Received: 17 March 2021 | Revised: 3 November 2022 | Accepted: 8 November 2022

DOI: 10.1111/mec.16782

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

The rhizosphere microbiome and host plant glucosinolates exhibit feedback cycles in *Brassica rapa*

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Funding information
National Science Foundation, Grant/Award Number: IOS-1444571 and IOS-1547796

Handling Editor: Loren Rieseberg

Abstract

The rhizosphere microbiome influences many aspects of plant fitness, including production of secondary compounds and defence against insect herbivores. Plants also modulate the composition of the microbial community in the rhizosphere via secretion of root exudates. We tested both the effect of the rhizosphere microbiome on plant traits, and host plant effects on rhizosphere microbes using recombinant inbred lines (RILs) of *Brassica rapa* that differ in production of glucosinolates (GLS), secondary metabolites that contribute to defence against insect herbivores. First, we investigated the effect of genetic variation in GLS production on the composition of the rhizosphere microbiome. Using a Bayesian Dirichlet-multinomial regression model (DMBVS), we identified both negative and positive associations between bacteria from six genera and the concentration of five GLS compounds produced in plant roots. Additionally, we tested the effects of microbial inoculation (an intact vs. disrupted soil microbiome) on GLS production and insect damage in these RILs. We found a significant microbial treatment × genotype interaction, in which total GLS was higher in the intact relative to the disrupted microbiome treatment in some RILs. However, despite differences in GLS production between microbial treatments, we observed no difference in insect damage between treatments. Together, these results provide evidence for a full feedback cycle of plant–microbe interactions mediated by GLS; that is, GLS compounds produced by the host plant "feed-down" to influence rhizosphere microbial community and rhizosphere microbes "feed-up" to influence GLS production.

KEYWORDS
glucosinolates, insect, microbiome, plant–soil feedback, rhizosphere

1 | INTRODUCTION

Plants serve as hosts to a variety of microorganisms. Bacterial communities found in the plant rhizosphere, the zone of soil immediately surrounding and influenced by plant roots, are complex and can harbour tens of thousands of microbial taxa (Berendsen et al., 2012). Rhizosphere microbes influence many aspects of plant fitness, including defence against insect herbivores (Badri et al., 2013; Berendsen et al., 2012; Hubbard et al., 2019; Pineda et al., 2010). Plants have evolved a variety of defence mechanisms, including production of primary and secondary metabolites, to deter insect feeding, and the host genetic, transcriptomic and metabolomic bases of these defences have been the subject of intensive research for several decades (Bennett & Wallsgrove, 1994; Chan et al., 2010; Kliebenstein, 2004; Pieterse & Dicke, 2007). Beyond host regulation of its own transcriptome, rhizosphere microorganisms have been

shown to stimulate changes in plant gene expression via jasmonic acid (JA) and ethylene (ET) signalling pathways, priming plant defences against insects and pathogens in a phenomenon known as induced systemic resistance (ISR) (Berendsen et al., 2012; Pangesti et al., 2016; Pieterse et al., 2014). Some microbes have even been shown to produce plant hormones including JA, further influencing these signalling pathways (Gimenez-Ibanez et al., 2016). Microbes also assist in nutrient uptake, which may influence production of defensive metabolites (del Carmen Martínez-Ballesta et al., 2013; Hiruma, 2019; Wetzel et al., 2016). While ISR and nutrient uptake are probably contributors to plant defence, the pathways by which rhizosphere microorganisms work in tandem with (or independently of) host defence mechanisms to influence insect herbivory are not fully understood.

One of the most well-studied classes of plant defensive compounds are glucosinolates (GLS), amino-acid-derived secondary metabolites produced primarily in the family Brassicaceae (Agerbirk & Olsen, 2012). GLS are distinguished by the amino acid precursor. The two major GLS classes are aliphatic and indolic, where aliphatic GLS are those derived from methionine, isoleucine and valine, and indolic GLS are those derived from tryptophan. A few GLS are also derived from phenylalanine and are known as aromatic GLS. GLS and their hydrolysis products deter herbivory, especially by generalist insects (Hansen et al., 2008; Hiruma, 2019; Hopkins et al., 2009), inhibit bacterial, nonhost fungal, and oomycete pathogens (Bednarek et al., 2011; Fan et al., 2011; Hiruma, 2019; Schlaeppi et al., 2010; Wang et al., 2013), and influence plant responses to abiotic stresses (Salehin et al., 2019). While GLS serve as constitutive defences, the expression level and composition of GLS can also be induced and regulated by several plant hormones including JA, salicylic acid (SA) and ET (Bennett & Wallsgrove, 1994; Guo et al., 2013; Pangesti et al., 2016; Schreiner et al., 2009, 2011). Additionally, as nitrogen-, sulphur- and glucose-containing compounds, GLS production is also influenced by the availability of these nutrients and further regulated by plant phosphate, potassium and iron levels (del Carmen Martínez-Ballesta et al., 2013; Guo et al., 2013; Hiruma, 2019; Samira et al., 2018).

