Title

Mutualistic rhizobia harbor genetic variation for traits related to parasite infection

Teaser Text

Microbial mutualists like nitrogen-fixing rhizobacteria influence their host traits. Past work indicates that different strains of rhizobia may influence their host plant's interactions with nematode parasites. But how does this influence compare to the genetic variation present in hosts? We explore the contribution of genetic variation across rhizobia strains to infection-related traits in their host and find that rhizobia contribute to genetic variation for parasite resistance and virulence in their host.

Abstract

Microbial mutualists partially determine many host traits, including traits related to infection by parasites. However, while microbial effects on host trait plasticity is fairly well established, whether microbial mutualists contribute to genetic variation in infection-related remains an open question . Here we paired 10 mutualistic *Sinorhizobium meliloti* rhizobacteria strains with 20 *Medicago truncatula* plant genotypes in an incomplete factorial design, and experimentally infected the plants with parasitic root-knot nematodes. We used this design to estimate rhizobia contributions to genetic variation in four infection-related traits: host resistance, parasite virulence, host tolerance, and mutualism robustness. We find that rhizobia contribute to genetic variation in host resistance and mutualism robustness, and to genetic variation in parasite virulence via genotype-by-genotype interactions with the host. Rhizobia did not contribute to variation in host tolerance. The influence of rhizobia strains on parasite resistance was partially explained by their effect on host root growth. These results underscores the influence that

microbial mutualists have on their host's response to parasite infection, and suggests that resource mutualists may impact host-parasite evolution.

Keywords

Genetic variation, parasitism, mutualism, host-microbe, plant pathology

Introduction

Many plant and animal traits are jointly determined by the genomes of the host and their microbial mutualists (Friesen et al., 2011, p. 20; Fronk & Sachs, 2022; Ma et al., 2020), meaning that many host traits are, in fact, symbiotic extended phenotypes (Batstone et al., 2022). The effects of microbial mutualists can even extend to the host's interactions with pathogens and parasites. Indeed, the protection conferred by microbial mutualists is the defining feature of defensive mutualisms (Hopkins et al., 2017). Recent studies have established that nutritional mutualists also affect parasite or pathogen resistance (Buxton-Martin et al., 2025; Hopkins et al., 2017; Koch & Schmid-Hempel, 2011; O'Keeffe et al., 2022; Yi et al., 2025), suggesting that microbial effects on defense may be a widespread feature of mutualisms. This observation raises an important evolutionary question: do microbial mutualists harbor heritable variation for infection-related traits in their hosts?

In defensive mutualisms, the symbiont's contribution to genetic variation in parasite resistance can rival that of the host (Martinez et al., 2018). Furthermore, both symbiont genotype and genotype-by-genotype interactions between host and endosymbiont can contribute to the level of protection conferred to the host. For example, the pea aphid *Acyrthosiphon pisum* can possess the maternally inherited defensive bacterial endosymbionts *Regiella insecticola* which confers a survival advantage when infected by the aphid-specific pathogenic fungus *Pandora neoaphidis*. Different strains of *R. insecticola* contribute to defense against *P. neoaphidis* and some combinations of *R. insecticola*

strain and *A. pisum* host can vary by different aphid and bacterial genotypes (Parker et al., 2017). In nutritional mutualisms, strains can also differ in their effect on parasite infection in their host. For example, in the nutritional mutualism between legumes and nitrogen-fixing rhizobia bacteria, we recently found that the host plant's response to parasitic nematode infection depended on which rhizobia strain it was inoculated with (Buxton-Martin et al. 2024).

Variation among microbial mutualist strains in their effect on infection-related traits implies that mutualists harbor genetic variation for infection-related traits. Directly testing this hypothesis requires estimating genetic variances in infection-related symbiotic extended phenotypes. To date, most studies investigating microbial mutualist effects on infection-related traits have employed a small number of mutualist strains (Burr et al., 2022; Buxton-Martin et al., 2025; Oliver et al., 2005; Oliver & Higashi, 2019; Patel et al., 2023; Yi et al., 2025). This approach is a powerful way to measure the extended phenotype of individual microbial mutualists. However, estimating the genetic variance of a symbiotic extended phenotype requires a quantitative genetics-style experiment with many mutualist strains. Furthermore, because genotype-by-genotype interactions between hosts and symbionts are pervasive in microbial mutualisms (Heath, 2010; Heath & Tiffin, 2007), symbiont extended phenotypes should be assayed on multiple host genetic backgrounds.

