Allelopathy-selected microbiomes mitigate chemical inhibition of plant performance

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Summary

Allelopathy is a common and important stressor that shapes plant communities and can alter soil microbiomes, yet little is known about the direct effects of allelochemical addition on bacterial and fungal communities or the potential for allelochemical-selected microbiomes to mediate plant performance responses, especially in habitats naturally structured by allelopathy. Here we present the first community-wide investigation of microbial mediation of allelochemical effects on plant performance by testing how allelopathy affects soil microbiome structure and how these microbial changes impact germination and productivity across 13 plant species. The soil microbiome exhibited significant changes to 'core' bacterial and fungal taxa, bacterial composition, abundance of functionally important bacterial and fungal taxa, and predicted bacterial functional genes after the addition of the dominant allelochemical native to this habitat. Further, plant performance was mediated by the allelochemical-selected microbiome, with allelopathic inhibition of plant productivity moderately mitigated by the microbiome. Through our findings, we present a potential framework to understand the strength of plant-microbial interactions in the presence of environmental stressors, in which frequency of the ecological stress is a key predictor of microbiome-mediation strength.

Introduction

Competition via allelopathy is a notable mechanism that structures plant communities (Hierro & Callaway, 2021; Inderjit et al., 2011). Allelopathy has a broad taxonomic distribution, as a recent meta-analysis shows that 72% of all plant families are capable of producing bioactive secondary

metabolites (allelochemicals; (Kalisz et al., 2021)). Allelopathy is also common across ecosystems including grasslands (da Silva et al., 2017; Ning et al., 2016), shrublands (Hewitt & Menges, 2008; Mahall & Callaway, 1991), and both temperate and tropical forests (Ooka & Owens, 2018), and is an important factor in both agricultural and invasion ecology (Bais et al., 2003). Meta-analysis has also shown that allelopathy reduces mean plant performance by 25% (Z. Zhang et al., 2021). These declines in plant fitness result from both direct effects of allelochemical inhibition as well as from indirect effects such as decreasing soil nutrient availability, or through important yet largely unexplored alterations in the soil microbial communities that interact with surrounding plant roots (Cipollini et al., 2012; P. Zhang et al., 2019).

Soil microbes play outsized roles in plant health and survival (Berendsen et al., 2012), and range from negative to positive effects on plant performance depending on environmental conditions (Hodge & Fitter, 2013; Trivedi et al., 2020). Recent studies indicate that soil microbiomes can increase plant performance under stressful environmental conditions through amelioration of abiotic and biotic stressors (David et al., 2020; Liu et al., 2020). Plant response to abiotic and biotic sources of stress can act as a cue, sometimes described as a 'cry for help', that encourages recruitment of microbial communities and functions that ultimately enhance the plant's capacity to combat stress and maintain fitness (Bakker et al., 2018). Abiotic stressors, such as abnormally high temperature or prolonged drought, can directly alter soil microbial community composition and shift allocation of plant carbon to mutualistic microbes in soil (Palta & Gregory, 1997). Despite the many potential beneficial microbial responses to this 'cry for help,' post-stress plant microbial interactions can also lead to decreased microbiome multifunctionality and increased pathogen loads in the rhizosphere (Hinojosa et al., 2019; Santos-Medellín et al., 2017). Allelopathy can similarly impose stress-induced shifts in microbiome composition, with some

studies indicating changes in functional capabilities (Lorenzo et al., 2013), and that allelochemicals may more strongly impact soil bacteria than fungi (Kong et al., 2008). For example, some soil microbes have been shown to degrade phenolic allelochemicals (Zhang et al., 2010), but little is known regarding the recruitment or increased reliance on these potentially beneficial microbes by plants. It is important to note that given this capacity for certain microbes to degrade allelochemicals, there are two potential pathways through which the soil microbiome can mediate plant performance responses to allelopathy: through allelopathy-selected compositional and/or functional shifts in the microbiome, or through the direct degradation or metabolization of the allelochemical (Fu & Oriel, 1999). Given the importance of microbiome functionality to plant health and stress resilience, understanding allelopathic effects on soil microbiomes is a crucial part of understanding plant community responses.

Our knowledge of microbial mediation of plant allelopathic interactions is notably limited in systems structured by native allelochemical inhibition. Much of what we know about the effects of allelopathy on plant-microbial interactions comes from studies of plant invasions. Allelopathy is very common among invasive species, with 51-67% of invasive plants reported to have allelopathic capacity (Kalisz et al., 2021; P. Zhang et al., 2019). Allelochemicals from invasive species have been shown to negatively impact bacterial abundance and community composition (Cipollini et al., 2012; P. Zhang et al., 2019), change microbial functionality in the rhizosphere (Qu et al., 2021), and ultimately alter plant-soil feedbacks in agriculture (Hu et al., 2018). It has been proposed and largely supported that naive native plant species are vulnerable to negative impacts of non-native allelopathic plants because they have not adapted to the novel chemicals introduced in their system (novel weapons hypothesis; Callaway & Aschehoug, 2000). The likely corollary to this hypothesis, discussed in Callaway & Hierro (2005) and Mishra *et al.*

(2013), is that plants in ecosystems *natively* structured by dominant allelopathic plants will have adaptations that minimize inhibition by allelopathy. We predict that plant-microbial interactions play a key role in this adaptation to allelopathy. For instance, allelopathy may select for soil microbiomes (through differential shifts in community members and associated functions) that mitigate or neutralize the inhibition of plant performance by allelochemicals (e.g., via increased beneficial interactions in stressful environments; (David et al., 2020)). Importantly, the strength of microbial mediation of allelochemical stress can fall along a continuum and may be dependent on plant species-microbe specificity (Revillini et al., 2016).

Given the known importance of soil microbiomes for plant health and the global impacts of allelopathy (David et al., 2018; Wardle et al., 1998), we conducted a study to determine the direct effects of allelochemical addition on the soil microbiome, as well as the subsequent effects on performance of native plants from a habitat naturally structured by allelopathy. We address three questions: 1) Can allelochemicals alter bacterial and fungal community structure and function in soil? 2) How does a history of persistent allelochemical-selection on the soil microbiome impact native plant performance responses? 3) Which allelochemical-altered soil microbes and microbiome functions underpin these changes in plant performance? We are interested in the potential for adaptation among native plant and soil microbial communities to allelopathy, a persistent and long-term stressor in this habitat, as there will have been consistent selection pressure for plant-microbial associations that are able to weather that stress. We predict that allelochemical addition to soils will more strongly alter bacterial than fungal communities due to previously noted bacterial susceptibility to allelochemicals (Lorenzo et al., 2013; Niro et al., 2016), and concomitant potential for greater fungal tolerance to allelochemicals (Barto et al., 2011). Moreover, we expect that changes in microbial abundance as a response to allelopathy and

relationships with increased plant performance will allow for identification of microbial consortia that are adapted to mitigate inhibitory effects of native allelopathy in this system. We intend for this work to function as a template for future research in allelopathic systems by identifying core sets of allelochemical-selected soil microbiota and attendant microbiome-mediation of allelopathy.

Materials & Methods

Study system

The Florida Scrub ecosystem has the highest rate of endemism in the southeastern US and hosts many threatened species (Dobson et al., 1997; Menges et al., 2008). This ecosystem exhibits a range of habitat types from open sand gaps and shrublands to mixed conifer flatwoods within a relatively small area (Abrahamson et al., 1984). Many of the rare and endemic plants in this ecosystem are found in the rosemary scrub habitat, where they occur in open sand gaps between the dominant, allelopathic shrub Florida rosemary (*Ceratiola ericoides* Michx.). Florida rosemary produces a suite of allelochemicals that can affect performance of other scrub species. Notably, Florida rosemary produces ceratiolin, a flavonoid that quickly decomposes into dihydrocinnamic acid (HCA) and negatively affects plant germination and root length for many herbaceous Florida scrub plant species (David et al., 2018; Fischer et al., 1994). Ceratiolin and derivative HCA are documented as the dominant allelochemicals found in litter and soil of the Florida scrub habitat (Jordan, 1990), and have been credited with contributing to the patchy structure of the ecosystem (Hewitt & Menges, 2008; Hunter & Menges, 2002).