Given the complex relationships between nutrient availability and induction by the JA, SA and ET signalling pathways, GLS levels and composition are probably influenced by microbes in the rhizosphere. While several studies have examined the effects of specific strains of plant-growth-promoting (PGP) bacteria on GLS production in *Arabidopsis thaliana*, the results are mixed with some studies showing either increased or decreased GLS with microbial inoculation and others showing no differences. The effect of rhizosphere bacteria on GLS composition and concentration probably depends on many factors including the strain of inoculum, life stage of the plant and environmental conditions (Brock et al., 2013, 2018; Pangesti et al., 2016; Witzel et al., 2017). On the other hand, research is limited as to how complex microbial communities that reflect those found in field settings affect GLS production. Hubbard et al. (2019) found that three different soil microbial communities led to different levels of insect herbivory in the wild mustard *Boechera stricta*, but no differences in GLS production. A better understanding of how the rhizosphere microbial community influences GLS production and, in turn, insect herbivory has implications for evolutionary dynamics. In particular, plant evolutionary responses will be reduced if microbial associates rather than host plant genetic variance disproportionately affect GLS phenotypes.

Plant–microbe interactions may exhibit a complete feedback cycle, in which microbes in the rhizosphere not only influence plant defence as described above but are also affected by the host plants. One mechanism by which plants modulate the composition of the rhizosphere microbiome is via secretion of root exudates, which can select for or inhibit particular microbes (Bais et al., 2006; Bulgarelli et al., 2013; Hu et al., 2018; Huang et al., 2019; Zhalnina et al., 2018). Several secondary metabolites, including GLS, have been shown to inhibit certain soil microorganisms and potential pathogens. In vitro inhibition of fungal and bacterial cultures by GLS compounds has been well documented, and the effect is dose-dependent and varies based on the organism as well as the compounds in question (Aires et al., 2009; Sotelo et al., 2015). Additionally, Bressan et al. (2009) demonstrated that production of a single exogenous GLS in *A. thaliana* had significant impacts on rhizosphere fungal and bacterial community composition. However, some microbes have developed resistance to GLS (Fan et al., 2011) and the role of segregating variation in GLS production on the composition of complex microbial communities remains largely unknown. The current research advances our understanding of mechanisms (metabolite exudation) by which plant hosts may modulate the rhizosphere microbiome to their advantage (Brachi et al., 2022; Hubbard et al., 2018).

The goal of the current study was to examine both microbial effects on secondary compounds and plant insect damage as well as plant effects on rhizosphere communities, both as mediated through GLS. Specifically, we tested whether naturally segregating variation in GLS production in roots of *Brassica rapa* correlated with differences in rhizosphere microbial community composition. We then tested the effects of intact vs. disrupted microbiome treatments on GLS production in host plant leaves and if these microbial community differences affect insect herbivory. More specifically, we were interested in ascertaining whether GLS mediate a complete feedback cycle between microbial communities and their host plants.

2 | MATERIALS AND METHODS

2.1 | Plant genotypes and growth conditions

Plants used in this study were recombinant inbred lines (RILs) of *Brassica rapa* developed from a cross between a yellow-sarson oilseed (R500) and a genotype of the rapid cycling Wisconsin Fast Plant (IMB211). These RILs are the product of at least eight generations of self-fertilization and single-seed descent resulting in an expected 99% homozygosity within these immortal lines. We selected 12 of these RILs based on multilocus single nucleotide polymorphism (SNP) genotype and leaf GLS phenotypes from previous field studies

(RE Kerwin, C Weinig, and DJ Kliebenstein; unpublished data): three of each possible combination of low or high predicted aliphatic and indolic GLS (high aliphatic and high indolic [HH], high aliphatic and low indolic [HL], low aliphatic and high indolic [LH] and low aliphatic and low indolic [LL]; Figure S1). Due to recombination and independent assortment, each of the six RILs per aliphatic or indolic category are unique multilocus genotypes, providing replication and limiting the potential for other, non-GLS, traits to impact microbial communities.

Replicate seeds of each RIL were sown into one of two treatments: an "intact" or "disrupted" microbiome treatment. While the disrupted treatment was recolonized via aerial microbes during the course of the experiment, it acts as a control treatment allowing for comparisons between plants growing with a functional microbial community and those growing without. RIL seeds were first sterilized in 70% ethanol for 1 min followed by 10% bleach for 10 min, and sown directly into a 1:1 mix (v/v) of Profile Greens Grade clay (Profile) and SunGro propagation mix (Sungro Horticulture). The soil matrix was autoclaved for 1 h, left to rest for 24 h and autoclaved for an additional hour. For the "intact microbiome" treatment, an inoculate of sieved soil was added at 5% by volume to the soil matrix. The soil used as inoculate was collected from Road 234 in the Medicine Bow National Forest near Laramie, WY (41.325837° N, 106.465902° W; 2720 m). This site is a disturbed road-side forest plot inhabited by the native *Boechera stricta* (Brassicaceae), and previous experiments in our laboratory using inocula of this soil microbial community have resulted in plant growth promoting effects in both *Boechera stricta* and *B. rapa* (MT Brock, CJ Hubbard, and C Weinig, unpublished data). Using sterile gloves and trowels, soil was collected from this site, sieved (2 mm) to homogenate and remove rocks, roots, etc., bagged in sterile whirl packs, and archived at −20°C. For the "disrupted microbiome" treatment, sieved soil from Road 234 was autoclaved for 40 min and added to the bulk soil mix at 5% by volume. Plants were watered daily with reverse osmosis (RO) water. All experiments were performed in greenhouses and field sites at the Agriculture Experimental Station in Laramie, WY, USA (41.3198° N, 105.5598° W; 2217 m).