If microbial mutualists strains harbor genetic variation for parasite resistance, they may significantly influence the evolution of infection-related traits in their hosts. The evolutionary potential of infection-related traits in hosts will depend on the total genetic variation in hosts (direct genetic variance; G_{Host}), in microbial mutualists (indirect genetic variance; $G_{Rhizobia}$), and genotype-by-genotype interactions between host and microbe (intergenomic epistasis; $G_{Host \times Rhizobia}$) (De Lisle et al., 2022; Wade, 2007). Therefore, focusing only on direct genetic variances in hosts (G_{Host}) could significantly underestimate the evolutionary potential of any infection-related trait whose expression is determined in part by microbial mutualists.

In this study, we tested whether microbial mutualists contribute to genetic variation in infection-related traits in a tripartite system involving the mutualism between legumes and nitrogen-fixing

rhizobacteria (rhizobia) and plant-parasitic root knot nematodes. We did so in an inoculation experiment with 20 *Medicago truncatula* Gaertn. plant genotypes and 10 *Sinorhizobium meliloti* rhizobia strains in an incomplete factorial design (Figure 1A). *M. truncatula* is an annual legume native to the Mediterranean (Cook, 1999) and forms a mutualistic symbiosis with nitrogen-fixing rhizobia, such as *S. meliloti*. Rhizobia are housed within nodules that develop from the roots of their host plant where they trade with the host plant for carbon-containing compounds. The northern root-knot nematode (*Meloiogyne hapla*) is an obligate endoparasite that form galls on plant roots, where it siphons nutrients from the host (Jones & Goto, 2011; Koltai et al., 2001). We previously found that *M. truncatula* plants inoculated with two different *S. meliloti* strains differed in their resistance to *M. hapla* infection (Buxton-Martin et al., 2025).

We focused on three infection-related traits: resistance, tolerance, and virulence (Figure 1B) (Bush et al., 1997). Resistance (the inverse of susceptibility) is the host's ability to prevent parasite infection, and is measured by infection intensity (the total number of parasites on a host). Tolerance is the fitness cost per parasite, measured as the slope of the relationship between infection intensity and fitness. Finally, virulence is the total fitness cost of infection, as measured as the difference in fitness between infected and uninfected hosts. All three infection-related traits are typically determined jointly by both the host's and parasite's genotype and differ in their implications for host-parasite interactions (Bruns et al., 2012; Jutzeler et al., 2024; Salvaudon et al., 2007). In addition to these three classic infection-related traits, we also measured mutualism robustness to parasite infection, defined as the impact of parasite infection on mutualist colonization (Figure 1B). This captures an often-overlooked cost of parasite infection: disrupted interactions with mutualists. For each of these four traits, we compared rhizobia and plant contributions to total genetic variance. Finally, we evaluated whether the rhizobial effect on infection intensity was related to their effect on host growth (i.e., resource mutualists increase plant size, and bigger plants host more parasites).

We found that rhizobia contributed significantly to genetic variance in resistance, virulence, and mutualism robustness. For some traits, their contribution rivaled that of the host. Rhizobia contributed to genetic variation in these traits via indirect genetic variance ($G_{Rhizobia}$) as well as intergenomic epistasis

between plants and rhizobia ($G_{Host\ x\ Rhizobia}$). A path analysis revealed that rhizobia primarily influence infection intensity indirectly via their effect on plant size, raising the possibility that mutualist effects on infection intensity are common in growth-promoting resource mutualisms.

Methods

Study system

Medicago truncatula Gaertn. is an annual legume native to the Mediterranean (Cook, 1999). *M. truncatula* plants form association with nitrogen-fixing rhizobia in the soil in the genus *Sinorhizobium*, through the successful exchange of compatible chemical cues and the migration of rhizobia into root tissues. Rhizobia are then housed within nodules that develop from the roots of their host plant. Within these nodules rhizobia fix nitrogen that is traded with the host plant for carbon-containing compounds. Many studies have investigated rhizobia strain effects on host interactions with other species (reviewed in Granada Agudelo et al., 2023), albeit across limited numbers of rhizobial strains.

