

Article

Effects of Soil Rhizobia and Drought on Plant–Vector–Pathogen Interactions on a Legume Host

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Abstract

Symbiosis between rhizobia and legumes can affect plant tolerance to abiotic and biotic stressors such as drought and herbivores. Yet few studies have assessed how soil rhizobia impact plants that face abiotic and biotic stress simultaneously. This is a major knowledge gap given that many aspects of plant growth and defense are affected by interacting stressors, and these interactions can affect legume–rhizobia symbiosis. Here we assessed rhizobia-mediated interactions between a legume host (*Pisum sativum*), a vector herbivore (pea aphid, *Acyrtosiphon pisum*), a plant virus (*pea enation mosaic virus*, PEMV), and soil water availability. We show that rhizobia promoted plant growth and mitigated osmotic stress caused by reduced soil water availability. Rhizobia also increased plant tolerance to PEMV under high soil moisture conditions but had no measurable effect on PEMV when plants were grown in soil with reduced water. To assess the mechanisms that mediated these complex interactions, we measured gene transcripts related to phytohormone signaling and found that salicylic acid, jasmonic acid, abscisic acid, and ethylene signaling were affected by interactions between rhizobia and water availability; each of these pathways affects PEMV transmission. Our study shows that beneficial effects of rhizobia on legumes are impacted by abiotic and biotic stress, and outcomes of symbiosis may be context-dependent in field settings.

Keywords: soil rhizobia; drought; pea aphid; PEMV; defensive genes; legume host



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1. Introduction

Soil microbes play a key role in agricultural ecosystems by altering plant responses to abiotic stressors such as drought, salinity, and nutrient deficiency [1,2]. Soil microbes also affect plant tolerance to biotic stressors that include plant pathogens and herbivores, by priming plant defenses and inducing systemic resistance [3,4]. Soil microbes in turn often increase biomass production across entire plant communities, with consequences for ecosystem health and crop productivity [5]. At the same time, both abiotic and biotic stressors can alter the structure and function of root-associated microbial communities and plant–microbe mutualisms to the detriment of plant health [6–8].

Soil rhizobia are a diverse group of soil bacteria that form specialized nodules on legume roots. Rhizobia fix up to 60 million metric tons of atmospheric nitrogen each year in global agroecosystems and are a primary reason that legumes are grown in rotation with cereal crops worldwide [9]. Symbioses between legume hosts and rhizobia often also

promote tolerance of legumes to pathogens by altering physical and chemical defenses and by heightening the efficacy of plant hypersensitive responses via programmed cell death [10–12]. However, abiotic and biotic stressors can hamper symbioses between rhizobia and legumes and, therefore, nitrogen fixation. For example, viral pathogen infection inhibits root nodule formation [11,13], while drought alters legume root architecture and nodules in ways that decrease atmospheric nitrogen fixation [14,15]. Yet, although plants are commonly attacked by multiple abiotic and biotic stressors at the same time in both natural and agricultural systems, relatively few studies have assessed the effects of soil rhizobia on protecting plant hosts from diverse combinations of stressors [16].

We predict that the interactions among legume plant hosts and rhizobia may affect plant tolerance to both abiotic and biotic stressors through plant-mediated mechanisms, as soil rhizobia can alter plant defenses against herbivores or pathogens by regulating phytohormones such as salicylic acid, jasmonic acid, and ethylene [5]. However, as plants produce many of the same hormones in response to abiotic and biotic stress [17,18], rhizobia may alter tolerance to abiotic and biotic stressors concurrently. Similarly, enhanced colonization of hosts by rhizobia has been observed in plants with reduced salicylic acid hormone levels, which often occurs on plants not under attack by pathogens [19,20]. Drought also affects plant tolerance to pathogens and herbivores by inducing phytohormone signaling [21]. These studies suggest interactions among plant hosts, rhizobia, and both abiotic and biotic stressors should be mediated by plant-mediated pathways. However, few studies have considered the diverse molecular mechanisms underlying interactions among multiple stressors, microbial mutualists, and legume hosts.