2.2 | Experiment 1: Effect of GLS on the rhizosphere microbiome

To investigate the effect of GLS production on the rhizosphere microbiome, 10 replicate pots of three plants of each *B. rapa* genotype were planted in each of the soil microbiome treatments in a randomized block design. Plants were grown in a greenhouse for 5 weeks, after which rhizosphere soil for microbial community characterization was collected from eight replicate pots and root tissue for GLS analysis was collected from six replicate pots. However, eight samples had insufficient root mass for GLS analysis. As described above, three replicate plants were grown in each pot, and the roots of plants within a pot were intertwined but could be separated to sample individual plants. Paired rhizosphere and root tissue samples were thus collected from replicate plants within the same pot. We chose to collect replicate samples from the blocks with the highest germination rates.

For GLS quantification, root tissue from one plant from each of six replicate pots of each genotype was rinsed with RO water, flash frozen in liquid nitrogen and stored at −80°C until samples could be freeze-dried over 24 h and bead-beaten. Up to 50 mg of ground root tissue was sent to the University of California at Davis for GLS quantification. GLS were extracted from the samples and quantified by HPLC (high-performance liquid chromatography) as previously described (Chan et al., 2011; Kliebenstein et al., 2001).

Rhizosphere samples were collected from a single plant from each of eight replicate pots of each genotype for characterization of the microbial community. To extract rhizosphere microbial DNA, bulk soil (>1 mm from the roots) was shaken from roots, and the remaining soil closely adhering (within 1 mm) to roots was defined as the rhizosphere. Roots with adhered rhizosphere soil were agitated for 15 min in phosphate-buffered saline solution (PBS) with 0.01% Silwet L-77 detergent. Roots and larger soil particles were removed by passing the solution through a steriflip filter (Millipore). The collected solution was centrifuged at 3000 rcf for 15 min. The supernatant was removed, and the soil pellet was stored at −80°C until DNA extraction. DNA was extracted from 250 mg or less of the soil pellet using the Qiagen Dneasy PowerLyzer PowerSoil kit (Qiagen) according to the manufacturer's instructions. Library preparation of the V4/V5 region of the 16 S rRNA gene was performed at the Marine Biological Laboratory using previously described methods (Newton et al., 2015) and sequenced using paired-end 250-nt reads on the Illumina MiSeq platform (Illumina). We used the package DADA2 in R to filter and trim based on quality, denoise, merge paired-end reads, and remove chimeras (Callahan et al., 2016; R Core Team, 2020). Taxonomy was assigned with DADA2 using the SILVA reference database (version 132; Quast et al., 2013). To test for differences in microbial community composition among genotypes and GLS concentration, we used the PHYLOSEQ (McMurdie & Holmes, 2013) and VEGAN (Oksanen et al., 2020) packages to perform PERMANOVA (permutational multivariate analysis of variance; via the *adonis* function) and principal coordinates analysis (PCoA) on Bray–Curtis dissimilarity matrices based on nonrarefied sequence data. We tested for differences in microbial diversity between samples by calculating Shannon's alpha diversity indices based on rarefied sequence data using PHYLOSEQ (McMurdie & Holmes, 2013).

Because ordination approaches test only for whole-community shifts in composition, we used a Bayesian Dirichlet-multinomial regression model (DMBVS) to test for potential effects of endogenous host plant GLS on individual rhizosphere bacterial genera (Wadsworth et al., 2017). DMBVS models the sequence counts with a Dirichlet-multinomial distribution where the γ parameters of the Dirichlet-multinomial are informed by regression models of the predictors. Because the modelling results in individual regression models for each bacterial genus, DMBVS uses spike and slab priors for regularizing the coefficients of the regression predictors, in our case GLS, to probabilistically identify the GLS compounds

associated with changes in microbial abundance. This enables us to evaluate thousands of regression models simultaneously and protect against type II statistical errors associated with identifying important GLS compounds in regression analysis. Here we report on GLS compounds that had a marginal posterior probability of inclusion (MPPI) of 1; that is, after burn-in, the GLS compounds we identified as important were included in each accepted Markov chain Monte Carlo (MCMC) sample. We ran the analysis for 1,000,001 MCMC steps, with a burn-in of 300,001, and thinned the chains to every 20th sample. Chains for each parameter were well mixed and free of autocorrelation. For priors, we used the parameters evaluated and recommended by Wadsworth et al. (2017). They found that a relatively flat beta distribution leads to a reasonable balance between false positive and false negative covariates included in the model. Following their advice for the penalization prior, we set the priors as $\alpha = 0.02$ and $\beta = 1.98$ in a beta distribution. This is a fairly weak prior that assumes ~1% of covariates will be included in the final model. For the regression parameter priors, we used diffuse priors, again following the guidance of Wadsworth et al. (2017). Using a normal distribution with a mean of zero and large variance such as 10 results in robust posterior distributions that are insensitive to the priors. One benefit of regularized regression comes from reducing the number of predictors in the final model that are correlated. In our final model, only two variables had a Pearson correlation coefficient greater than 0.5, I3M and 4MOI3M. These two compounds showed a correlation of 0.66. In the final models, I3M and 4MOI3M were only selected by the model when they had opposing effects on microbial abundance.

We performed regression analysis both at the genus level (presented in the text) and the amplicon sequence variant (ASV) level (presented in the Supporting Information) and included only six GLS compounds that were detected in greater than 10% of root tissue samples. To account for a potential effect of block, we tested the model including block as a covariate. However, this did not affect the associations between GLS and bacterial taxa, and we therefore present a simplified model including only GLS and microbe associations. Additionally, GLS did not exhibit a significant block effect, making it unlikely that microsite heterogeneity within the greenhouse influenced associations between microbial taxa and GLS.

