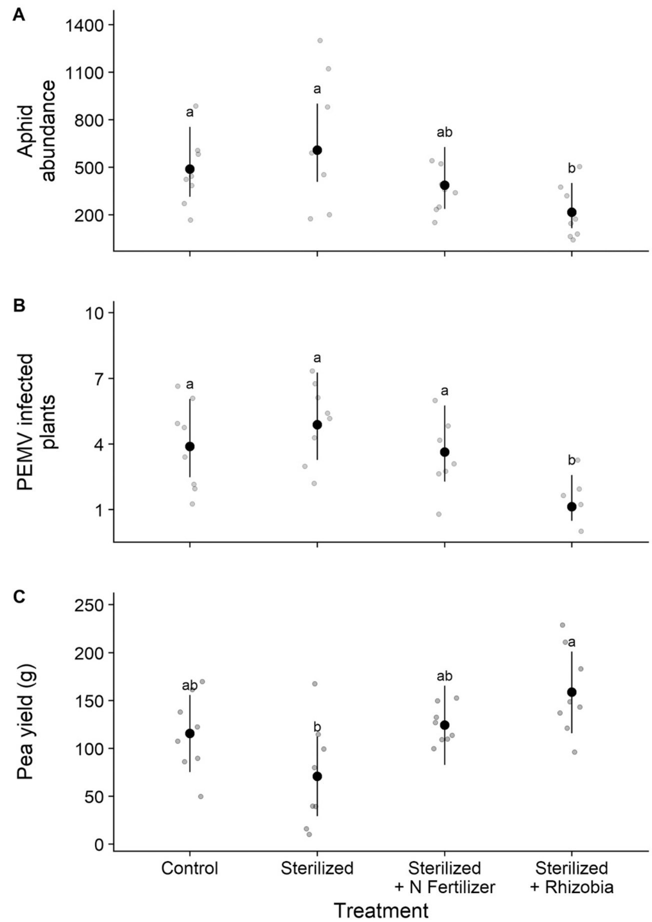

Fig. 2. Effects of the soil treatments on (A) aphid abundance, (B) the number of Pea enation mosaic virus (PEMV) infected plants, and (C) pea yield (g/plant). Small grey points are the raw data, solid black points are estimated marginal means and whiskers are 95 % confidence intervals from GLMMs (A & B) and LMMs (C). Different letters above the whiskers denote significant differences among the treatments (Sidak, α = 0.05).

variables. This coding matrix was designed *a priori* to model selection and reflected the condition of soil treatments moving linearly from "sterilized" to the "rhizobia-treated" condition. In the cases of sterilized and sterilized plus nitrogen fertilization treatments, this matrix was set to "0" to reflect no rhizobia. As the control soil was not sterilized, it contained some naturally occurring rhizobia, which was confirmed before the experiment. However, naturally occurring rhizobia differ from commercially available inoculum, both in genetics and concentration and the plant's ability to form symbiotic nitrogen-fixing associations (Thilakarathna and Raizada, 2017). Our greenhouse study showed significantly higher nodulation for plants in treatments with rhizobia inoculation compared to control untreated soil (see Fig. S1; *Field experiment* section). For these reasons, we represented the rhizobia treatment in models as 0 for sterilized soil and sterilized + nitrogen fertilizer soil, 1 for control soil, and 2 for sterilized + rhizobia soil.

Using these parameters, we built our initial structural equation model to reflect complete mediation, where soil predictors affected aphids, aphids affected PEMV prevalence, and PEMV prevalence affected yield (Fig. S3). Our model selection then combined knowledge from our hypotheses about indirect effects of soil treatments on PEMV and pea yield with tests of directed separation to identify partial mediation linkages that improved model fit and were theoretically supported (Lefcheck, 2016). Model fit was assessed by AIC and Fisher's C (Shipley, 2009).