Recent studies show that there are distinct soil microbiomes in rosemary scrub compared to surrounding flatwoods habitat (Hernandez et al., 2021), and that many of the rare, endemic plants occurring in the rosemary scrub are strongly influenced by interactions with the soil

139 microbiome (David et al., 2018, 2020). For this study, we used soils from 10 sites at Archbold
140 Biological Station (Venus, FL, USA; 27.18° N, 81.35° W) and collected seeds of 13 perennial,
141 herbaceous plant species from across Archbold (Table S1) that vary across a spectrum of life
142 history traits.
143
144 *Allelochemical treatment of the microbiomes*
145 To capture the abiotic variation in our study system, we chose soils to test allelopathic effects
146 across a realistic sampling range of two important metrics in the system (Menges et al., 2017). We
147 collected soils from 10 Florida rosemary scrub patches (i.e., open habitat patches dominated by *C.*
148 *ericoides* that occur at relatively high elevations above the water table; Table S2) with a range of
149 fire histories -- time since fire and total number of fires experienced within the last 52 years. We
150 collected ~5 kg of soil from open sand gaps (at least 3 meters from *C. ericoides* to minimize the
151 effects of ambient environmental HCA) at each of the 10 sites, and then stored soils for two days
152 before applying the allelochemical treatment. HCA concentrations have been shown to decrease
153 rapidly with increasing distance (>2 meters) from the host plant in this ecosystem (Quintana-
154 Ascencio and Menges 2000). The allelochemical addition treatment was performed using 250 ppm
155 hydrocinnamic acid (3-phenylpropionic acid; HCA) diluted in ultrapure H_2O. HCA concentration
156 was selected based on previous studies from the field that identified natural concentrations of HCA
157 ranging from 15-418 ppm (Jordan, 1990), and a manipulative study that found 250 ppm HCA
158 effectively impacted plant performance (David et al., 2018). 1250 mL of soil from each site was
159 equally split among sterilized aluminum trays (34 cm x 24 cm x 7 cm, n = 20) to receive the control
160 (ultrapure H_2O) or allelochemical addition (HCA+) treatment. Each tray was soaked with 50 mL
161 (4% volume) using a sterile 2 L pump sprayer of either treatment three days per week in a

temperature-controlled environment (25ºC) for 5 weeks, leading to a total HCA concentration of 150 ppm.

Soil microbiome extraction, amplification, sequencing, and bioinformatic processing

DNA was extracted from homogenized soil samples after the allelochemical addition treatment concluded (n = 20; 10 soil sources and 2 allelochemical treatments) using the DNeasy PowerSoil Pro QIAcube HT Kit (Qiagen, Carlsbad, CA, USA) with an adapted protocol without QIAcube (see Supplemental Methods for more detail; Revillini et al., 2021). DNA was quantified with a Qubit 4 fluorometer (Qiagen, Carlsbad, CA, USA), and normalized to 5 ng/µL. Libraries were prepared for sequencing using a two-step dual indexing protocol (Gohl et al., 2016). PCR was targeted for archaeal/bacterial (16S) and broad fungal (ITS2) ribosomal DNA (rDNA) using primer pairs 515F-806R and ITS7o-ITS4, respectively. Index and Illumina flowcell sequences were added in second-step PCR. All targeted amplicon products were pooled in equimolar quantities, and sent to the Duke University Microbiome Core Facility (Durham, NC, USA). Libraries were sequenced on a MiSeq Desktop Sequencer (v3, 300 bp paired end; Illumina, Inc., San Diego, CA, USA).

Paired-end molecular sequence data was processed using QIIME2 v2021.4 (Bolyen et al., 2019). Briefly, denoising was performed with the DADA2 algorithm (Callahan et al., 2016), which removes chimeric sequences and truncates 16S and ITS amplicon forward and reverse sequences to an equal length. Naive Bayes classifiers were constructed using the Greengenes database v13.8 (99%) and the UNITE database v7.2 (99%) for archaeal/bacterial and fungal amplicons, respectively, and then amplicon sequence variants (ASVs) were classified using the sklearn algorithm (Pedregosa et al., 2011). Multiple sequence alignments were performed using

mafft v7 (Katoh & Standley, 2013), an unrooted tree was created using FastTree2 (Price et al., 2009), and then the midpoint root method was used to create a rooted tree for phylogeny-based analyses (e.g., weighted UniFrac). ASVs that were not present in at least two samples were filtered out, and diversity metrics and dissimilarity matrices were calculated using the QIIME2 commands *diversity core-metrics-phylogenetic* (sampling depth = 6,500) and *diversity core-metrics* (sampling depth = 9,000) for archaea/bacteria and fungi, respectively. All microbiome data from QIIME2 was read into R v4.1 (R Core Team, 2020) using the qiime2R package v0.99.6 (https://github.com/jbisanz/qiime2R).

Allelochemical-selected microbiome-plant performance experiment

To determine the magnitude of microbial effects on plant performance in the rosemary scrub and how these effects depend on the microbiome's exposure to the dominant allelochemical (HCA) found in rosemary scrub soils (Fischer et al., 1994), we conducted a $2 * 2$ factorial growth room experiment manipulating microbiome presence (presence vs. absence) and allelochemical selection on the microbiome (control vs. HCA+) replicated using soil microbiomes collected from 10 rosemary scrub patches (see Supplemental Methods for more detail). We first sterilized half of the soil from each allelochemical treatment by autoclaving three times (121°C, 2 hr). The 13 rosemary scrub plant species (Table S1) were each grown in sterilized pots (66 mL) inoculated with soil microbiomes from all 40 factorial combinations of soil source, allelochemical treatment, and microbiome presence. Each pot was filled with 50 mL of sterilized background rosemary scrub soil and topped with 10 mL of inoculum from one of the 40 treatment combinations. To ensure that the majority of soil in each pot had similar abiotic properties, and thus the only manipulation was the different soil microbiomes present in the inocula, background soil in this experiment was

collected from a single large open sand gap at Archbold >5 m from Florida rosemary and autoclaved 4x at 121°C. After seeding directly into inoculum soil, a 2 mL 'cap' of sterile, background soil was added to prevent seed desiccation. The number of seeds sown per pot (Table S1) reflected previously determined differences in germination rates among these plant species (David et al., 2020; Revillini et al., 2021), and all pots were thinned to one plant shortly after germination. Overall, our experiment included 10 microbiome sources in each of the four allelochemical × microbial treatment combinations, each with three replicates for each of the 13 plant species (except for *Liatris ohlingerae*, which had 5 microbiome sources due to lack of seed), totaling 1500 pots. All pots were watered with ~2 mL sterile water daily for one month and subsequently every other day. Plants were grown under full spectrum lights (~162 µmol/m2/s PAR), with a 14:10 hr (light:dark) schedule until harvest ~5 months after the start of the experiment (Table S1). Germination percentages were determined based on species-specific seeding rates per pot (Table S1). Shoot and root biomass were determined after oven drying at 50℃ until reaching constant mass. Root-to-shoot biomass ratios were calculated to determine plant allocation responses.

Data analysis

To identify a baseline for soil microbiome organization after allelochemical addition, we calculated the core microbiome for both bacteria and fungi using the *core* function from the 'microbiome' package in R (http://microbiome.github.com/microbiome). The core microbiome here represents taxa with a >0.1% relative abundance detection threshold that also occur in >60% of all samples that experienced allelochemical addition (Busby et al., 2017). The allelochemical effects on bacterial and fungal alpha diversity metrics (ASV richness, Shannon's H, Pielou's

evenness, and Faith's phylogenetic diversity) were assessed using paired t-tests, with allelochemical addition as the factor of interest and microbiomes paired by soil source. To determine allelochemical effects on bacterial (weighted uniFrac) and fungal (Bray-Curtis dissimilarity) community composition, a PERMANOVA stratified by soil source was performed using the *adonis2* function in R package vegan v2.5-7 (Oksanen *et al.*, 2020). To identify which microbial taxa responded strongly to allelochemical addition, analysis of differential microbial relative abundances from allelochemical control ('reference' factor level) to samples that underwent allelochemical addition was performed using the *DESeq* function in R package DESeq2 v1.32 (Love *et al.*, 2014).

We used the PICRUSt2 algorithm (Douglas et al., 2020) to calculate the predicted bacterial metagenome based on our 16S reads in order to assess the effect of allelochemical addition on important functional genes in nutrient release or transfer. We targeted analyses on genes associated with important carbon (C), nitrogen (N), and phosphorus (P)-cycling functions. Paired t-tests were performed on individual genes (*e.g.*, nifQ or amoA) or sums of gene sets that comprise functional pathways for nitrite reduction (nirBDK), phosphonate (organic P) cleavage and transport (phnCDEJ), as well as phosphate transport (ugpACQ), to identify increases or decreases in predicted bacterial function after allelochemical addition.

To understand how allelochemical effects on the soil microbiome contributed to plant performance responses, we constructed linear mixed models. Our models considered how plant performance responded to the presence or absence of soil microbiomes and whether or not soils experienced allelochemical addition. All 13 plant species were included in analyses of germination rates, but two species with the lowest germination rates, *Hypericum cumulicola* and *Paronychia chartaceae*, were excluded from analyses of productivity or biomass allocation due to insufficient

degrees of freedom. To meet the assumption of homogeneity of variances across species, z-scores were calculated for all plant response metrics within each plant species prior to analysis. Germination percentages were arcsine-square root transformed prior to z-score calculations to improve normality. Using these data, we first ran global models for all plant species combined. Terms in these models included microbiome presence (presence vs. absence), allelochemical selection on the microbiome (control vs. HCA+), and their interaction as well as plant species identity and interactions between plant species and all of the other terms. We also included a random effect of soil collection site. After finding significant interactions with plant species identity in the global models, we constructed follow-up general linear models for each of 11 plant species individually. These models included the same microbiome and allelochemical main effects and their two-way interaction term. We conducted linear mixed models using the *lmer* function in R and model output was determined using Type III sums of squares, which are independent of the input order of predictor variables.