To compare the fine-scale DMBVS modelling with more traditional methods, we first performed PERMANOVA using microbial data from only those taxa that were significantly associated with GLS compounds in the DMBVS model. These two approaches generally support the effects of GLS compounds on the relative abundance of taxa; GLS compounds that influence a greater number of taxa in the DMBVS model are more significant in the PERMAOVA framework. We also used R/ANCOMBC (Lin & Peddada, 2020), which utilizes linear regression to test the abundance of genera on GLS compounds. Results show that four of the five GLS compounds (3-butenyl, I3M, 4MOI3M and phenylethyl) influence numerous microbial genera (Table S5; Figure S3) and corroborate the significance and directionality of 11 of the 17 associations identified by DMBVS (Figure S4). In using ANCOM-BC2 for statistical confirmation, we noted that this approach identified significant connections between four GLS compounds (3-butenyl, I3M, 4MOI3M and phenylethyl) and 24 bacterial genera (Figure S3). Given the greater sensitivity of the DMBVS and its multivariate hierarchical structure, which enables testing multiple predictor and multiple response variables simultaneously, we present these results in the primary text but provide PERMANOVA and ANCOM-BC2 results for reference in the Supporting Information (Tables S1, S2 and S5; Figures S3 and S4). We further note that while cross-validation across statistical approaches is important (Nearing et al., 2022), resulting statistical associations define hypotheses that require testing through direct validation.

2.3 | Experiment 2: Effect of the rhizosphere microbiome on GLS and insect damage

A separate experiment was performed to investigate the effect of intact vs. disrupted soil microbial communities on GLS production and susceptibility to insect damage. We planted 10 replicate pots with two plants per pot of each *B. rapa* RIL in each soil microbial treatment (intact vs. disrupted; planted as described above) in a randomized block design. On the third week of growth, plants were placed outside for 2–6 h each day to allow them to adjust to UV light exposure in preparation for relocating to the field. After 3 weeks of growth but prior to relocating, we collected 7-mm leaf discs from the second and third true leaves of one plant from each of six replicate pots from each RIL in the LL and HH GLS categories (R500 × IMB211; RILs 30, 36, 183, 284, 337, 357) from both the intact and disrupted microbial treatments. Leaf tissue was flash frozen and stored at −80°C. Up to 50 mg of ground frozen leaf tissue was sent to the University of California at Davis for GLS characterization as described above. On June 28, pots were placed in the field adjacent to the greenhouse where flea beetles (*Phylotreta* spp.) had been previously observed. Plants were grown in the presence of flea beetles for ~16 days after which the third and fourth true leaves were collected from up to nine replicated plants of each genotype and microbial treatment. Leaves were scanned using an Epson V700 scanner, and WINRHIZO was used to quantify leaf damage as the fraction of leaf area removed or dead, based on colour classification. We used two-way ANOVAs and mixed effects models with block as a random intercept using the R packages LME4 (Bates et al., 2015) and LMERTEST (Kuznetsova et al., 2017) to determine the effects of genotype (i.e., RIL) and microbial inoculation on GLS concentrations and leaf damage. Figures were created using GGPLOT2 (Wickham, 2016).

3 | RESULTS

3.1 | Influence of host GLS production on rhizosphere microbial community (experiment 1)

We obtained GLS data from 64 root samples. Total GLS levels in root tissue ranged from 0 to 0.53 nmol mg^{-1}. We detected two aliphatic GLS compounds (3-butenyl and 4-pentenyl), three indolic GLS

(1-methoxyindol-3-ylmethyl [1MOI3M], 4-methoxyindol-3-ylmethyl [4MOI3M] and indol-3-ylmethyl [I3M]) and one aromatic GLS (phenylethyl). Two additional compounds, 4-methylsulfinylbutyl (4MSO) and 4-hydroxyindol-3-ylmethyl (4OHI3M), were each detected in only two and four samples respectively and were not used in analysis. Significant differences in total, aliphatic and indolic GLS production in roots were observed between genotypes (all $p < .0001$, $n = 64$; Figure 1). Average aliphatic GLS (3-butenyl and 4-pentenyl) among genotypes varied nearly 30-fold from 0.0068 to 0.2 nmol mg^{-1} while average indolic GLS (1MOI3M, 4MOI3M and I3M) ranged ~6-fold from 0.014 to 0.093 nmol mg^{-1}. The aromatic compound phenylethyl GLS was detected in five of the 12 RILs, with averages ranging from 0 to 0.034 nmol mg^{-1} (Figure 1).

For the microbial sequencing data, we retained 16,071,465 high-quality, nonchimeric reads from 22,418,992 raw reads across 96 samples. Reads represented 5327 bacterial ASVs that mapped to taxa from 27 bacterial phyla. Microbial communities consisted predominantly of taxa from the phylum Proteobacteria (52.9%), followed by Bacteroidetes (31.8%) and Actinobacteria (8.2%). Based on PCoA and PERMANOVA of Bray–Curtis dissimilarities, no significant differences in microbial community were observed between genotypes ($p = .17$), or based on concentrations of total, indolic or aliphatic GLS (all $p > .56$), indicating that there was no common response of the majority of microbial community members to host plant genotype or GLS phenotype. Alpha diversity measures also did not show an association with total, indolic or aliphatic GLS concentrations (all $p > .52$).