For the subset of soil samples analyzed immediately after

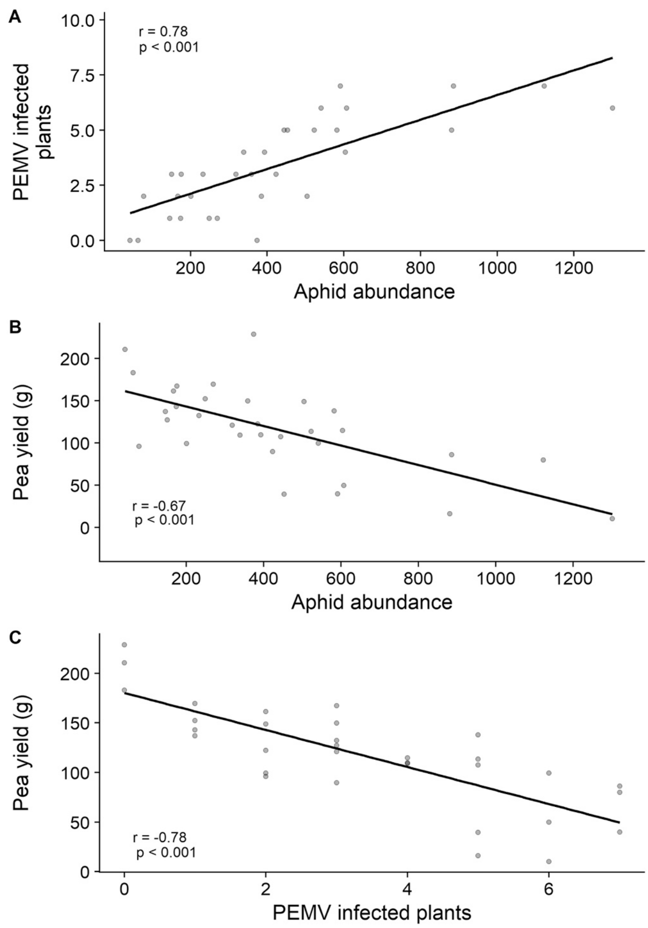

Fig. 3. Observed correlations between (A) aphid abundance and PEMV incidence, (B) aphid abundance and pea plant yield, and (C) PEMV incidence and pea plant yield.

autoclaving, NO_3^- content, NH_4^+ concentration, and microbial respiration were assessed to compare control and sterilized soils using Welch's two-sample *t*-tests. Similar analyses were conducted for the soil from each replicate at the end of the experiment using ANOVA as there were four treatments (control, sterilized, sterilized + rhizobia, sterilized + nitrogen fertilizer) and the response variables met parametric assumptions. Posthoc means separation was conducted with Šidák tests.

3. Results

3.1. Effects of soil treatments on aphids, PEMV, and yield

In accordance with our pre-experiment trial, all plants from the experiment grown in the untreated field soil (control treatment) produced nodules (mean = 17.50, SD = 3.55), although not as many as in sterilized + rhizobia treatments (mean = 28.00, SD = 12.30) (t_8 = −2.33; $P < 0.05$) (Fig. S4); plants in sterilized and sterilized + nitrogen fertilizer treatments did not produce any nodules (Fig. S4). In addition, the soil tested immediately after autoclaving had 93 % lower microbial respiration than controls (t_{10} = 16.9, $P < 0.0001$) (Fig. S5), indicating autoclaving was largely effective at removing naturally occurring microbes.

In the field experiment, soil treatments had highly significant effects on aphid abundance (χ^2 = 13.6, df = 3, P = 0.0030), PEMV incidence (χ^2 = 15.8, df = 3, P = 0.0010) and pea yield (χ^2 = 17.7, df = 3; $P < 0.001$) (Fig. 2). Yields ranged from 10.2 to 228.9 g/m^2 (mean = 117.3, SE = 9.01), lower than regional averages on commercial farms of 180–270 g/m^2 (Schroeder et al., 2018). Plants grown in sterilized soil had the lowest yield (Fig. 2). In contrast, adding rhizobia to sterilized soil resulted in the fewest aphids and the lowest PEMV incidence, while producing the highest yield (Fig. 2), indicating strong positive direct and indirect effects of rhizobia.

All of our response variables were significantly correlated (Fig. 3). PEMV incidence was positively related to aphid abundance (r = 0.78, $P < 0.001$), and yield was negatively correlated with aphid abundance (r = -0.67, $P < 0.001$) and PEMV incidence (r = -0.78, $P < 0.001$) (Fig. 3). In turn, structural equation models more precisely determined if soil treatments directly affected yield, or if effects on yield were indirectly mediated by aphids or PEMV. Our initial model with only direct links (treatments → aphids → PEMV → yield) had relatively poor fit (Fisher's C_{14} = 23.5, P = 0.052, AIC = 4591). As we hypothesized treatments may indirectly affect both PEMV incidence and yield, we used d-separation tests to show that two out of seven links representing these indirect pathways were missing in the initial model. Incorporating these links improved the model fit drastically (Fisher's C_{12} = 8.79, P = 0.72, AIC = 4583) (Fig. 4). Only one direct link from the initial model (sterilized → aphids) was non-significant and its removal did not alter model fit or interpretation. For parsimony, we excluded this link from the final model (Fig. 4).