To identify the relationships between plant performance responses and the microbiome responses to allelochemical addition each of the five measured plant performance metrics were regressed on the 23 bacterial and fungal ASVs (aggregated at the lowest taxonomic level and with 'unidentified' ASVs removed), and also on five predicted bacterial functional genes that were observed to change after allelochemical addition (nifQ, amoA, nir, ugp, and phn). Collection site (patch) and plant species identity were set as random effects. The Benjamini-Hochberg procedure was used to account for multiple comparisons.

Results

Allelochemical-selected microbiome

We identified 44 core bacterial ASVs and 42 core fungal ASVs in the allelochemical-selected microbiome. For bacteria, the allelopathic core microbiome was dominated by two taxa in the Burkholderiaceae -- *Burkholderia tuberum* (34% of identified ASVs) and *Burkholderia byrophila* (29%) -- with the remainder of core microbiome taxa coming from the Solibacteraceae, Mycobacteriaceae, and Nitrosphaeraceae (Table S3). Notably, four bacterial families in the control bacterial core microbiome fell below the core thresholds for soils experiencing allelochemical addition, and taxa in the Burkholderiaceae emerged only with allelochemical addition, becoming the second most prevalent allelopathic core member (Figure 1). Of the 42 ASVs in the fungal core microbiome, 19% were from the genus *Talaromyces*, and the remaining ASVs were fairly equally distributed across 11 identified genera (Table S3). We observed the appearance of two new genera in the core fungal microbiome with allelochemical addition, *Gelasinospora* and *Chaetomium*, as well as increases in the prevalence of taxa in the *Gibberella* and *Veronaeopsis* (Figure 1).

Allelochemical addition also significantly shifted overall bacterial community composition (pseudo-F = 2.35, P = 0.002; Figure 2a), but did not significantly affect overall fungal community composition (pseudo-F = 1.31, P = 0.08). However, differential abundance analysis of both bacteria and fungi revealed highly-responsive taxa that significantly increased or decreased in relative abundance after allelochemical addition. Bacterial ASVs in the families Solibactereaceae (Acidobacteria) and Acetobacteraceae (Alphaproteobacteria) increased with allelochemical addition, with a 22 $\log_2$-fold change (LFC) for both, while abundance of three ASVs in the Thermogemmatisporaceae decreased by ~20 LFC (Figure 2b). Of the 65 total bacterial ASVs that shifted after allelochemical addition, the majority (24 and 15 ASVs) were identified as two species: *Burkholderia tuberum* and *Burkholderia bryophila*, respectively.

Differential abundance analysis showed that 19 fungal ASVs also significantly responded to allelochemical addition (18 fungal taxa across eight genera plus one 'unidentified'; Figure 2c). Changes in abundance of fungal taxa after allelochemical addition were comparable in strength to LFCs observed in the more allelochemical-responsive bacterial community (-23 to +21 LFC). Two taxa in the genera *Gibberella* and *Paraphaeosphaeria* and an unidentified taxon in the order Eurotiales all increased in abundance by approximately +20 LFC, and the putative plant pathogen, *Pseudopithomyces*, had the largest decrease after allelochemical addition (Figure 2c).

Of the 11 bacterial functional genes/gene sets associated with C, N, and P cycling we examined, five were significantly affected by allelochemical addition; nifQ, amoA, nir, phn, and ugp (Figure 3). Allelochemical addition increased predicted gene abundances for nitrogen fixation (nifQ; $P = 0.003$) and decreased predicted gene abundances for ammonia oxidation (amoA; $P = 0.039$), nitrite reduction (nirB, nirD, and nirK; $P = 0.004$), phosphate transport (ugpA, upgC, and upgQ; $P = 0.004$), and phosphonate uptake and breakdown (phnC, phnD, phnE, and phnJ; $P = 0.022$).

Plant performance responses

Across all plant species, effects of allelochemical addition on productivity were microbially-mediated, where allelochemical-selected microbiomes mitigated negative impacts to total plant biomass (Table 1). Both shoot and total biomass were significantly reduced by allelochemical addition ($P = 0.019$ and $P = 0.026$, respectively), but in the case of total biomass this was significantly mitigated by the soil microbiome ($P = 0.034$; Figure 4a) whereas microbial mitigation was marginal for shoot mass ($P = 0.073$; Figure 4a). Plant species varied significantly in their response to the microbiome treatment across all plant performance metrics that we examined ($P \leq$

0.009), but only for root:shoot ratio was there significant interspecific variation in the degree to which allelochemical treatment modulated this response ($P = 0.016$), ranging from a 106% decrease (for *Balduina angustifolia*) to a 137% increase (for *Liatris tenuifolia*) in root biomass investment when the microbiome was present to mediate allelopathic effects. Surprisingly, individual plant species models revealed only one species with a significant allelochemical*microbiome treatment interaction (Figure 4b). In *Balduina angustifolia*, the allelochemical*microbiome interaction was significant for three plant performance responses: both aboveground shoot biomass and total plant biomass were significantly higher when a microbiome was present to alleviate the effects of allelochemical addition ($P < 0.0001$; $P = 0.027$, respectively), while investment in roots was significantly lower when the microbiome was present to mediate allelopathic effects ($P < 0.0001$).

Relationships between microbiomes and plant performance responses

Of the 23 microbial taxa and four bacterial functions significantly affected by allelochemical addition, we identified 6 microbial taxa (three bacterial and three fungal) and two bacterial functions that had significant relationships with at least one of the five measured plant responses: germination, root biomass, shoot biomass, total biomass, or root:shoot biomass ratio (Figure 5). The fungal species *Exserohilum rostratum,* which increased after allelochemical addition, had the strongest positive relationship with both shoot biomass and total biomass ($t = 2.27$, $P = 0.03$; $t = 2.12$, $P = 0.034$, respectively), while the bacterial genera *Rhodoplanes* and *Bacillus*, which decreased and increased after allelochemical addition, respectively, had the strongest negative effects on shoot biomass and root biomass ($t = -3.15$, $P = 0.009$; $t = -2.14$, $P = 0.032$, respectively). The two predicted bacterial functions nir and ugp, both of which decreased after allelochemical

addition, also explained variation in plant performance that was significant, but moderate (Figure 5). Nir genes, which code for nitrite reduction, had slight negative effects on germination ($P = 0.012$), while ugp genes, which code for organic P solubilization, had slight positive effects on germination and negative effects on root:shoot biomass ratio ($P = 0.024$, $P = 0.025$, respectively).

Discussion

Allelopathy strongly affected soil microbiome structure and predicted functions in this study. The allelopathic chemical derived from the dominant native shrub (*C. ericoides*) in this system altered the core microbiome, bacterial composition, and relative abundances of bacterial and fungal taxa. Allelochemical-treated microbiomes also showed evidence of functionally important changes, such as notable increases in abundance of putative beneficial bacteria (i.e., Burkholderiales) and putative fungal pathogens (Figure 2) as well as shifts in predicted bacterial functional genes including an almost seven-fold increase in the abundance of the nifQ gene, coding for N_2-fixation. We found significant, but weaker effects of allelochemical-altered microbiomes on plant performance responses (productivity) in the manipulative growth experiment. Similar to previous studies, the microbiome exhibited a net positive effect on plant productivity, one of the most important performance metrics for perennial germinants in this habitat (Menges & Kohfeldt, 1995), in the presence of allelopathy (Cipollini et al., 2012; Mishra et al., 2013). Our study explicitly reveals the link between allelochemical-altered soil microbes and plant performance. While previous studies have identified allelopathy-induced shifts in microbiomes and/or plant performance, the vast majority could only infer microbiome-mediation (but also see Hu et al. 2018). However, the effect sizes we observed tended to be fairly modest, such that when combined

with the smaller sample sizes within individual species, only one of the 11 species (*Balduina angustifolia*) registered a significant effect. Our study predominately investigated the effect of allelochemical-induced compositional shifts in the microbiome on plant performance (as the allelochemical was not added to the plants themselves, but was incorporated as a prior treatment to the inoculum). Microbial mitigation of allelopathic effects may be stronger if microbial degradation of allelochemicals reduces the direct effect of allelopathy in addition to this indirect compositional change. We also suspect the *lack of novelty* of the allelochemical weapon in this ecosystem has led to previous adaptive responses of these plants that allow them to tolerate allelochemical-induced shifts in the microbiome (i.e., representing a stable community exhibiting weak-neutral responses; (Shade et al., 2012)). The novelty of an ecological weapon has a direct relationship with the frequency of a stressor, where high frequency would present a more common weapon and low frequency would represent a more novel weapon. We propose that the strength of microbial mitigation or exacerbation of plant responses to disturbance is negatively related to the frequency of the ecological stressor in question (Figure 6).