Because PCoA of Bray–Curtis dissimilarity identifies only coarse-grained changes in complex communities, we used a Bayesian Dirichlet-multinomial regression approach to model the relative abundance of individual microbial genera as a function of GLS concentration. We found several highly supported associations (MPPI of 1) between five of the six GLS compounds analysed and six bacterial genera (*Chryseobacterium*, *Stenotrophomonas*, *Pedobacter*, *Pseudomonas*, *Phyllobacterium* and *Paenarthrobacter*) from three different phyla (Proteobacteria, Actinobacteria and Bacteroidetes; Figure 2). Analysis at the ASV level returned similar results (Figure S2). Furthermore, 11 of the 17 GLS–bacterial genera associations were supported by ANCOM-BC2 analyses (Table S5; Figures S3 and S4), and indeed ANCOM-BC2 identified a further 30 GLS–bacterial associations.

3.2 | Influence of soil microbial treatment on GLS production and insect herbivory (experiment 2)

We obtained GLS data from 69 leaf samples. Total GLS concentrations in leaf tissue ranged over 800-fold, from 0.014 to 9.6 nmol mg^{-1}, among individuals. We detected three indolic GLS (1MOI3M, I3M and 4MOI3M), and three aliphatic GLS (3-butenyl, 4MSO and 5-methylsulfinylpentyl [5MSO]). We grouped the aliphatic GLS into two subcategories: alkenyl (3-butenyl and 4-pentenyl) and MSO (4MSO and 5MSO). MSO GLS are the precursors used by the AOP2 enzyme to make the respective alkenyl GLS; 4MSO is the precursor for 3-butenyl and 5MSO is the precursor for 4-pentenyl. The most prevalent compound was 3-butenyl GLS, which constituted 90% of total GLS detected. The effect of microbial inoculation on GLS production varied by plant genotype and GLS compound (Figure 3). The interaction of microbial treatment × genotype was significant for the alkenyl GLS ($p = .017$) as well as the overall total GLS ($p = .013$) and aliphatic total ($p = .014$)—as these were strongly driven by 3-butenyl. The genotypes producing high levels of the alkenyl GLS in comparison to the other RILs in the disrupted treatment expressed even higher levels of these compounds in the intact microbial treatment. This difference was significant for RIL 357. On the other hand, genotypes producing high levels of MSO GLS in the disrupted microbial treatment produced lower levels in the intact microbiome, and this difference was significant for RIL 30.

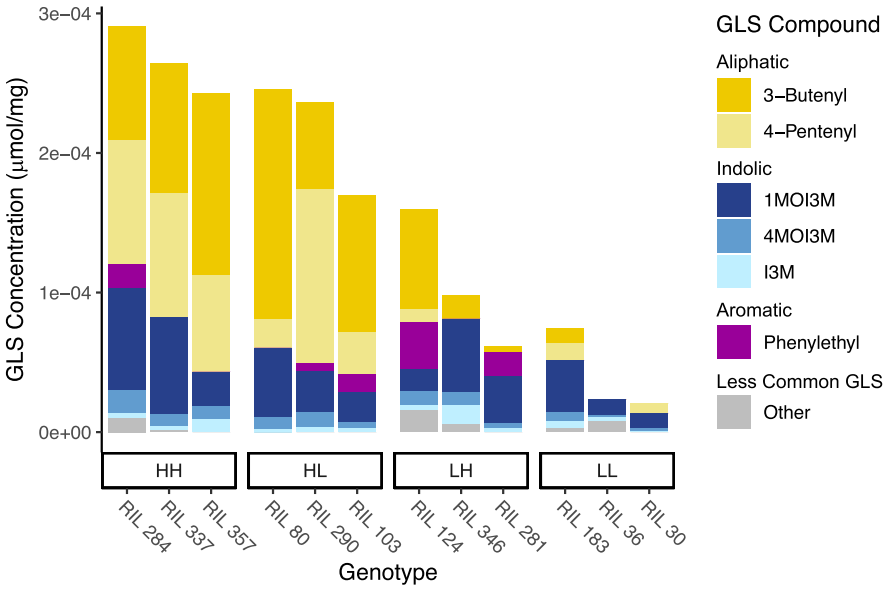

FIGURE 1 Concentrations of GLS compounds detected in roots of each RIL. Aliphatic compounds (3-butenyl and 4-pentenyl) are shown in yellow, indolic compounds (1MOI3M, 4MOI3M and I3M) are shown in blue, and phenylethyl, an aromatic compound, is shown in purple. Compounds detected less frequently are shown in grey and include aliphatic, indolic and aromatic compounds. The designations along the *x*-axis indicate predicted GLS category (HH: High aliphatic, high indolic; HL: High aliphatic, low indolic; LH: Low aliphatic, high indolic; and LL: Low aliphatic, low indolic)