The parasitic root knot nematode *Meloidogyne hapla* is an obligate endoparasite with an extremely large host range that includes *M. truncatula* (Opperman et al., 2008). Female *M. hapla* burrow through intracellular spaces of host roots until they reach a suitable infection site in the vascular cylinder. They then pierce plant cells with their stylet mouthpiece and inject effector proteins that suppress host immune responses and induce cell differentiation producing giant cells from which the nematode siphons nutrients. A gall forms around these infection sites (Jones & Goto, 2011). We previously showed that plant maternal families (hereafter, “genotypes”) and plants inoculated with different rhizobia strains differ in resistance to this parasite (Buxton-Martin et al., 2025; Wood et al., 2018).

We chose 20 accessions of *M. truncatula* from the French National Institute for Agriculture Research (INRAE) germplasm library. We haphazardly chose these accessions across the range of standing genetic variation in *M. truncatula*, including representative diversity across structured

populations as well as resistance to nematode infection and nodulation with rhizobia reported in Wood *et al*. (2018) (see Supplemental for details). We chose 10 strains of *S. meliloti* that were isolated from *M. truncatula* (STable 2). Using FASTANI (Jain et al., 2018), these strains showed a minimum average nucleotide identity of no less than 98% (SFigure 3), indicating all strains belong to a single species. The sources of all *S. meliloti* strains can be found in Supplemental. We sourced *M. hapla* line LM from the Department of Entomology and Plant Pathology at North Carolina State University, which was originally collected in France (Chen & Roberts, 2003).

Experimental design and data collection

Following the protocol of Garcia *et al.* (2006), we grew *M. truncatula* in 66 mL Cone-tainer pots (Stuewe & Sons, Inc., Tangent, OR, USA) within a growth chamber at the University of Pennsylvania. Plants were assigned to treatments such that plant genotypes encountered 2-4 rhizobia strains, and each rhizobia strain encountered 5 or 6 host genotypes (Figure 1A). We replicated this experimental design across nematode-infected and nematode-uninfected treatments. Plants were inoculated with nematodes and rhizobia 14 days after planting. Each treatment (plant genotype-rhizobia strain combination) initially included 7-8 replicates, to ensure that at least 4-5 replicates survived to harvesting. See Supplemental for details regarding inoculation, growth conditions, and sample preparation.

To prevent rhizobial or nematode contamination, each rack of plants contained a single rhizobia treatment and nematode treatment. We distributed rhizobia strain and nematode treatments across different shelves and within the growth chamber in an incomplete block design and randomized plant genotypes within racks. We included shelf location within growth chambers as a fixed-effect blocking variable in our statistical models.

Plants were harvested 52-56 days after planting. Approximately 5-40 nodules were dissected from plant roots during harvesting for a separate experiment and the number of nodules removed was recorded. To harvest, roots were washed in water, and roots and shoots were separated with scissors.

Shoots were dried for a minimum of 48 hours in a drying oven at 60 °C and root systems were frozen in -20 °C for later processing.

After drying, shoots were weighed to the nearest tenth of a milligram. To quantify parasitic nematode infection, we dyed the root systems of nematode-infected plants with red food coloring following the methods outlined in Kandouh *et al.* (2019), which stains mature nematode egg sacs. Roots were scanned on an Epson Perfection V850 Pro scanner at 1200 dpi with high contrast background correction following methods outlined in York (2023). Nodules, galls, and nematode egg sacs were manually counted from these images using ImageJ (Schneider et al., 2012). After scanning, roots were dried for >48 hours in a drying oven and weighed to the nearest 0.0001 gram.

Variance component analyses: Quantifying rhizobia contributions to genetic variation in infection-related traits

To estimate the contribution of rhizobia strains to variation in resistance, virulence, tolerance, and mutualism robustness, we used variance component analyses (Figure 1B). We fit two models for each trait except virulence: one with (Figures 2, 4, 5) and one and without (SFigures 5-7) root biomass as a covariate, to capture rhizobia strain effects on infection intensity measured as parasite density (i.e. number of galls per root biomass) and total infection intensity (i.e. number of galls per individual plant). We primarily present models with parasite density but include results that do not correct for root biomass in the supplemental materials (SFigures 5-7). Results were qualitatively similar for most analyses.