Microbial composition shifted distinctly with allelochemical addition, indicating strong direct effects of allelochemical addition on soil microbiomes. Soil bacteria were notably more responsive to allelochemical addition than fungi, as has been found in previous studies (Kong et al., 2008), and also appear to have shifted towards a structure and functions that would promote greater plant growth. In particular, we found significant increases in Burkholderia (many putative N-fixers), as well as *Rhidopila globiformis* (Acetobactereaceae), a nitrogen-fixer that may contribute to alternative N_2-fixation via the vanadium-dependent nitrogenase pathway (Imhoff et al., 2018). In contrast, fungi exhibited increases in multiple putative pathogens after allelochemical addition. These included an increase in prevalence of *Gibberella*, a known fungal pathogen (Bai

et al., 2021). Interestingly, there was also an increase in the dark septate endophytic genus *Veronaeopsis*, which has been shown to mitigate infectivity of other fungal pathogens (Khastini et al., 2012). Thus, the increased relative abundance of this taxon may indicate a fungal mechanism for reducing allelochemical-induced stress to plant roots. These shifts reveal increased dominance for putative beneficial bacteria and putative pathogenic fungi among their respective soil consortia, and this apparent positive-negative balance of representative microbial taxa might contribute to the weakly positive microbial mediation effect of the soil microbiome on plant performance responses in our manipulative growth experiment (Vandenkoornhuyse et al., 2015).

Functional changes to soil microbiomes after allelochemical addition indicate a range of responses to allelopathy that also likely contributed to the neutral-to-positive microbial mediation of plant performance observed here. Multiple bacterial functional genes shifted after allelochemical addition, with increases in potential N-fixation via the nifQ gene, which donates molybdenum to nifH for biosynthesis of the FeMo nitrogenase enzyme (Hernandez et al., 2008). On the other hand, we observed decreases in ammonia oxidation, nitrite reduction, phosphonate reduction, and phosphate transport that suggest a suppressive effect of allelopathy on bacterial N and P cycling belowground (Figure 3). While these results are predicted using the PICRUSt2 algorithm, which can underestimate certain gene frequencies (Toole et al., 2021), they still indicate a functional mechanism – via increased N_2-fixation – that may have contributed to the mitigation of allelopathic stress on plant productivity found in our across plant species analysis (Figure 4). To build on these findings, we advocate for future research exploring differential responses of bacterial and fungal functions to allelopathy using targeted methods such as metagenomics or quantitative stable isotope probing to assess impacts on microbiome functional responses and subsequent plant-microbial interactions (Hungate et al., 2015).

Significant relationships between allelochemical-responsive microbial taxa (6 out of 23) and bacterial functions (2 out of 5) and plant performance may help identify individual microbial taxa that could be important for the resilience and persistence of the rare, endemic plants in this system (Figure 5). Interestingly, our results relating plant performance with specific members and functions of the microbiome revealed that not all taxa or functions considered putatively beneficial or inhibitory influence host performance as expected. For instance, the fungal taxon with the strongest positive effects on shoot and total biomass, *Exserohilum rostratum*, is a putative plant pathogen that causes root rot across many plant families (Sharma et al., 2014). Though the majority of research indicates that this species negatively impacts plant productivity, a recent study found an *E. rostratum* variant that was beneficial for plant growth in sunchokes (Khaekhum et al., 2021) suggesting that this taxon can act as a mutualist under certain conditions. Negative microbial relationships with plant performance were also surprising, because many were found for taxa known to contribute to plant-growth promotion including those in the Burkoholderiaceae and Rhodoplanes (Adesemoye et al., 2009; Anzuay et al., 2021; Carrión et al., 2018). These relationships between members of the microbiome and plant performance metrics suggest that: 1) many allelochemical-responsive microbial taxa and functions may play outsized roles impacting plant performance, 2) putative functional categorizations of members of the soil microbiome are likely oversimplified, and 3) functional relationships between individual plants and members of the soil microbiome should be studied further to identify patterns of context-dependency across systems experiencing disturbance.

We predicted that the nature of the allelopathy stress in this study system – functioning as a persistent stressor – would lead to beneficial plant-microbial interaction responses, and our results supported this prediction. Allelopathy is persistent in the rosemary scrub, leading to

438 increased opportunities for plant and microbial adaptation via increased interaction frequency (i.e.,
439 familiarity) compared to infrequent disturbances. We propose that stress frequency is critical in
440 determining the strength of the plant-microbial interaction response (Figure 6). More specifically,
441 we expect that microbial mediation of plant response to stress becomes more muted with increased
442 stress frequency, as the community experiences persistent selection for greater stability (weaker
443 interactions) in the face of such a common stressor. This is in contrast to effects observed from
444 novel or infrequent disturbances (e.g., species introductions, fire, drought) on plant-microbial
445 interactions. For instance, our research was conducted in a fire-dependent system (Menges &
446 Kohfeldt, 1995), where fire is a naturally occurring disturbance with a return interval of ~16 years
447 (Menges, 2007). In a previous study testing the ability of soil microbiomes to mediate plant
448 performance responses to prescribed fire with many of the same plant species used here, we
449 showed much stronger mediation effects of the post-fire soil microbiome on plant performance
450 (Revillini et al., 2021). This difference in the strength of microbial-mediation of allelopathic vs.
451 fire stress within the same ecosystem could be a feature of their local adaptation to the dominant
452 and *persistent* allelochemical stress as opposed to relatively infrequent fire disturbance (Figure 6).
453 To test this prediction, future research should strive to identify the continuum under which plant-
454 microbial interactions respond to stressors along a frequency gradient. Finally, to more broadly
455 confirm our results regarding plant-microbial interaction responses to allelochemical addition, we
456 feel it would be valuable to investigate the total effects of the source allelopathic plants
457 (incorporating roots and the full suite of phytochemicals) on microbial mediation of plant
458 responses in future experimental manipulations.

459 Microbial resistance and resilience to stress, resulting legacies in soil, and microbiome-
460 mediation of plant responses to stress are still emerging lines of research in soil ecology (Bakker

et al., 2018; Kiesewetter & Afkhami, 2021; Philippot et al., 2021), but it is becoming apparent that the strength and frequency of ecological stressors should be considered a major contributing factor to the functional relationships between the soil microbiome and aboveground communities. We have shown that plant-microbial interaction responses to persistent allelopathy stress are subtle and neutral-to-positive for plant performance. In a system natively structured by allelopathy of a dominant plant, the effects of allelochemicals in soil can function as a method of chemical warfare that directly alters the soil microbiome and plant performance, and also have their effects mediated by the soil microbiome. While previous studies have begun to identify patterns of microbial resistance and resilience to disturbance in a broad global sense (Rocca et al., 2018; Shade et al., 2012), our research focuses this field by directly testing the link between the stress-selected microbiome and plant performance responses. This work suggests that the soil microbiome has great potential to mitigate plant responses to abiotic stress, and emphasizes the importance of future work identifying functional roles of the soil microbiome that mediate environmental stress.

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Competing interests

The authors declare no competing interests with regard to the preparation of this manuscript.

Data Availability Statement

All raw plant and soil data will have been submitted to the Dryad database, and all molecular data used for microbiome analyses have been submitted to the NCBI SRA.

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661 **Table 1.** Significant results from linear mixed models of z-score standardized plant performance
662 responses. Model predictor terms are column headers, P-values are presented when $P \leq 0.05$. ↟ =
663 P-values presented are from performance responses prior to within plant species standardization.

Response	Plant species↟	Microbiome	Allelochemical (Allelo)	Microbiome x Allelo	Plant x Microbiome	Plant x Allelo	Plant x Microbiome x Allelo
Germination	<0.0001	-	-	-	0.008	-	-
Root Biomass	<0.0001	-	-	-	0.0004	-	-
Shoot Biomass	<0.0001	-	0.02	-	< 0.0001	-	-
Total Biomass	<0.0001	-	0.029	0.034	0.0001	-	-
Root:Shoot	<0.0001	-	-	-	0.005	0.002	0.016

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665 **Table 2.** Individual plant species general linear model results. For each performance response,
666 only plant species with a significant effect are presented ($P \leq 0.05$). Shading in gray indicates a
667 negative main effect on plant performance, while main effects without shading indicate a positive
668 main effect.