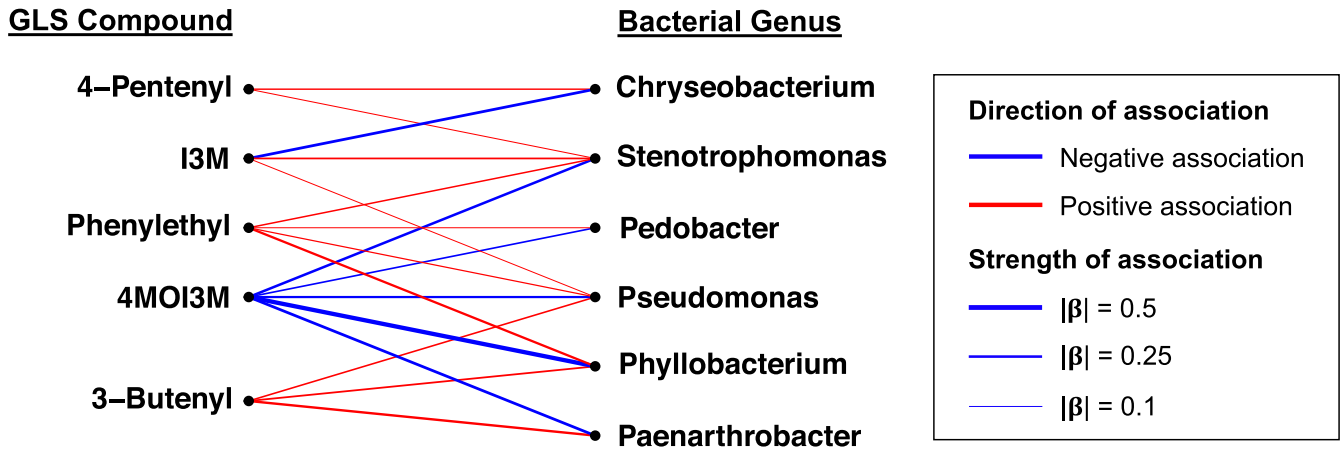

FIGURE 2 Associations between GLS compounds and rhizosphere microbial taxa with MPPI = 1. Microbial taxa were aggregated by genus for this analysis. Positive associations are shown in red while negative associations are shown in blue. The width of the line is proportional to the magnitude of association

We found no effect of microbial treatment on insect damage (Figure 4) for any genotype (n = 168). While genotypes exhibited significant differences in flea beetle damage, these differences were not associated with differences in GLS concentrations.

4 | DISCUSSION

Recent research has unveiled the importance of the rhizosphere microbiome in plant health (Berendsen et al., 2012; Hubbard et al., 2019; Pineda et al., 2010) and that host plant phenotypes also shape the microbial community in the rhizosphere (Bulgarelli et al., 2013; Hu et al., 2018). However, the details of these complex interactions have yet to be unravelled. To address feedback cycles in plant–microbe interactions, we tested both the effect of the rhizosphere microbiome on plant defence and the effect of the host plant's GLS phenotype on rhizosphere microbes.

The first objective of our study was to investigate the effects of variation in GLS production on the rhizosphere microbial community using *Brassica rapa* RILs. We found that differences in root GLS production among our 12 RILs did not lead to coarse-grained differences in microbial community composition or diversity as shown by PCoA of Bray–Curtis dissimilarity. However, Bayesian Dirichlet-multinomial regression modelling revealed fine-scale associations between the concentration of GLS compounds in roots and the relative abundance of several microbial taxa in the rhizosphere. In our experiment, each pot contained a common homogeneous potting mix and was inoculated with the same starting microbial community when seeds were planted, thus identifying effects of host plant GLS on the trajectory of microbial community composition rather than other micro-environmental edaphic effects. Additionally, given that our plants are RILs, variation at GLS loci should be independent of variation segregating elsewhere in the genome. While it is possible that unmeasured factors that correlate with GLS explain microbial community composition, we hypothesize that associations between GLS and microbial taxa represent a direct effect of GLS on these microbes, due to the common inoculation schedule, the otherwise controlled growth conditions in the greenhouse and the magnitude of segregating host genetic variation in GLS.

4MOI3M GLS was negatively associated with five bacterial genera in our DMBVS analyses, a result supported by many significant negative associations detected via ANCOM-BC2 (Table S5; Figure S3). Indolic GLS, especially 4MOI3M, and their hydrolysis products have been shown to play important roles in inhibition of both beneficial (Anthony et al., 2020; Nongbri et al., 2012) and pathogenic fungi (Bednarek et al., 2011; Clay et al., 2009; Hiruma, 2019) as well as oomycete pathogens (Schlaeppi et al., 2010), suggesting this compound inhibits soil microorganisms. While studies of the effects of this compound on soil bacteria are more limited, our results suggest that 4MOI3M may also play an important role in inhibiting bacteria. By contrast, the alkenyl GLS tested were each positively associated with several microbial taxa. In leaf tissue, concentrations of these alkenyl GLS increased in response to intact microbiomes in some of our RILs. As such, the positive association may represent stimulation of alkenyl GLS production by these taxa, a pattern that has also been demonstrated in other aliphatic GLS (Brock et al., 2013; Pangesti et al., 2016). Alternatively, the positive association may reflect that higher concentrations of alkenyl GLS select for bacteria that can degrade these compounds for use as a carbon and energy source. For example, high alkenyl GLS concentrations enhanced the growth of the plant growth-promoting bacterium *Kosakonia radicincitans* DSM 16656 on Brassicaceae plant extracts (Schreiner et al., 2009). Phenylethyl was also positively associated with four bacterial genera, most probably indicating induction of GLS production (Schreiner et al., 2011). Finally, I3M formed several positive associations with *Pseudomonas*, *Pedobacter* and *Stenotrophomonas* as well as a relatively strong negative association with *Chyseobacterium*. These probably represent a mixture of induction and direct effects, and more research is required to determine the mechanisms of these associations.