Here, we examined interactions among a legume host (pea, *Pisum sativum*), soil rhizobia (*Rhizobium leguminosarum* biovar. *viciae*), a vector herbivore (pea aphid, *Acyrtosiphon pisum*), and a vector-borne virus (pea enation mosaic virus, PEMV). These species commonly co-occur in the inland Western United States (Washington, Idaho, Montana). In experiments, we assessed how interactions among the biotic organisms affected plant defense and PEMV transmission in environments with or without reduced water. Through a combination of manipulative experiments and gene expression studies, we show that the benefits of rhizobia for plant tolerance to biotic stressors are affected by water stress. Our study suggests that the outcomes of plant–microbe symbioses are affected by complex interactions between abiotic and biotic stressors, both above- and belowground.

2. Materials and Methods

2.1. Study System

Across much of Eastern Washington and Northern Idaho States of the USA, dryland agricultural systems are dominated by cereal and legume crops. In legumes such as pea, lentil, and garbanzo bean, pea aphids, PEMV, and rhizobia co-occur. Populations of *A. pisum* emerge in the spring and feed on weedy legumes such as clover and vetch, before migrating to legume fields when they become suitable. The pea aphid is the primary vector of PEMV, and when this pathogen reaches high levels in peas, yields can be reduced by 40% or more [22]. Peas associate with rhizobia, and these interactions can affect the tolerance of pea plants to aphids and PEMV [12,23]. However, peas are grown across regions with highly variable precipitation, from Central Washington where precipitation averages only 20 cm a year to regions with 50 cm or more [12]. Variation in water availability might not only affect pea–rhizobia mutualisms directly but might also affect interactions with aphids and viruses through plant-mediated mechanisms [17,18].

Here, we assessed whether interactions between soil rhizobia and plant hosts were affected by pea aphid vectors, PEMV infection, and soil water availability. All aphids were reared in greenhouses on *P. sativum* host plants (cv. Banner) that were kept at 16:8 h

light/dark with 22:17 °C light/dark. We reared both viruliferous and non-viruliferous *A. pisum* colonies. Both these colonies originated from the same field population of viruliferous *A. pisum* in 2012, which has been supplemented with additional aphids over time to maintain genetic diversity. To create the non-viruliferous colony that had the same genetics as the viruliferous colony (but without the PEMV pathogen), 50 viruliferous *A. pisum* adults were put in Petri-dishes for 3 d. Because the PEMV virus is not transmitted from adult to offspring, all *A. pisum* nymphs born were non-viruliferous [22]. After the initial non-viruliferous offspring were produced, they were reared on uninfected plants.

2.2. Experimental Design and Data Collection

Our experiment was conducted in greenhouses with plants grown in potting mix soil (Sunshine[®] LC1, Sungro Horticulture, Agawam, MA, USA). The design was a $2 \times 2 \times 3$ factorial with treatments applied sequentially at different times. There was a total of 144 replicates per block, with 12 replicates for each of the 12 treatments in the $2 \times 2 \times 3$ design. For the first 2 wk, 72 of the initial 144 plants were inoculated with soil rhizobia; the other 72 were uninoculated. We inoculated the potting soil mixture with pea-specific rhizobia (*R. leguminosarum* biovar. *viciae*) by mixing N-Charge, a peat-based inoculant, with *P. sativum* seeds following the manufacturer's protocol (Verdasian Life Sciences, Cary, NC, USA). Following treatments, plants were grown for 2 wk in a greenhouse (conditions as before) with 75% soil moisture. To monitor soil moisture levels, soil was measured twice a week by taking a soil fresh weigh sample, drying it for 48 h at 100 °C to remove any moisture, after which soil dry weight and soil moisture water content was measured. Water was added at a rate of approximately 50 mL every 2 days to achieve 75% soil moisture.