To test whether rhizobia strains contributed to variation in resistance to nematode infection, we fit our data from nematode-infected plants to mixed-effects models with number of galls as the response variable and rhizobia strain, host genotype, and the genotype-by-strain interaction as random effects. In this analysis, a significant effect of rhizobia strain or significant genotype-by-strain interaction indicates that rhizobia contribute to genetic variation in resistance via indirect genetic effects ($G_{Rhizobia}$) or

intergenomic epistasis ($G_{Host \times Rhizobia}$) respectively. Experimental block was included as a fixed effect, and we fit a negative binomial error distribution (Lindén & Mäntyniemi, 2011).

Virulence is measured as the difference in fitness between infected and uninfected hosts. To test whether rhizobia strains contributed to variation in virulence, we fit our data to a mixed-effects model with shoot biomass, our host fitness proxy, as the response variable and rhizobia strain, host genotype, genotype-by-strain, and all three of these effects interacting with nematode infection status as random effects. Experimental block and nematode infection status were included as fixed effects. In this analysis, a significant effect of infection-by-strain indicates rhizobia strain effects on virulence and a significant effect of infection-by-strain-by-genotype indicates that rhizobia contribute to virulence via indirect genetic effects ($G_{Rhizobia}$) or intergenomic epistasis ($G_{Host \times Rhizobia}$) respectively.

Tolerance is the relationship between infection intensity and host fitness. To test whether rhizobia strains contributed to variation in tolerance, we fit our data to a mixed-effects models with shoot biomass as the response variable and rhizobia strain, host genotype, and both of these effects interacting with the number of galls as random effects. Unlike our other analyses, because each genotype-by-strain combination had 4-8 replicates, we did not have the statistical power to confidently estimate slopes for specific host genotype-rhizobia strain combinations and therefore did not include genotype-by-strain or infection-by-genotype-by-strain effects in our model. For random effects interacting with the number of galls, we included random slopes and no random intercept for the number of galls. We included the number of galls and experimental block as fixed effects. In this analysis, a significant interaction between number of galls and rhizobia strain indicates that rhizobia strain contributes to genetic variation in tolerance via indirect genetic effects ($G_{Rhizobia}$).

Mutualism robustness is measured as the difference in mutualism colonization between infected and uninfected hosts. To test whether rhizobia strains contributed to variation in mutualism robustness, we fit our data to mixed-effects models with the number of nodules as the response variable and rhizobia strain, host genotype, genotype-by-strain, and all three of these effects interacting with nematode infection status as random effects. Experimental block and nematode infection status were included as

fixed effects. In this analysis, a significant effect of infection-by-strain or infection-by-strain-by-genotype indicates rhizobia strain effects on mutualism robustness via indirect genetic effects ($G_{Rhizobia}$) or intergenomic epistasis ($G_{Host \times Rhizobia}$) respectively. We used a negative binomial distribution error (Lindén & Mäntyniemi, 2011).

We implemented our data analysis in R. We fit mixed-effects models to data using the glmmTMB package (Brooks et al., 2017), and used the Satterthwaite's degrees of freedom method implemented in the lmerTest package (Kuznetsova et al., 2017) to estimate p-values and infer statistical significance. We used the DHARMa package (Florian, 2016) to check that model residuals were normally distributed and homoscedastic. We tested significance of random effects with likelihood ratio tests. We used the MuMIn package (Bartoń, 2025) to calculate the conditional and marginal R^2 values for each model and used the trigamma method when possible per the package author's suggestion.