The final structural equation model supported negative effects of the nitrogen fertilization and rhizobia treatments on aphid abundance, although the rhizobia effect had greater magnitude ($\beta_{nitrogen} = -0.39$, $P < 0.001$; $\beta_{rhizobia} = -0.64$, $P < 0.001$). Aphid abundance directly increased PEMV incidence ($\beta_{aphids} = 0.42$, $P < 0.001$), and both nitrogen fertilizer and rhizobia treatments had an indirect negative effect on PEMV mediated by lower aphid abundance. However, the rhizobia treatment also had a direct negative effect on PEMV incidence that was independent of aphid abundance ($\beta_{rhizobia} = -0.49$, P = 0.011) (Fig. 4). Pea yield was negatively affected by both PEMV incidence ($\beta_{PEMV} = -0.68$, $P < 0.001$) and the sterilized soil treatment ($\beta_{sterilized} = -0.26$, P = 0.039) (Fig. 4). These results suggest rhizobia increased plant tolerance to biotic stressors, and pea yield, in ways that extended beyond what was provided by nitrogen alone.

3.2. Relationship between soil treatment and soil properties

Although levels of NO_3^- ($t_{10} = -0.97$, P = 0.36) and NH_4^+ (t_{10} = 1.28, P = 0.25) measured immediately after soil was sterilized were not different from untreated soil, soil at the end of the experiment was affected by treatments (Fig. 5). Soil NO_3^- varied significantly with soil treatment (F_3 = 33.5, $P < 0.001$) and was highest in the sterilized + nitrogen fertilizer treatment (Fig. 5A). Soil NH_4^+ levels also varied

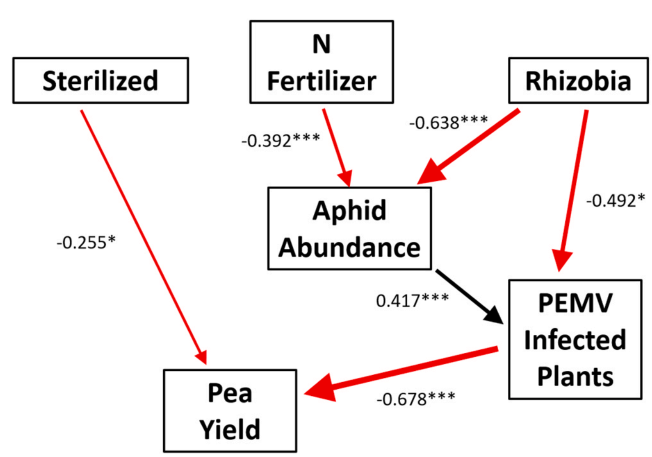

Fig. 4. Path diagram of the best fit structural equation model, where all paths are significant. Red arrows indicate negative paths, whereas black arrows indicate positive paths. Standardized path coefficients are shown, and significance level is denoted by asterisks: * $P < 0.05$, * ** $P < 0.001$.