After 2 wk of plant growth, plants in both the rhizobia and no-rhizobia treatments were further randomly sub-divided into full and low soil water availability treatments, with 36 plants in each rhizobia \times water availability combination. In the full soil water availability treatment, the soil moisture content was maintained at 75% soil moisture per pot (10 \times 10 \times 10 cm), whereas the soil moisture for the low water treatment was maintained at 38% [21,24]. During the experiment, fully watered plants received the amount of water lost from fully watered soil after drying (50 mL) every 2 days to maintain a 75% soil moisture, and the low water treatment was set at 25 mL of water every 2 days to maintain 38% soil moisture. In preliminary experiments we found that water levels below 38% soil moisture induced severe stress of plants and was unfeasible. Water treatments continued for 1 wk, after which plant height and number of nodes were measured before aphid treatments were applied. We added water every other day until final samples were collected.

The final sub-division of plants assessed effects of pea aphids and PEMV on plant traits (Figure 1) with treatments: (i) no *A. pisum*, (ii) sham: ten 5-day-old non-viruliferous adults that fed for 24 h, and (iii) PEMV: ten 5-day-old PEMV-viruliferous adults that fed for 24 h. Plants from rhizobia and water availability treatments were each randomly allocated into aphid treatments, with 12 plants in each unique rhizobia \times water \times aphid treatment (144 plants total in each block). After feeding, *A. pisum* was removed using an aspirator. All treatments were conducted on individual *P. sativum* plants in mesh dorms (0.6 \times 0.6 \times 0.6 m). After insects were removed, plants were allowed to develop infection for 3 d before we harvested tissue to assess viral titer, defense expression, and 'osmotic potential' (i.e., water availability). Three days is a suitable amount of time for plants to develop symptoms from PEMV after inoculation by aphids and allowed us to compare results to prior studies [12,22,23]. Tissue from the aboveground plant was collected, flash frozen, and stored in a -80 °C freezer. The experiment was repeated in two blocks.

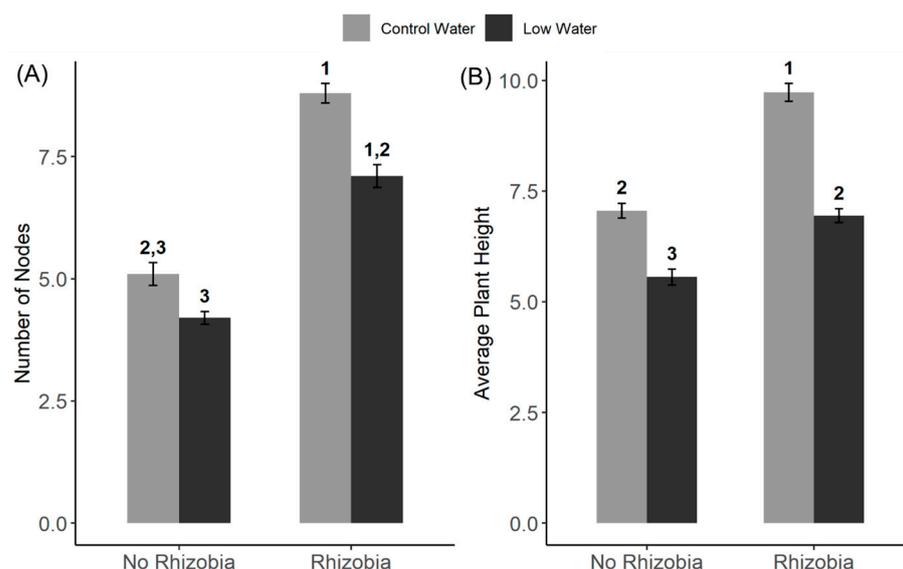


Figure 1. Effect of rhizobia and water treatments on (A) number of plant nodes and (B) plant height. Bars and error bars were based on predicted marginal means and standard errors ($n = 24$ per treatment). Bars labeled with different numbers (1, 2, 3) are significantly different (Tukey HSD).

For measuring PEMV-1 and PEMV-2 titer, plant tissue samples were wrapped in foil, frozen in liquid N₂, and snap chilled in dry ice. Samples were then ground into a powder in liquid N₂ with sterilized mortar and pestles. A quantity of 50–100 mg of homogenized tissue was used for total RNA extraction with Promega SV RNA kits (Promega, Madison, WI, USA), where cDNAs were synthesized from 1 µg of total RNA using Bio-Rad iScript cDNA kits. To conduct the qRT-PCR, we used PEMV-1 and PEMV-2 specific primers (Table 1). Reactions had 3 µL of ddH₂O, 5 µL of iTaq Univer SYBR Green Supermix, 1 µL of diluted primer mix (forward and reverse 10 µM), and 1 µL of diluted (1:25) cDNA template. The qRT-PCR procedure had 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. We used a dissociation step to create the melting curves (55 °C for 10 s, and then 0.5 °C for 10 s until 95 °C). The accumulation of PEMV-1 at two time points (3 and 7 d after infection) were calculated using the delta–delta Ct method [11,22].