Path analysis: Distinguishing direct and indirect effects of rhizobia on infection intensity

Resource mutualists like rhizobia could affect the intensity of parasite infection (i.e., the number of parasites on a host) directly, or indirectly via their effects on host growth, which impacts the amount of host tissue available to infect. We therefore used path analysis to ask whether the effect of rhizobia on infection severity was mediated by root biomass. Our path analysis used the following models:

Outcome model: Number of galls ~ Root biomass + Host genotype + Rhizobia strain

Mediating model: Root biomass ~ Host genotype + Rhizobia strain

To test for significant effects in the overall path analysis, we used a negative binomial GLM implemented with the glm.nb function from the MASS package in R (Venables & Ripley, 2002) to fit our outcome models that had gall number as the response variable (Lindén & Mäntyniemi, 2011) and a Gaussian linear model implemented in the lm function from the STAT package (Bolar, 2019) to fit the mediating model. To estimate standardized path coefficients we used a Gaussian distribution for both models fit with the lm

function. We used the piecewiseSEM package (Lefcheck et al., 2025) to test and structure our path analysis. We used sum-to-zero contrasts in all our models. To test for the significance of direct and indirect effects in the per strain path analyses, we used the boot function in the boot package (S) et al., 2025) to perform bootstrapping analysis with 10,000 resamples to acquire confidence intervals for the product of path coefficients and the proportion of meditation using the Gaussian models.

Results

Resistance

Our resistance model (Figure 2) explained 41.4% of the total variation in the number of galls (R^2_C). Of the total variation in the number of galls, 10.28% was explained by fixed effects (R^2_M) leaving 31.12% explained by our random effects. Of the variation in the number of galls explained by random effects, our variance component analysis attributed 86% to host genotype ($p < 0.0001$), 14% to the genotype-by-strain interaction ($p = 0.0610$), and no significant variation to rhizobia strain ($p = 0.1730$). These results indicate that host genotype was the largest determinant of resistance but that genotype-by-strain interaction does contribute a significant variation to resistance. Our variance component analysis that did not correct for root biomass indicated that only host genotype contributed to variation in gall numbers.

Virulence

Our virulence model (Figure 3) explained 92.6% of the total variance in shoot biomass (host fitness) (R^2_C). Of the total variance in shoot biomass, 27.51% was explained by fixed effects (R^2_M) leaving approximately 65.09% explained by our random effects. Of the variation in shoot biomass explained by random effects, 5% was attributed to the infection-by-strain interaction ($p = 0.048$). The infection-by-genotype interaction and infection-by-genotype-by-rhizoba-strain interaction explained 3%

and 8% of the variance, respectively, but neither was statistically significant ($p = 0.133$ and $p = 0.222$ respectively). These results indicate that rhizobia strains contribute to variation in the fitness consequences of nematode infection, but that their effect depends on the genotype of their host plant.

Tolerance

Our tolerance model that corrected for root biomass (Figure 4) explained 77.18% of the total variation in shoot biomass (host fitness) (R^2_C). Of the total variation in shoot biomass, 69.57% was explained by fixed effects (R^2_M) leaving approximately 7.61% explained by our random effects. Of the variation in shoot biomass explained by random effects, our variance component analysis attributed no variance to effects interacting with nematode infection severity (number of galls). Instead, all variation in host fitness was attributed to host genotype (100%, $p < 0.0001$). Qualitatively similar results were found from our tolerance model that did not correct for root biomass ($R^2_C = 0.2253$; $R^2_M = 0.0697$). These results indicate that rhizobia strains do not contribute to variation in tolerance.

Mutualist robustness

Our mutualism robustness model that corrected for root biomass (Figure 5) explained 46.86% of the total variation in nodule counts (R^2_M). Of the total variation in nodule counts, 11.71% was explained by fixed effects (R^2_C) leaving approximately 35.15% explained by random effects. Of the variation in nodule counts explained by random effects, 12% was attributed to the infection-by-genotype interaction ($p = < 0.0001$) and 16% to the infection-by-genotype-by-strain interaction ($p = 0.0480$). No variance was attributed to the infection-by-strain interaction ($p = 0.5409$). Our mutualism robustness model that did not correct for root biomass showed qualitatively similar results. These results indicate that individual host genotype and rhizobia strain combinations contribute to variation in mutualism robustness to parasite infection, but that the rhizobia effect depends on the genotype of its host.