Response	Plant species	Microbiome	Allelochemical	Microbiome x Allelo
Germination	*Chapmannia floridana*	F = 8.32, P = 0.003	F = 3.95, P = 0.046	
	Eryngium cuneifolium	F = 5.41, P = 0.019	F = 8.33, P = 0.003	
	Polygonella robusta	F = 4.61, P = 0.031		

Root Biomass	*Chamaecrista fasciculata*	F = 27.27, P < 0.0001		
	Eryngium cuneifolium	F = 3.84, P = 0.049		
	Lechea cernua		F = 3.85, P = 0.049	
	Pityopsis graminifolia		F = 4.8, P = 0.028	
	Polygonella robusta	F = 3.8, P = 0.05		
Shoot Biomass	*Balduina angustifolia*	F = 5.42, P = 0.019	F = 13.09, P = 0.0002	F = 19.14, P < 0.0001
	Chapmannia floridana	F = 23.69, P < 0.0001		
	Lechea cernua	F = 4.85, P = 0.027		
	Pityopsis graminifolia	F = 4.98, P = 0.025		
	Polygonella robusta	F = 22.87, P < 0.0001	F = 4.75, P = 0.029	
Total Biomass	*Balduina angustifolia*			F = 4.85, P = 0.027
	Chamaecrista fasciculata	F = 14.68, P = 0.0001		
	Eryngium cuneifolium	F = 4.5 P = 0.033		
	Polygonella robusta	F = 10.89, P = 0.0009		
Root:Shoot	*Balduina angustifolia*		F = 13.216, P = 0.0002	F = 18.75, P < 0.0001
	Chamaecrista fasciculata	F = 27.9, P < 0.0001		
	Chapmannia floridana	F = 12.09, P = 0.0005		
	Eryngium cuneifolium			F = 3.82, P = 0.05
	Lechea cernua		F = 5.17, P = 0.022	
	Polygonella robusta	F = 5.1, P = 0.023		

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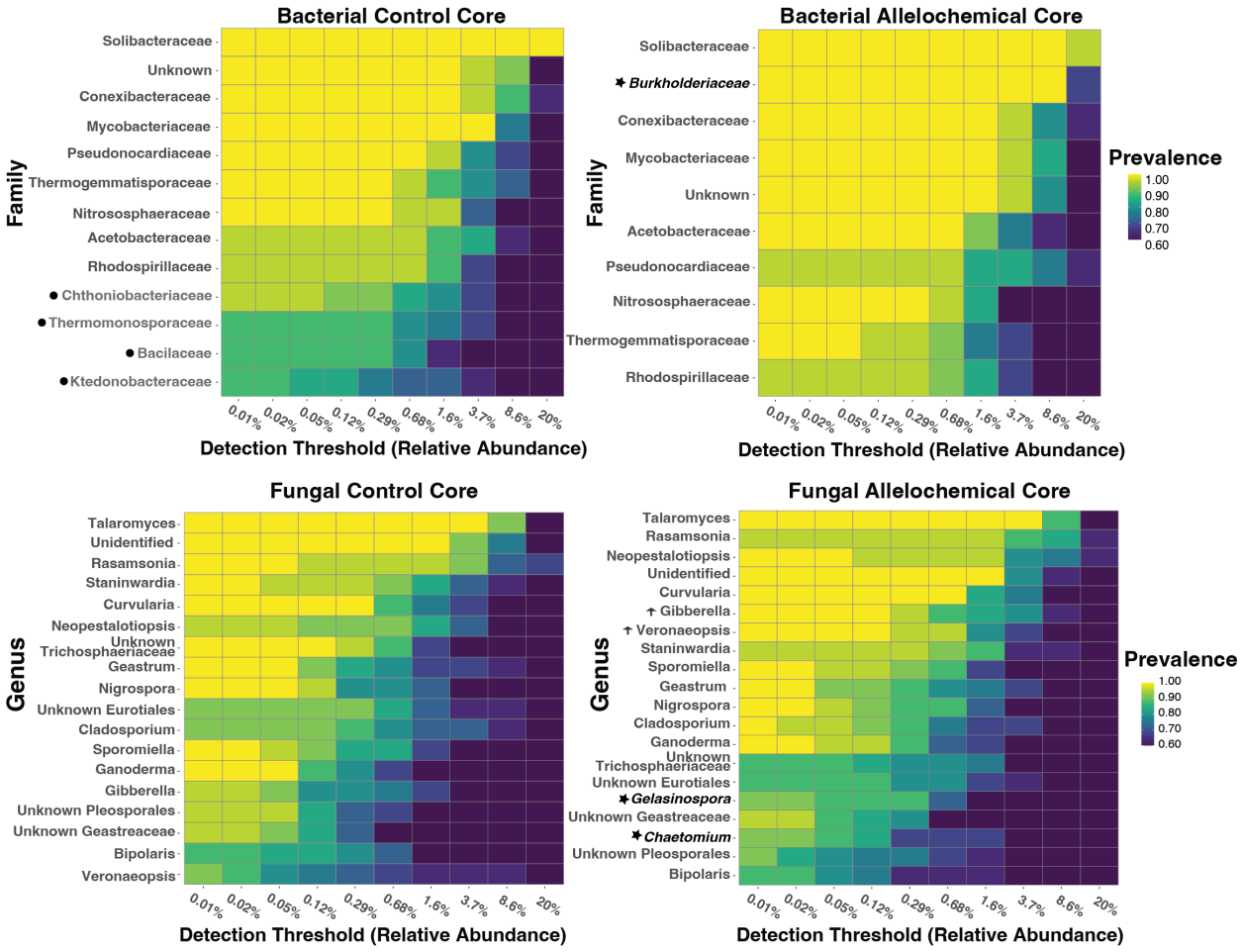


Figure 1. Core microbial taxa without allelochemical addition (left) and with the addition of hydrocinnamic acid (right). Colored by prevalence and organized by relative abundance detection thresholds for core bacterial families (top) and core fungal genera (bottom). ★ = addition to core microbiome after allelochemical treatment; ● = removal from control core microbiome after allelochemical treatment; ↑ = increase in prevalence after allelochemical treatment.

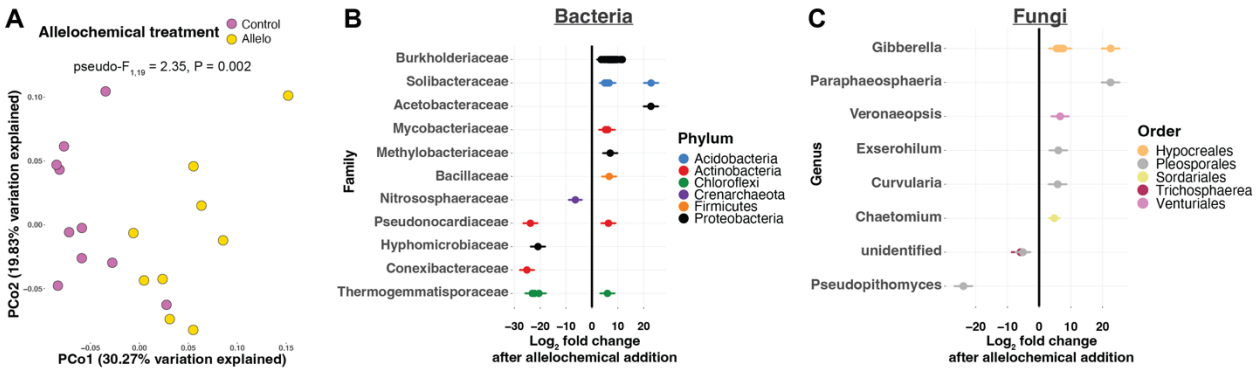
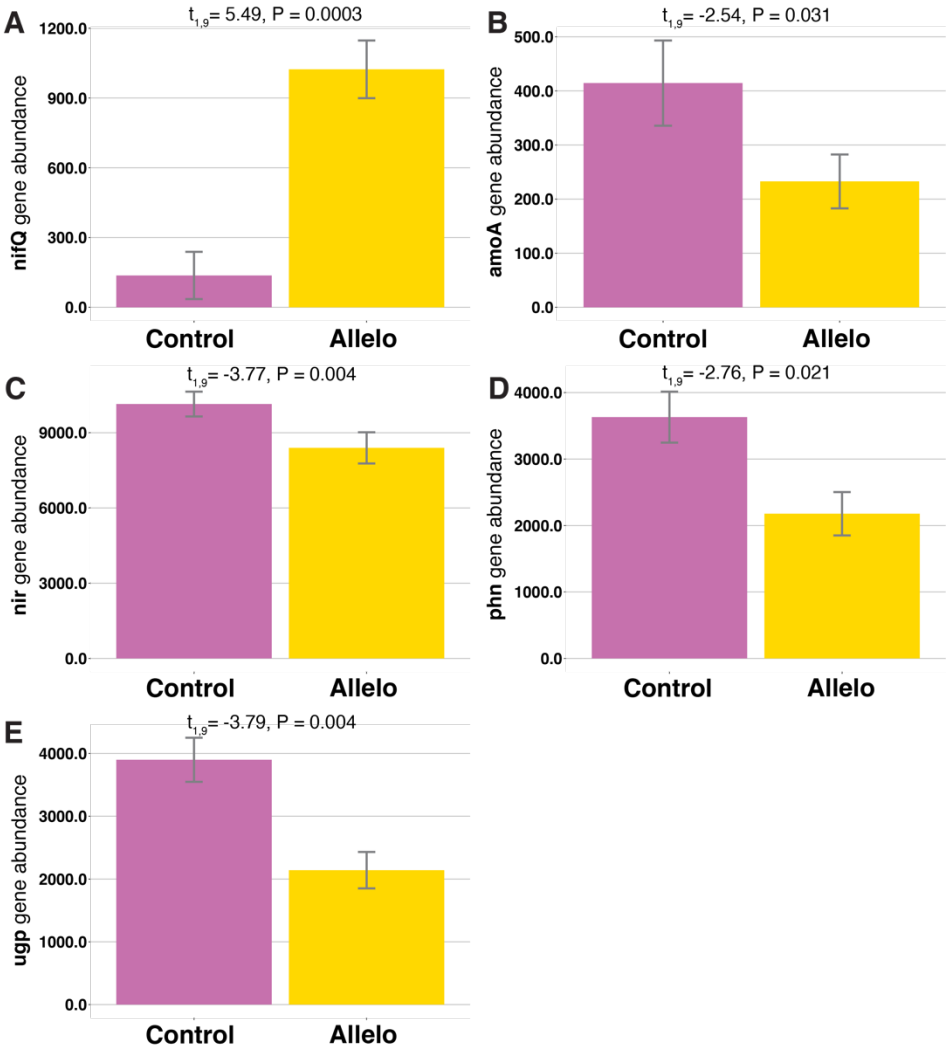


Figure 2. Microbial responses to allelochemical addition in this experiment. (a) Principal coordinate analysis of bacterial community composition (weighted UniFrac) colored by allelochemical treatment. Bacterial composition is significantly different after allelochemical addition ('Allelo'). Significant $\log_2$ fold change (LFC) of bacterial abundance at the family level, colored by bacterial phylum (b), and of fungal abundance at the genus level, colored by fungal order (c). Points represent mean LFC and lines represent standard error from DESeq2.