Table 1. Primers used in this study.

Gene	Primer Sequences	NCBI No.	Amplicon Size (bp)
PEMV-1 FP PEMV-1 RP	GCAATCCTACAGGACCTTCATA CTCATCGTCTCCGTGTCATC	HM439775.1	121
PEMV-2 FP PEMV-2 RP	TGCTAGGAGAGGTGGAGATATG GCAATTGAGTAGGGTGGGTA AAA	JF713436.1	130
PsPR1 FP PsPR1 RP	TGGGGCAGTGGTGACATAAC TGCGCAAACAACCTGAGTA	LT635896	178
PsLox2 FP PsLox2 RP	GCAACCAAGTGACGAAGTCTA GGAGACCCGATTGTAAGGTATTT	PsCam 059875	95
PsACS2 FP PsACS2 RP	GGCATAGTAATTTGAGGTTGAGCC GCCCAACATTTAAAGGACCTATTA	AF016459	103
PsAO3 FP PsAO3 RP	TTATAGGACACAGGCTAGCTCAGCA TGACACAAGCTTATTCAGCATGACA	EF491600.1	127
Psβ-tubulin FP Psβ-tubulin RP	GTAACCCAAGCTTTGGTGATC ACTGAGAGTCCTGTACTGCT	X54844.1	203

To assess effects of phytohormones on interactions between rhizobia, *A. pisum*, PEMV, and soil water availability, we measured relative expressions of four gene transcripts in plants harvested 3 d after aphids were applied. The gene *Pathogenesis-related protein 1 (PR1)* is associated with salicylic acid and triggers systemic acquired resistance—mediated defense against plant pathogens. The gene *Lipoxygenase 2 (LOX2)* is expressed upstream of jasmonic acid biosynthesis to promote herbivore defense [23]. We also assessed *1-aminocyclopropane-1-carboxylic acid synthases 2 (ACS2)* and *Aldehyde oxidase 3 (AO3)*, which are genes associated with ethylene and abscisic acid biosynthesis, respectively [23]. AO3 is also involved in the production of reactive oxygen species that provide tolerance against pathogens [25]. All gene transcripts were chosen from the literature on peas, and sequences were obtained from NCBI or Pea Marker Database [26]. Gene specific primers for qRT-PCR were designed using the IDT Primer Quest Tool (Table 1).

To assess whether rhizobia mediated plant osmotic stress in response to aphids, PEMV, and water availability, we measured osmotic potential from the plant stem near the second node with a pressure bomb (PMS instrument company, Albany, Oregon, USA). A pressure bomb increases pressure around the leafy shoot until sap is forced out to measure water movement in plants (osmotic potential) [27]. We only measured at the second node as the stem was small enough to fit into the pressure bomb, and although the water content may vary at other parts of the plant, this allowed us to have a uniform measurement across all plants tested. The pressure at which sap begins to ooze out from the cut end of the stem was measured from the air pressure gauge of the pressure bomb for samples collected at both time points (50 to 60 samples) but was measured one at a time.

2.3. Data Analysis

Analyses were conducted in R v. 4.3.3 [28]. We used linear regression to assess whether rhizobia, soil water availability, and their interaction affected plant height and nodes; height was tested with a Gaussian distribution and node count with a Poisson distribution. We checked models for assumptions of normality as well as for overdispersion. We also assessed effects of rhizobia, soil water, aphids, and all two- and three-way interactions on osmotic potential using linear regression. We then used generalized linear models to test if rhizobia, soil water availability, and their interaction affected PEMV-1, PEMV-2, and each relative gene transcript. Analyses for viral titer and gene expression were run on cycle threshold values (Ct), and $2^{-\Delta\Delta Ct}$ (relative expression) was calculated using parameters from the model. Estimated marginal means of Ct values and standard errors were generated with “emmeans” [29], and control water treatments without rhizobia or aphids were used as reference values.