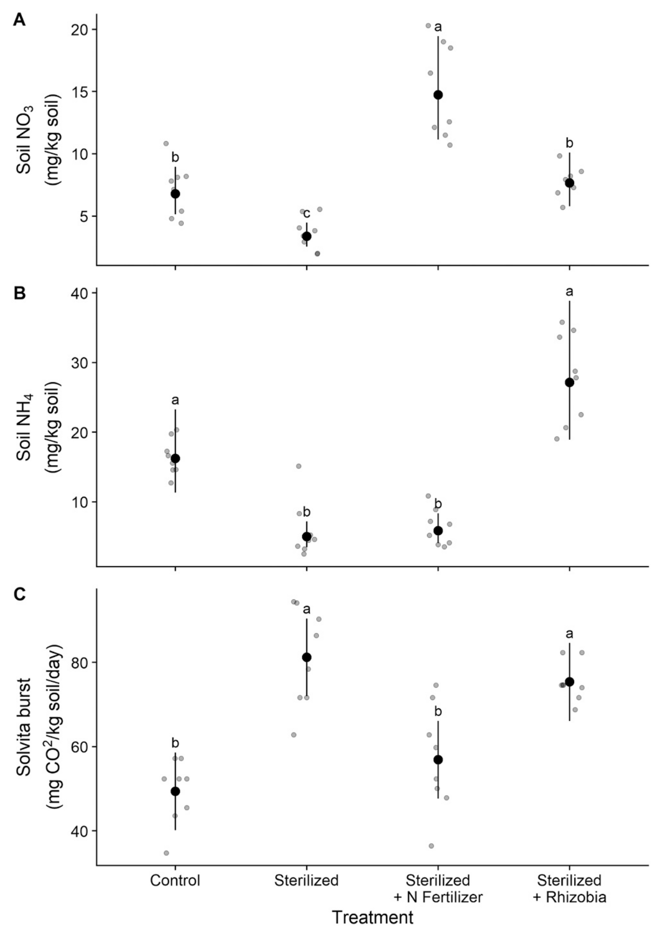

Fig. 5. Effects of the soil treatments on soil properties at the end of the experiment, including (A) NO_3^- (mg kg^{-1} soil), (B) NH_4^+ (mg kg^{-1} soil), and (C) microbial respiration (mg CO_2-C g^{-1} soil). Small grey points are the raw data, solid black points are estimated marginal means and whiskers are 95 % confidence intervals from one-way ANOVAs. Different letters above the whiskers denote significant differences among treatments (Sidak, α = 0.05).

significantly with treatment ($F_3 = 36.2$, $P < 0.001$) and were highest in the sterilized + rhizobia treatment. The untreated control soil also had significantly higher NH_4^+ levels than the sterilized and sterilized + nitrogen fertilizer soils (Fig. 5B). Microbial respiration varied significantly with soil treatment ($F_3 = 17.1$, $P < 0.001$) with sterilized soil having the lowest levels and sterilized + rhizobia soil having the highest levels (Fig. 5C).

4. Discussion

Our results supported three of our four initial predictions, in that rhizobia boosted plant yield while also increasing tolerance to aphids and PEMV, and that these benefits exceeded what was provided by nitrogen fertilizer. At the same time, manipulation of soil microbes by autoclaving greatly decreased plant yield. The effects of added rhizobia and nitrogen fertilizer on yield were primarily indirect, and mediated by negative impacts of these additions on either aphids and/or PEMV. However, the benefits of rhizobia on pea yield exceeded those of nitrogen fertilizer for two primary reasons. First, the addition of rhizobia reduced aphid abundance more than the addition of nitrogen fertilizer. Second, rhizobia had a direct negative impact on PEMV that was independent of aphid abundance, while the nitrogen fertilizer treatment did not.

Our trials showed a major benefit of rhizobia and nitrogen fertilizer was a negative impact on aphid abundance. This was somewhat surprising, as terrestrial ecosystems are often nitrogen-limited, and added nitrogen can benefit herbivores (Butler et al., 2012; Elser et al., 2000). Yet, nitrogen fertilizer can alter the protein:carbohydrate ratio of plants in ways that reduce herbivore performance (Le Gall et al., 2020). Moreover, nitrogen fertilizer tends to have more minimal effects on herbivore fitness in the field than the lab, with natural environments buffering benefits of fertilization on herbivores (Butler et al., 2012). Uptake of atmospheric nitrogen by rhizobia also increases defensive metabolites and toxic alkaloids in plants that reduce herbivore growth, and these effects are not seen with fertilizer (Basu et al., 2022; Campbell and Vallano, 2018; Irmer et al., 2015). Additionally, aphids that feed on pea plants with rhizobia have reduced uptake of key amino acids (Basu et al., 2022). While our mesocosms prevented colonization, other studies show addition of rhizobia can promote ant abundance and 'tending' of aphids (Keller et al., 2018); rhizobia can also increase nectar and benefit parasitoids (Bustos-Segura et al., 2024). For these reasons, benefits of rhizobia on aphid suppression and crop yield might be greater if we conducted our experiment in an open field where ants and parasitoids were present.