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687 **Figure 3.** Predicted bacterial functional genes that responded significantly to allelochemical
688 addition (Allelo). Bars are mean predicted gene abundance with standard error. A nitrogen fixation
689 gene, nifQ, increased with allelochemical addition (a), and amoA, responsible for ammonia
690 oxidation, decreased after allelochemical addition (b). Sums of genes responsible for nitrite
691 reduction (nirB, nirD, nirK) are presented for 'nir' (c), sums of genes responsible for the uptake
692 and breakdown of phosphonates (phnC, phnD, phnE, phnJ) are presented for 'phn' (d), and sums
693 of genes responsible for phosphate transport (ugpA, ugpC, ugpQ) are presented for 'ugp' (e).

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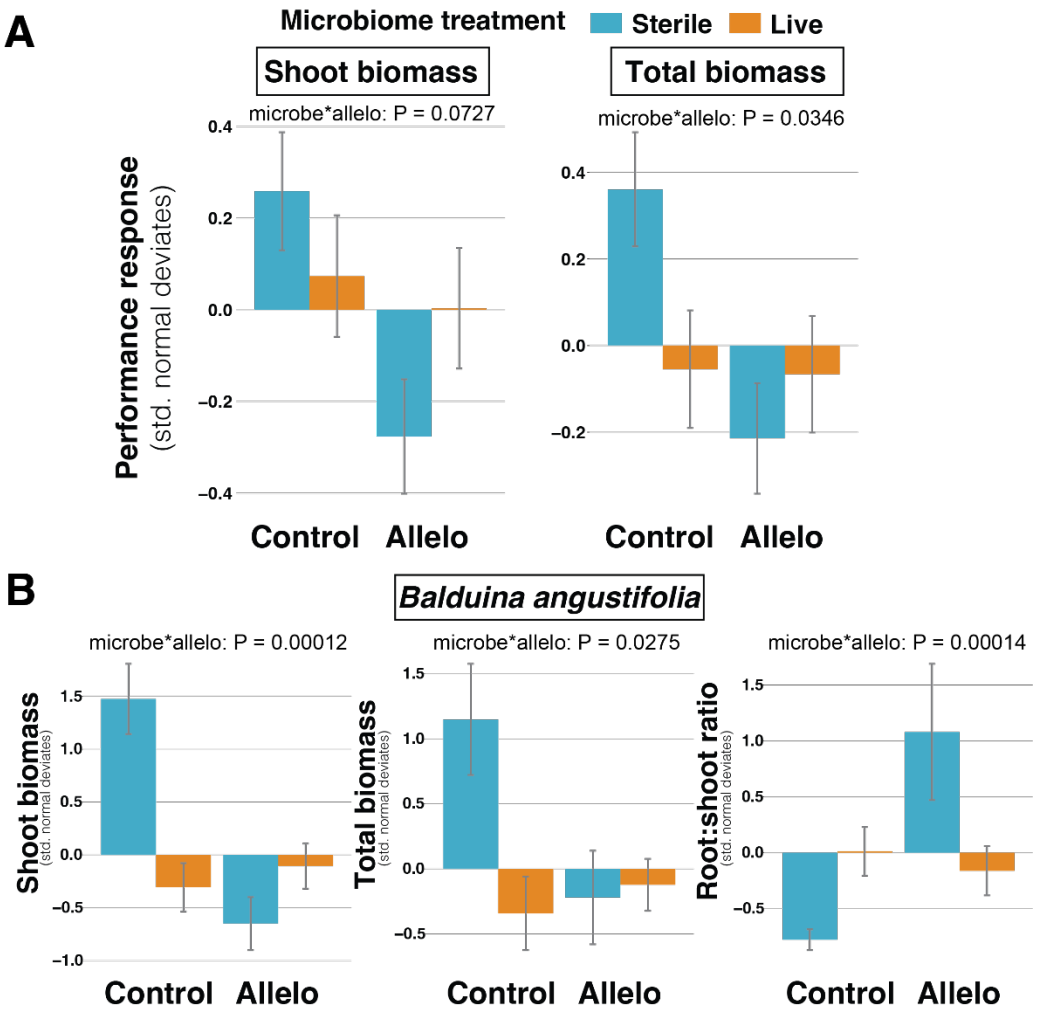


Figure 4. Plant performance responses to allelochemical addition (Allelo) treatments, colored by microbiome treatment. (a) Plant shoot biomass and total biomass responses for all plant species combined (n = 11). Overall, total biomass exhibited microbiome-mediated effects of allelochemical addition. (b) Examples of microbial-mediation of allelochemical effects in *Balduina angustifolia*. Shoot and total biomass of *B. angustifolia* were less inhibited by allelochemical addition in the presence of a microbiome. The allocation of biomass to roots was significantly lower when the microbiome was present to mediate allelochemical effects. All data

were converted to z-scores prior to analysis to standardize results within each plant species and are presented as standard normal deviates from the mean.

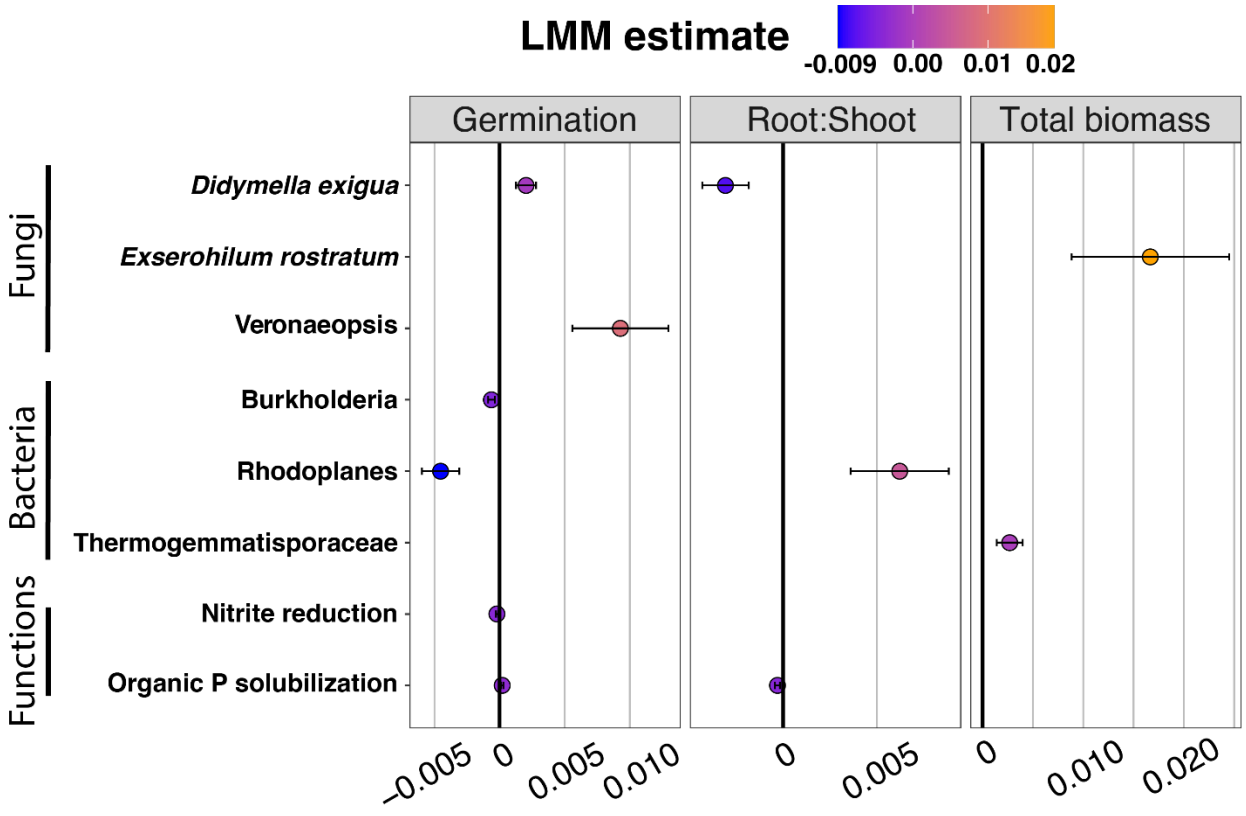
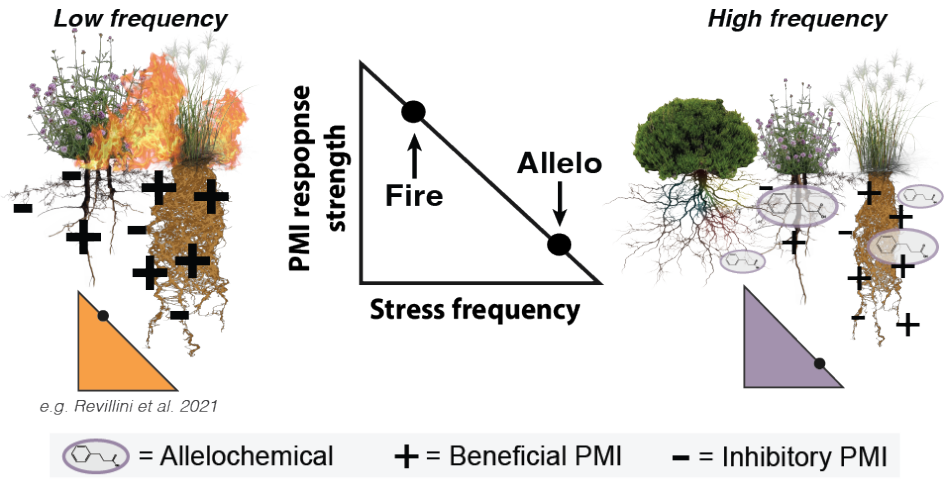