3. Results

3.1. Effects of Rhizobia and Water Availability on Plant Traits

In the full water treatment, plants grown with rhizobia had more nodes ($Z = 3.10$, $df = 39$, $p = 0.001$) and were taller ($t_{52} = 9.84$, $p < 0.001$) than plants without rhizobia (Figure 1A,B). In the low water treatment, rhizobia promoted plant height but not the number of nodes (Figure 1A,B). Increased soil water availability increased plant height regardless of rhizobia ($t_{52} = -6.39$, $p < 0.001$) but soil water availability did not affect the number of nodes with any rhizobia treatment ($Z = -0.93$, $df = 39$, $p = 0.35$) (Figure 1A,B). The low soil water availability treatment lowered plant osmotic potential significantly, with the effects independent of rhizobia or aphid treatments ($t_{38} = -6.51$, $p < 0.001$, Figure 2). Rhizobia did not generally affect plant osmotic potential in the full water treatment or when PEMV aphids were not present. However, rhizobia mitigated the effects of low water and PEMV on plant osmotic potential ($t_{38} = 3.86$, $p < 0.001$) (Figure 2).

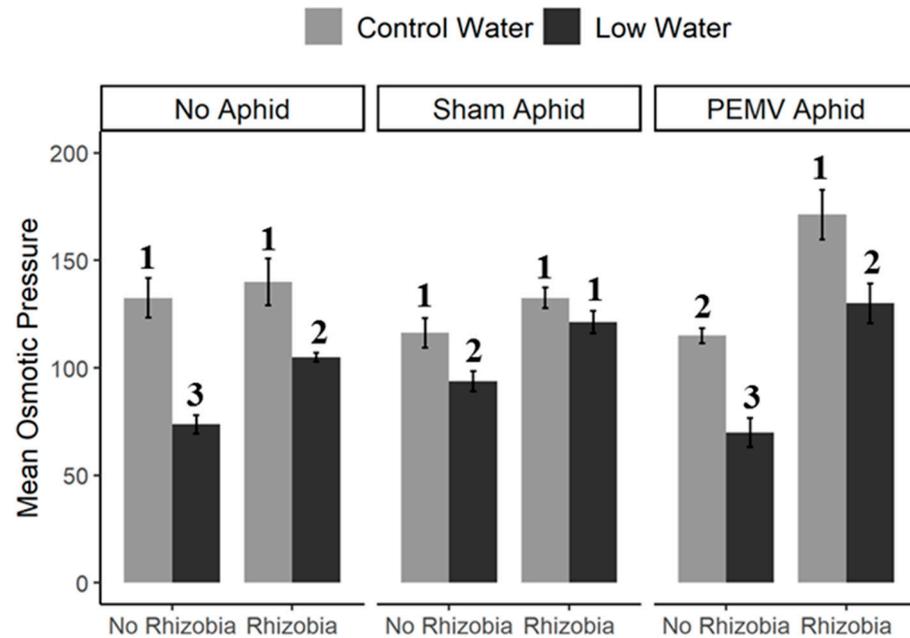


Figure 2. Effects of aphid treatments on osmotic potential for plants with or without rhizobia at two water levels ($n = 24$ per treatment). Bar height and error bars are based on predicted marginal means and standard errors. Bars with different numbers (1, 2, 3) are significantly different (Tukey HSD).

3.2. Effects of Soil Treatment and Drought on Viral Accumulation

When rhizobia were absent from the soil, plants grown with low water availability had significantly lower titer of PEMV-1 and PEMV-2 than plants grown in the full water availability treatment (PEMV-1: $t_{23} = 4.96, p < 0.001$; PEMV-2: $t_{23} = 4.96, p = 0.001$, Figure 3). However, when rhizobia were present in the soil, plants grown in either the low or full water availability treatments had similar levels of PEMV-1 and PEMV-2 (Figure 3). When plants were grown in the full water availability treatment, rhizobia lowered the titer of PEMV-1 and PEMV-2 more than when plants were grown in the low water availability treatment (PEMV-1: $t_{23} = 3.05, p = 0.006$; PEMV-2: $t_{23} = 2.87, p = 0.02$, Figure 3).