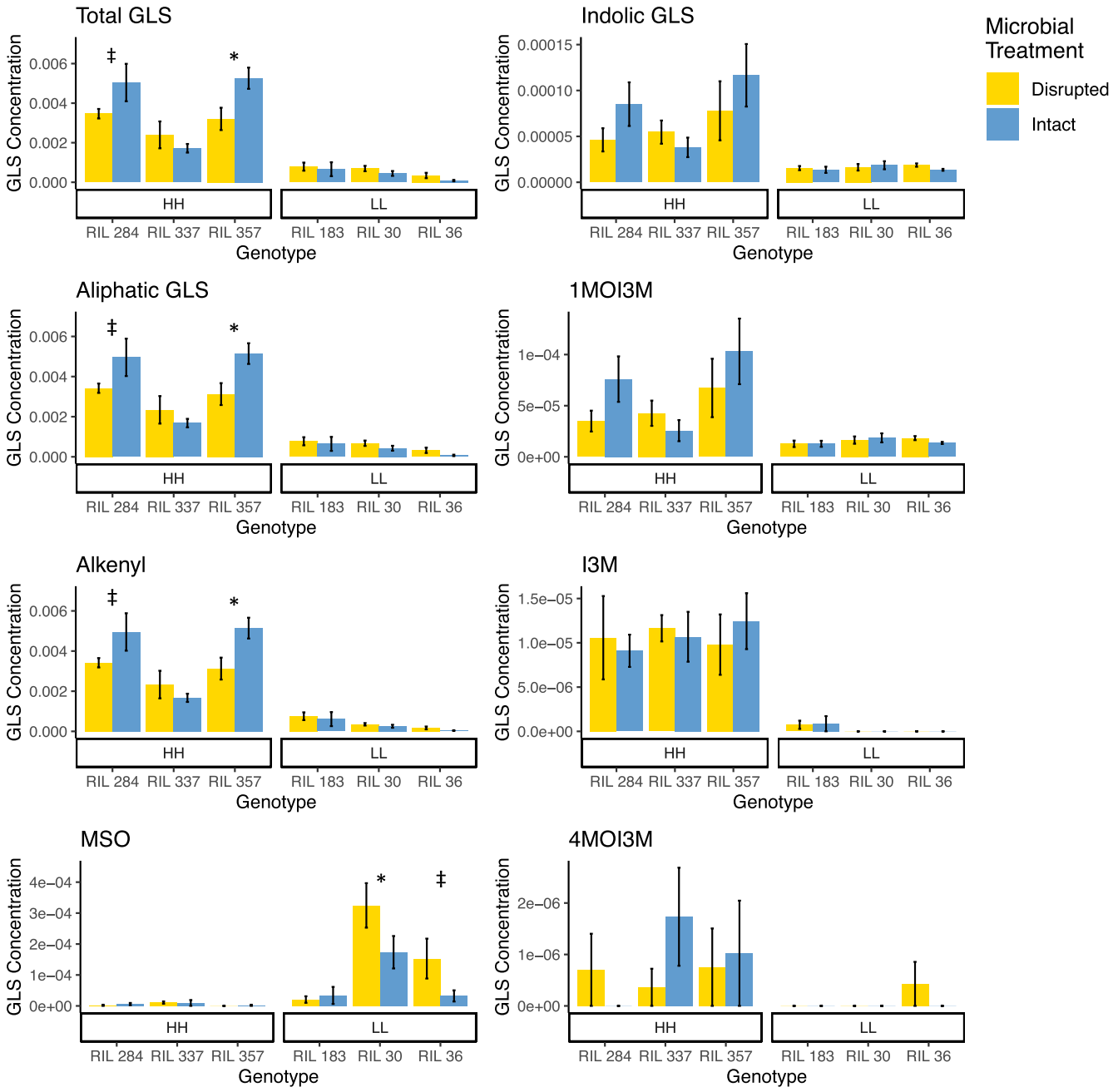

FIGURE 3 GLS concentrations ($\mu mol\, mg^{-1}$) detected in leaves of each of six RILs for plants grown in disrupted (yellow) and intact (blue) soil microbial treatments. GLS compounds shown on the left (alkenyl and MSO) are aliphatic while compounds shown on the right are indolic (1MOI3M, I3M,and 4MOI3M). "*" denotes a significant difference in GLS between microbial treatments where $p < .05$, and "‡" indicates a marginally significant difference where $.05 < p < .1$

Our second objective was to examine the effects of the rhizosphere microbiome on GLS production and insect damage. Many recent studies have found an effect of soil microbial community on plant metabolite production and insect herbivory (Badri et al., 2013; Hubbard et al., 2019; Pineda et al., 2010). In this study, the effect of microbial treatment on GLS production depended on both the plant genotype and GLS compound in question. Specifically, the RILs producing high levels of total GLS produced even higher levels in the intact microbiome as compared to the disrupted treatment. This pattern was driven by the alkenyl GLS (3-butenyl and 4-pentenyl) while the opposite pattern was observed for MSO GLS (4MSO and 5MSO). The RILs varied in whether alkenyl or MSO GLS were the predominant GLS and accordingly these genotypes differed in whether GLS was induced by the microbial treatment. The production of alkenyl or MSO GLS is controlled by structural variation altering the expression of the AOP2 enzyme that catalyses the conversion of MSO to alkenyl GLS. This suggests that variation at the *AOP2* locus between these RILs may be connected to the response of GLS production to microbial treatment. Likewise, 1MOI3M GLS showed a similar, although nonsignificant, pattern in which RILs 284 and 357 exhibited