Figure 5. Significant ($P < 0.05$) linear mixed-effects model (LMM) estimates between three plant performance responses and relative abundance of microbial taxa and predicted bacterial functions that responded significantly to allelochemical addition.



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715 **Figure 6.** Potential framework explaining the relationship between stress frequency and post-
716 disturbance plant-microbial interaction (PMI) responses. We propose that infrequent stressors can
717 strongly affect belowground communities (as in Revillini et al. 2021), which leads to equally
718 strong effects on microbial mediation of plant performance (size of PMI), while a frequent stressor
719 ultimately results in moderate-to-weak microbial mediation of plant performance (this study). Size
720 of PMI interaction (+/-) is relative to microbial mediation effect under different stress conditions.

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Supplemental Tables and Figures

Supplemental Table 1. Significant results from linear mixed models of z-score standardized plant performance responses. Model predictor terms are column headers, P-values are presented when $P \leq 0.05$. ↟ = P-values presented are from performance responses prior to within plant species standardization.

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Root Biomass	<0.0001	0.205	0.204	0.235	0.0004	0.125	0.311
Shoot Biomass	<0.0001	0.876	0.021	0.074	< 0.0001	0.12	0.137
Total Biomass	<0.0001	0.2273	0.029	0.034	0.0001	0.55	0.353
Root:Shoot	<0.0001	0.478	0.832	0.608	0.005	0.002	0.016

Supplemental Table 2. Individual plant species general linear model results. For each performance response, only plant species with a significant effect are presented in bold ($P \leq 0.05$). Shading in gray indicates a negative main effect on plant performance, while main effects without shading indicate a positive main effect.

Response	Plant species	Microbiome	Allelochemical	Microbiome x Allelo
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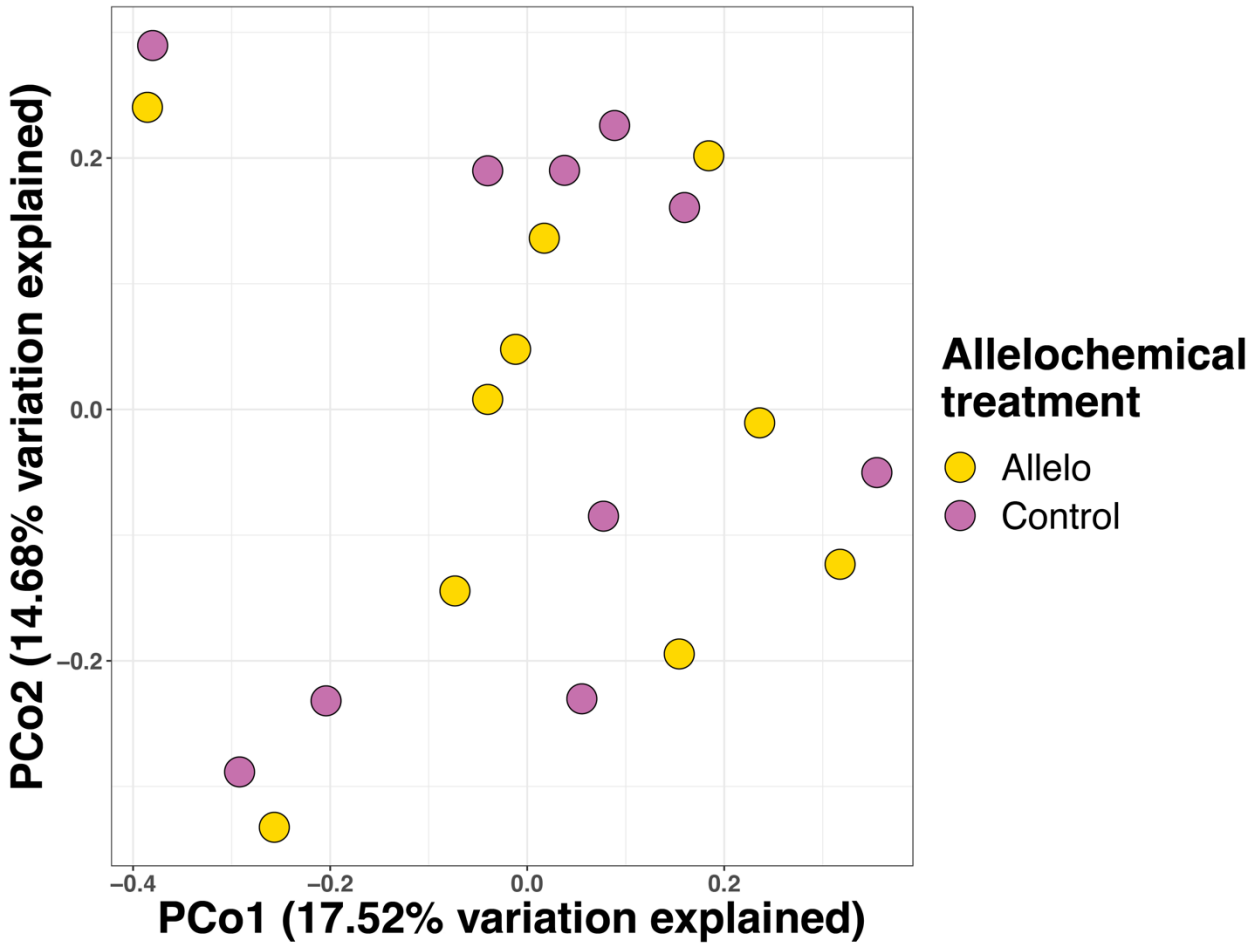
	Eryngium cuneifolium	**F = 5.41, P = 0.019**	**F = 8.33, P = 0.003**	F = 0.107, P = 0.743
	Polygonella robusta	**F = 4.61, P = 0.031**	F = 0.127, P = 0.721	F = 1.314, P = 0.251
Root Biomass	*Chamaecrista fasciculata*	**F = 27.27, P < 0.0001**	F = 0.026, P = 0.870	F = 0.370, P = 0.542
	Eryngium cuneifolium	**F = 3.84, P = 0.049**	F = 0.305, P = 0.580	F = 0.642, P = 0.422
	Lechea cernua	F = 1.624, P = 0.202	**F = 3.85, P = 0.049**	F = 2.873, P = 0.09
	Pityopsis graminifolia	F = 0.292, P = 0.588	**F = 4.8, P = 0.028**	F = 0.337, P = 0.561
	Polygonella robusta	**F = 3.8, P = 0.05**	F = 0.152, P = 0.696	F = 0.189, P = 0.663
Shoot Biomass	*Balduina angustifolia*	**F = 5.42, P = 0.019**	**F = 13.09, P = 0.0002**	**F = 19.14, P < 0.0001**
	Chapmannia floridana	**F = 23.69, P < 0.0001**	F = 0.017, P = 0.895	F = 0.467, P = 0.494
	Lechea cernua	**F = 4.85, P = 0.027**	F = 0.20, P = 0.885	F = 0.209, P = 0.646
	Pityopsis graminifolia	**F = 4.98, P = 0.025**	F = 0.732, P = 0.392	F = 0.090, P = 0.764
	Polygonella robusta	**F = 22.87, P < 0.0001**	**F = 4.75, P = 0.029**	F = 0.093, P = 0.759
Total Biomass	*Balduina angustifolia*	F = 3.06, P = 0.08	F = 1.37, P = 0.24	**F = 4.85, P = 0.027**
	Chamaecrista fasciculata	**F = 14.68, P = 0.0001**	F = 0.294, P = 0.587	F = 0.057, P = 0.81
	Eryngium cuneifolium	**F = 4.5 P = 0.033**	F = 0.015, P = 0.901	F = 0.054, P = 0.815
	Polygonella robusta	**F = 10.89, P = 0.0009**	F = 1.95, P = 0.161	F = 0.147, P = 0.700
Root:Shoot	*Balduina angustifolia*	F = 0.124, P = 0.724	**F = 13.216, P = 0.0002**	**F = 18.75, P < 0.0001**
	Chamaecrista fasciculata	**F = 27.9, P < 0.0001**	F = 1.69, P = 0.193	F = 0.488, P = 0.484
	Chapmannia floridana	**F = 12.09, P = 0.0005**	F = 0.126, P = 0.722	F = 0.243, P = 0.626
	Eryngium cuneifolium	F = 0.348, P = 0.555	F = 0.441, P = 0.506	**F = 3.82, P = 0.05**
	Lechea cernua	F = 0.584, P = 0.444	**F = 5.17, P = 0.022**	F = 0.216, P = 0.641
	Polygonella robusta	**F = 5.1, P = 0.023**	F = 3.30, P = 0.069	F = 0.027, P = 0.866