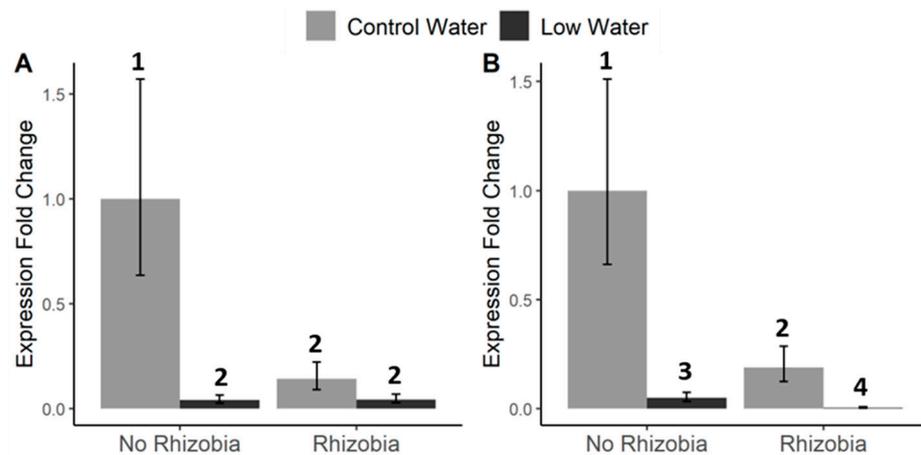


Figure 3. Relative accumulation of (A) PEMV-1 and (B) PEMV-2 in pea plants grown with different rhizobia and soil water availability treatments ($n = 24$ per treatment). Fold change in PEMV-1 and PEMV-2 titers were compared to controls based on parameter estimates from generalized linear models. Bars labeled with different numbers (1, 2, 3, 4) are significantly different (Tukey HSD test).

3.3. Effects of Rhizobia, Drought, Aphids, and PEMV on Plant Gene Expression

Though expressions of *AO3*, *LOX2*, and *PR1* were not significantly altered in the aphid-free treatment group, *ACS2* expression was significantly upregulated in both rhizobia-

inoculated and -uninoculated plants following exposure to drought conditions when aphids were absent (*ACS2*: $F_{3,12} = 8.38$, $p = 0.003$, Figure 4). Investigation into gene expression changes following exposure to non-viruliferous aphids revealed that *AO3* was significantly upregulated in all plants exposed to drought while *PR1* and *LOX2* were significantly upregulated in plants without rhizobia following drought exposure (*AO3*: $F_{4,14} = 10.07$, $p < 0.001$; *LOX2*: $F_{4,14} = 3.31$, $p = 0.042$; *PR1*: $F_{4,14} = 4.10$, $p = 0.021$, Figure 4). Finally, gene expression changes following exposure to aphids vectoring PEMV showed *AO3* was significantly upregulated in all fully watered plants as well as plants without rhizobia exposed to drought, *ACS2* and *LOX2* were significantly upregulated in all plants exposed to drought, and *PR1* was significantly upregulated in all fully watered plants (*LOX2*: $F_{4,14} = 9.74$, $p < 0.001$; *PR1*: $F_{4,14} = 4.98$, $p = 0.010$; *ACS2*: $F_{4,14} = 9.15$, $p < 0.001$, Figure 4).