While nitrogen fertilizer reduced PEMV indirectly by reducing aphid abundance, rhizobia reduced PEMV both indirectly and directly. The lack of direct effects of nitrogen fertilizer on PEMV are not surprising, as reviews show fertilizer often has limited impacts on pathogens (Mur et al., 2017). In contrast, although rhizobia have been shown to decrease susceptibility of plants that were mechanically inoculated with a virus in a lab (Avis et al., 2008), our study is among the first to show effects of rhizobia on viral incidence in a field setting. Rhizobia can increase plant physical defenses, which might reduce herbivore penetration and virus transmission even without changes in vector abundance (Basu et al., 2021, 2022). Inoculation of plants with rhizobia also often increases salicylic acid hormone levels, a key defense pathway (Basu et al., 2021). The greater physical and chemical defense in rhizobia treatments may reduce PEMV incidence.

Our study did reject one of our initial predictions, which was that the direct benefits of rhizobia on plant yield would exceed the indirect benefits. There may be several key reasons for this finding. First, aphids and PEMV are highly damaging to pea yields, with studies showing that PEMV can reduce yields by over 40 % even when aphids are not highly abundant (Elbakidze et al., 2011); the indirect effects of reducing these stressors may thus exceed any direct benefits on yield. Second, potential costs for plants that associate with rhizobia may counteract any of the yield benefits. Plants have to expend resources to manage mutualisms and to 'sanction' poorly-performing microbes (Kiers and Denison, 2008; Wendlandt et al., 2025); these factors may cause tradeoffs between microbial benefits to yield and resources used to manage the mutualisms. Third, there is often a tradeoff between plant defenses against biotic stressors and plant growth (Huot et al., 2014). It is possible that the increased plant defenses promoted by rhizobia resulted in a lack of effect on plant growth (Huot et al., 2014) and rhizobia may have greater direct impacts on yield in environmental contexts where biotic stressors are not prevalent.

Immediately after treatments were established, autoclaving reduced microbial respiration without changing $NO_3^-$and NH_4^+ levels. However, at the end of the experiment, soil that had added fertilizer had significantly higher levels of NO_3^- than other treatments, while treatments with rhizobia (both untreated field soil and sterilized soil with added rhizobia) had significantly higher levels of NH_4^+. The higher levels of NO_3^- in the fertilizer treatment were not a surprise, since this treatment received ammonium nitrate fertilizer, with some of the residual nitrate in the soil likely coming from the fertilizer, and some ammonium in the fertilizer being converted to nitrate by nitrification in the soil (Brady and Weil, 2008). However, the NO_3^- levels in all four treatments were within the range of normal concentrations seen in agricultural soils (2–15 mg kg^{-1} soil) (Collins, 2012; Sullivan et al., 2021). Additionally, a defining feature of rhizobia-legume symbioses is the secretion of NH_4^+ by rhizobia (Patriarca et al., 2002; Schulte et al., 2021), which was reflected in the soil with added rhizobia having the highest levels of NH_4^+ and well above typical concentrations seen in agricultural soils (Horneck et al., 2023; Sullivan et al., 2021). Moreover, the rhizobia-treated soil had the highest microbial respiration and appeared to have benefits for both tolerance to aphids and PEMV as well as pea yield. This evolved symbiosis between rhizobia and legumes may thus promote benefits more than nitrogen alone.

5. Conclusions

Overall, our study adds to the body of literature that suggests that factors influencing plant yield are complex and interdependent. Insect herbivores, soil nutrition, plant pathogens, and soil-borne microbes may impact yield directly and by influencing each other through indirect pathways. Consequently, the net influence of soil composition on plant yield may be highly dependent on ecological context, such as the relative prevalence of pests and pathogens in the agroecosystem. The relative economic importance of these pest, pathogen, and other threats also varies between cropping systems, and threats may be more damaging in certain contexts. In turn, discerning the relationships between these different factors is key to understanding the response of plants to varying levels of soil nitrogen availability. Regardless, our results suggest that soil-inhabiting microbes such as rhizobia might provide considerable benefits to plant health and productivity both directly and by indirectly reducing the impacts of multiple aboveground stressors.

CRediT authorship contribution statement

Charlton Akaisha: Writing – review & editing, Methodology. **Chisholm Paul:** Writing – original draft, Methodology, Formal analysis, Conceptualization. **Crowder David:** Writing – review & editing, Supervision, Resources, Conceptualization. **Oeller Liesl:** Writing – review & editing, Project administration. **Anderson Riley:** Writing – review & editing, Formal analysis. **Reganold John:** Writing – review & editing, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2025.109552.

Data availability

Data will be made available on request.

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