Rhizobia effects on nematode infection intensity are primarily mediated by their effect on plant size

In our path analysis (Figure 6), we found that rhizobia strains varied in their influence on root biomass ($p<0.0001$) but not on gall numbers ($p=0.1381$) (Figure 2). Gall numbers increased with root biomass ($\beta=0.3151$, $p<0.0001$). These results indicate that after correcting for the differences in host genotype, larger roots are associated with greater number of galls, and rhizobia differ in their effect on root growth. These path analysis results cohere with the difference between variance component analyses that did (Figure 2) and did not (SFigure 5) correct for root biomass and indicate that their effect on plant growth is one mechanism through which rhizobia strains influence nematode infection severity.

Discussion

Here we report that nitrogen-fixing rhizobia contribute to genetic variation in infection-related traits in their host plant. These effects manifested as differences in parasite virulence among plants inoculated with different rhizobia strains (i.e., $G_{Rhizobia}$), as well as intergenomic epistasis for resistance (i.e., $G_{Host \times Rhizobia}$) (Table 1). In addition to these canonical infection-related traits, we also found that rhizobia contribute to genetic variation in the robustness of mutualism to parasite infection. Remarkably, variation in parasite virulence was not determined by host genotype but instead by rhizobia strain and host-genotype-by-rhizobia-strain effects. The rhizobia effect on resistance is partially explained by a core property of this nutritional mutualist: its effect on plant growth. These results add to the growing literature on the ecological and evolutionary significance of symbiotic extended phenotypes, establishing that microbial effects on host traits can cascade to interactions between host and parasites. Our study raises the possibility that many nutritional mutualists influence the evolutionary potential of infection-related traits in their hosts.

Rhizobia contributed to genetic variation in resistance and virulence

Rhizobia contributed significantly to genetic variation in two of the three canonical infection-related traits in our study: resistance (total parasite load) and virulence (the difference in fitness between infected and uninfected hosts). The contribution of rhizobia to genetic variation in resistance was small, and tied up in intergenomic epistasis ($G_{Host \times Rhizobia}$). Overall, variation in resistance was primarily governed by the host genome (G_{Host} = 86% of total genetic variance; Figure 2). By contrast, rhizobia contributed substantially to genetic variation in virulence. Our variance component analyses attributed 5% and 18% of variation in host fitness to the nematode infection-by-rhizobia strain interaction ($G_{Rhizobia}$) or the infection-by-genotype-by-strain interaction ($G_{Host \times Rhizobia}$) (Figure 3). Only 3% of variation in virulence was attributed to the host alone (infection-by-genotype interaction,i.e., (G_{Host}). In other words, rhizobia contributed to nearly 90% of total genetic variation in virulence. These results show that rhizobial strain identity is a primary determinant of the virulence of nematode infections in *M. truncatula*.

Significant genotype-by-genotype interactions (GxG) for resistance and virulence are consistent with the established prevalence of intergenomic epistasis in mutualisms (Batstone et al., 2022; Heath, 2010; Heath & Tiffin, 2007). Intergenomic epistasis is hypothesized to contribute to the maintenance of genetic variation in mutualisms, because it creates a situation where the symbiont genotype favored by selection varies across host genetic backgrounds (and vice versa) (Heath & Stinchcombe, 2014). Our study suggests that intergenomic epistasis may be a general feature of symbiotic extended phenotypes, extending beyond mutualism traits to host-parasite interactions. It is therefore possible that GxG between hosts and symbionts contribute to the maintenance of variation in infection-related traits as well. Ultimately, though, the evolutionary ramifications of GxG depend on how hosts and symbionts partner in wild populations (De Lisle et al., 2022). If hosts and symbionts partner non-randomly, intergenomic epistasis could contribute to additive genetic variance in host traits, because a symbiont's extended phenotype will only be expressed on a subset of host genetic backgrounds. In principle, non-random association could arise from partner choice, which is common in the legume-rhizobia mutualism (Simms

& Taylor, 2002). However, to the best of our knowledge, the covariance between host and symbiont genotypes in facultative mutualisms is rarely measured (De Lisle et al., 2022).