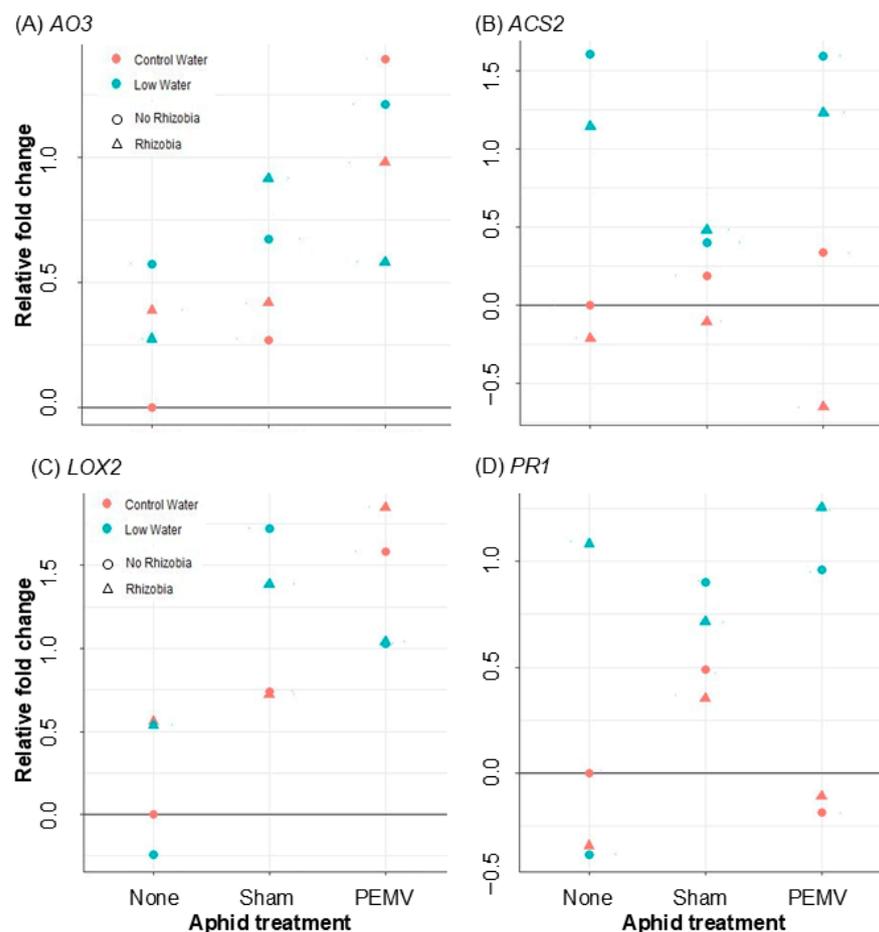


Figure 4. Fold change in relative expression of the (A) *AO3*, (B) *ACS2*, (C) *LOX2*, and (D) *PR1* gene transcripts in plants exposed to one of three aphid treatments, two water availability treatments, and two soil rhizobia treatments ($n = 24$ per treatment combination). Water availability treatments are labeled with different colors and rhizobia treatments are labeled with different shapes.

4. Discussion

Our study shows that soil rhizobia increased plant tolerance against an aphid-borne virus, although the effects were muted for plants grown with low compared to full water availability. This interaction between rhizobia and soil water may be driven by the observation that PEMV did not accumulate in plants with low water availability, with or without rhizobia. This provides evidence that plants grown with low water availability may respond with defenses that affect subsequent herbivore or pathogen attacks. Pea plants grown in rhizobia-inoculated soil also had increased plant height and nodulation, and rhizobia mitigated effects of low water availability on plant osmotic pressure. Our

study provides evidence that soil rhizobia functions as a keystone soil microbe that alters plant tolerance to stressors by altering plant defensive chemistry [11]. However, the effects of rhizobia on plant defensive chemistry were strongly mediated by soil water availability, showcasing the complexity of plant–insect–pathogen–microbe interactions.

Our results are consistent with other studies that show that soil rhizobia promote plant growth and tolerance to herbivores, pathogens, and drought [15,30]. Legumes grown with rhizobia often have reduced cellular, oxidative, and toxicity-related responses to stress [31,32]. Yet, low water availability also promoted tolerance to PEMV, and rhizobia only promoted tolerance to PEMV in fully watered plants. Low water availability can also slow transmission of insect-borne viruses like turnip yellow virus and soybean mosaic virus [16,33]. In contrast, aphid-borne transmission of cauliflower mosaic virus and turnip mosaic virus increased in *Brassica rapa* with low water availability [34]. These results show that the effects of water availability on virus transmission depend on the magnitude and direction of change in plant traits under stress [35,36].