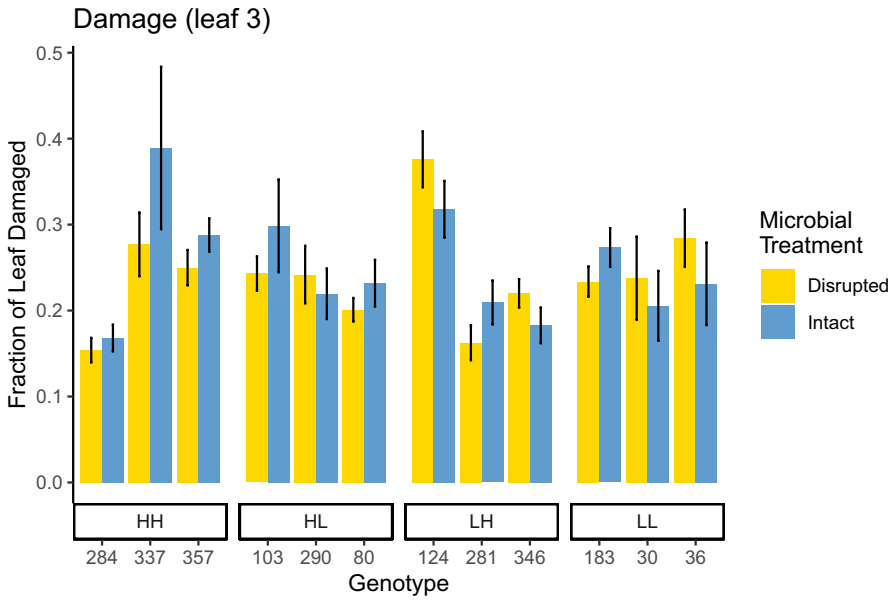

FIGURE 4 Insect damage on the third true leaf for plants of each of 12 RILs grown in disrupted (yellow) and intact (blue) soil microbial treatments. The designations along the *x*-axis indicate predicted GLS category (HH: High aliphatic, high indolic; HL: High aliphatic, low indolic; LH: Low aliphatic, high indolic; and LL: Low aliphatic, low indolic)

higher GLS in the intact microbiome relative to the disrupted microbiome treatment. It is possible that 1MOI3M production is also induced by the intact microbiome.

Despite differences in GLS produced in an intact and disrupted soil microbiome observed in some RILs, there were no differences in insect damage between the two microbial treatments. Additionally, differences in insect damage were observed between genotypes, but these differences did not relate to variation in GLS levels. It is probable that the effects of microbial community on plant defence mechanisms vary based on environmental factors, plant host species and genotype, and attributes of the soil microbial communities in question. The effect of GLS as plant defensive compounds depends on the combination of compounds produced and the insect herbivore in question. Generalist herbivorous insects tend to be inhibited by GLS compounds, while specialized insects feeding on GLS-producing members of the family Brassicaceae may preferentially feed on plants with high GLS levels (Hopkins et al., 2009). Some insect species, including *Phyllotreta striolata*, have even been reported to sequester GLS to aid in their own protection against natural predators (Beran et al., 2014; Hopkins et al., 2009). We believe the majority of damage in this study was caused by *Phyllotreta* sp. (flea beetles), but other insect herbivores such as grasshoppers were also observed in the field. A mixture of specialist and generalist insect herbivores probably confounded the association between insect damage and GLS level, leading to the lack of association. GLS levels are also induced by insect herbivory (Hopkins et al., 2009; Pangesti et al., 2016) and, while we measured GLS levels before the plants were subjected to insect damage, we do not know how GLS levels may have changed in response to early insect damage and may have influenced insect feeding later in the field experiment.

Modulation of the soil microbiome via root exudates represents a means by which plants influence their local environment and can enhance individual performance and the performance of future generations (Hu et al., 2018). Plant–microbe or plant–soil feedback cycles also play an important role in plant performance, diversity and community structure (Bennett et al., 2017; Teste et al., 2017). Our study identified both positive and negative associations between rhizosphere microbial taxa and GLS concentrations in roots, as well as an effect of soil microbial treatments on GLS production. Together, these results provide evidence for the role of GLS in a plant–microbe feedback cycle; that is, GLS compounds produced by the plant "feed-down" to influence the rhizosphere microbial community and, in turn, these rhizosphere microbes "feed-up" to influence GLS production. While plant genotype is commonly expected to affect GLS phenotypes, our results indicate that microbial communities (in a "feed-up" manner) may affect the expression of GLS and hence the opportunity for natural selection. The potential for an iterative feedback cycle where host plants modulate the microbial community via GLS exudation suggests the potential for complex evolutionary dynamics in plant secondary compounds as well as complex patterns of microbial community succession.

AUTHOR CONTRIBUTIONS

E.D., M.B., D.K. and C.W designed the research, E.D., M.B., E.K., B.L. and H.M. performed the research, E.D., M.B., W.J.C., D.K. and C.W analysed the data, and all authors participated in writing the manuscript.

ACKNOWLEDGEMENTS

This research was supported by NSF grant IOS-1444571 to C.W., L.M. and M.T.B., NSF grant IOS-1547796 to C.W., and the Wyoming Research Scholars Program. We are grateful to Monique Weaver, Sara Jean O'Neill, and Emma Fox-Fogle for their help in these experiments and to R. E. Kerwin and D. J. Kliebenstein for access to their unpublished data.

CONFLICT OF INTREST

The authors do not have any conflicts of interest to report.

DATA AVAILABILITY STATEMENT

Data are available on Dryad (https://doi.org/10.5061/dryad.c2fqz617d) (DeWolf et al., 2021) and the University of Wyoming Data Repository (https://doi.org/10.15786/21482388).

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SUPPORTING INFORMATION

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How to cite this article: DeWolf, E., Brock, M. T., Calder, W. J., Kliebenstein, D. J., Katz, E., Li, B., Morrison, H. G., Maïgnien, L., & Weinig, C. (2023). The rhizosphere microbiome and host plant glucosinolates exhibit feedback cycles in *Brassica rapa*. *Molecular Ecology*, *32*, 741–751. https://doi.org/10.1111/mec.16782