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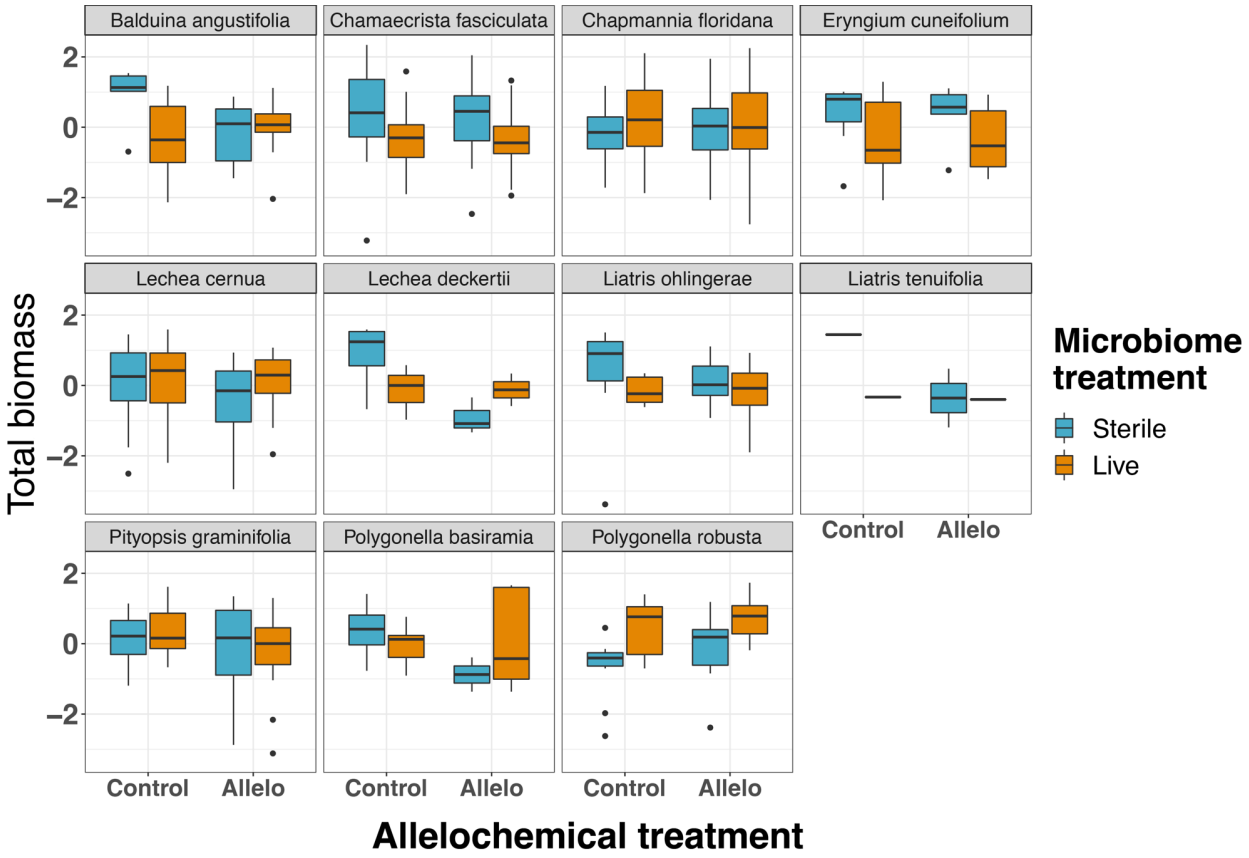
746 **Supplemental Figure 1.** Principal coordinate analysis of Bray-Curtis dissimilarity for fungal

747 communities that experienced allelopathy (‘Allelo’) or control treatments.

748

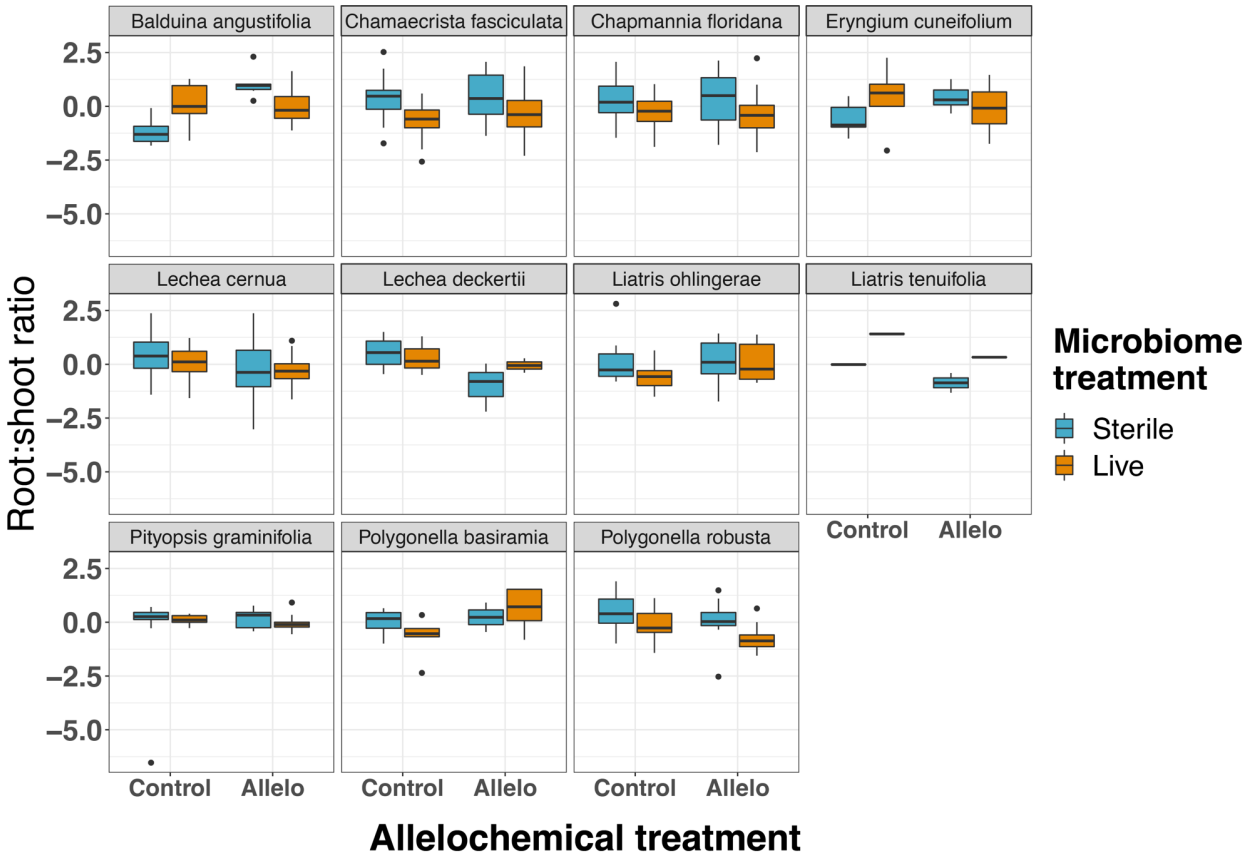


749

750 **Supplemental Figure 2.** Total biomass (z-score) response of all plant species in the study under

751 sterile and live soil conditions that experienced allelochemical or control treatments.

752



753

754 **Supplemental Figure 3.** Root-to-shoot ratio (z-score) response of all plant species in the study

755 under sterile and live soil conditions that experienced allelochemical or control treatments.