While ABA activity is primarily associated with improving plant tolerance in response to drought conditions, ABA also plays a role in limiting the systemic spread of viruses via callose deposition at plasmodesmata [17,37]. The induction of drought conditions may prime plant ABA activity, leading to a quicker ABA-mediated anti-viral response when infection occurs. This was supported by observations that the rate of systemic viral spread is reduced in drought conditions [36]. Furthermore, while ABA plays a role in the regulation of nodulation activity, our results indicate that when plants are exposed to aphid herbivory, the presence of rhizobia is associated with an increase in the expression of ABA in both drought-exposed and fully watered plants (Figure 4) [38]. Our findings indicate that ABA likely plays a role in both drought and viral response signaling and that these mechanisms may be influenced by the presence of rhizobia.

Ethylene also plays a diverse role in plant defenses and interacts with other phytohormones to regulate these responses [39]. The enzyme ACS is vital for the formation of ethylene and is upregulated following the perception of abiotic stressors, notably water scarcity [40], and we found that *ACS2* was significantly upregulated when exposed to drought conditions (Figure 4). Though plant ethylene levels are often influenced by interactions with soil microbe communities, our results show that the presence of rhizobia had a negligible role in modifying the expression of *ACS2* compared to plants lacking rhizobia, indicating that *ACS2* activity may be independent of soil microbe interference [41].

Our study also assessed jasmonic acid and salicylic acid hormones. Jasmonic acid has been found to primarily be involved in defense responses towards herbivory, though they can also be associated with abiotic stress response pathways. While salicylic acid has been largely associated with instigating defense responses following pathogen infection, they too can be involved in abiotic stress signaling [42,43]. Our results indicate that expression patterns for both *LOX2* and *PR1*, genes associated with jasmonic and salicylic acid production, respectively, are similar between no aphid and non-viruliferous aphid treatments. Plants that were not exposed to aphid herbivory showed no change in the expression of either gene with water or rhizobia treatment, whereas *LOX2* and *PR1* expressions were increased in plants exposed to a combination of pea aphid herbivory, drought, and the absence of rhizobia (Figure 4). Exposure to drought has been found to increase plant susceptibility to aphid herbivory, and this heightened level of susceptibility may in turn drive increased jasmonic acid expression when herbivory occurs [44]. Increases in salicylic acid levels in high-water treatment plants following aphid herbivory has been recorded in other studies, indicating that anti-pathogenic responses of salicylic acid activity may be limited to conditions where water availability is optimal [45].

In natural and agricultural ecosystems, legumes are often simultaneously exposed to water deficiency, insect herbivory, pathogen infection, and variation in abundance and composition of soil microbial communities. Our analysis of defense gene transcripts suggests that interactions among these abiotic and biotic factors were partially mediated by alterations in plant defense hormone signaling. For example, ample water availability exerted positive effects on the expression of salicylic acid, a hormone involved in pathogen defense, following herbivory and infection [23]. Low water availability also promoted jasmonic acid signaling, a hormone involved in herbivore defense, following herbivory [11]. Soil water availability also interacted with rhizobia to affect plant hormones such as ethylene and abscisic acid, which broadly modulate plant responses to stress [46–49].

Our study shows that soil water availability and soil rhizobia mediated interactions among pea plants, pea aphids, and PEMV by affecting growth, osmotic balance, and expression of defense genes related to phytohormones. Soil rhizobia promoted plant growth by maintaining osmotic potential under water stress (drought) and promoted tolerance to aphids and PEMV when plants were fully watered. At the same time, stressors such as low water availability that limit plant growth overall may actually have beneficial effects when considered in other contexts, such as indirectly promoting tolerance to pathogens. Increased assessments of the effects of soil microbial communities on plant quality and pathogen tolerance under variable environment conditions could greatly improve our understanding of vector-borne pathosystems. Manipulation of soil microbes may also provide a tactic to manage devastating herbivores and plant pathogens that they transmit, though our results suggest benefits of rhizobia are highly context-specific.

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Conflicts of Interest: The authors declare no conflicts of interest.

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