In addition, pervasive genotype-by-genotype interactions could explain why the measured effect of rhizobia on root-knot nematode infection varies across studies. Past work in our lab found conflicting results about rhizobial strain effects on nematode infection severity. Buxton-Martin *et al.* (2025) found that *Medicago* plants inoculated with different rhizobia strains differed in resistance to nematodes, whereas Yi *et al.* (2025) found no effect of rhizobia on nematode resistance. Variation in strain effects across host genetic backgrounds may explain the discrepancy between these studies. This point underscores the importance of assaying symbiotic extended phenotypes across a representative range of genetic variation in both host and symbiont.

Finally, we observed no strain effects on host tolerance. Increased tolerance or resistance are the two strategies that hosts evolve in response to evolutionary pressure from parasites, each with differing evolutionary consequences. Increased resistance selects for increased virulence, potentially leading to an evolutionary arms race whereas tolerance does not (Vorburger & Perlman, 2018). Examples of mutualist symbionts that protect their hosts via increasing tolerance appear to be rare or underrepresented in the literature (Vorburger & Perlman, 2018) and so the lack of rhizobial effects on host tolerance may support this notion that microbial effects on host tolerance may be rare. However, resource availability can influence tolerance (Budischak & Cressler, 2018; Yi et al., 2025; Yuan et al., 2023), making this lack of rhizobial effect on tolerance surprising. We hypothesize that this may be due to the similarities in molecular pathways that nematodes and rhizobia use to interact with hosts.

Rhizobia contribute to genetic variation in mutualism robustness to parasite infection

Previous research found that, in addition to stealing resources from their host, parasitic nematodes decrease nodulation with rhizobia. The magnitude of this effect varied across plant genetic backgrounds,

indicating that mutualism robustness is a heritable plant trait (Wood et al., 2018). In the present study, we replicated this result, finding significant host genetic variation for mutualism robustness (infection-by-host genotype interaction for nodulation; Figure 5). Furthermore, here we found that mutualism robustness is also influenced by genotype-by-genotype interactions between plants and rhizobia (infection-by-genotype-by-strain interaction for nodulation; Figure 5). Rhizobia thus contain genetic variation for mutualism robustness in the face of nematode infection, indicating that rhizobia evolution may influence mutualism robustness. Our results build on the importance of context dependence in mutualisms (Chamberlain et al., 2014; Heath & Tiffin, 2007; Parker et al., 2017), and emphasize that parasite infection may be an important environmental factor shaping mutualism expression and evolution (Carlson et al., 2025).

The genetic and physiological basis of strain effects on parasite infection

Mutualist effects on parasite infection can arise from two underlying mechanisms: immune crosstalk between mutualism and parasitism, or resource competition or facilitation (Carlson et al., 2025). While our experiment cannot directly discriminate between immune- and resource-mediated mechanisms, our path analysis (Figure 6) provides a basis for hypothesizing about the biological processes that may mediate the effects we observed. We used this analysis to evaluate whether the rhizobia effect on nematode resistance is mediated by the rhizobia effect on plant growth (root biomass), consistent with a resource-mediated link between rhizobia and parasite resistance. Our results are consistent with this hypothesis: rhizobia differed in their effect on root biomass, and root biomass was associated with parasite load, suggesting that the rhizobia effect on parasite load is associated with the resources they provide. This suggests that strain effects on parasite resistance may arise from genotype-by-genotype interactions between plants and rhizobia for resource- or growth-related biological processes.

By contrast, the direct path linking rhizobia and parasite load was not significant. One interpretation of this result is that immune crosstalk between rhizobia and nematodes does not

substantially influence nematode resistance. Furthermore, in our variance component analyses that corrected for plant size (i.e., analyses that included root biomass as a covariate), we still detected a significant rhizobia contribution to genetic variation in resistance, via GxG between rhizobia and host. Together, these observations suggest that, at least for some strains on some plant genetic backgrounds, the rhizobia effect on parasite resistance is not fully explained by the resources they provide. This inference is also supported by our recent transcriptomic study showing that rhizobia strains promote differential expression of genes enriched for immune functions in nematode galls (Buxton-Martin et al., 2025).

Ultimately, deciphering the biological basis involved in rhizobial strain effects on infection-related traits is important because these mechanisms differ in their implications for host-parasite evolution. In defensive mutualisms, the mechanisms by which defensive symbionts affect virulence (Armitage et al., 2022; Vorburger & Perlman, 2018) and the costs associated with harboring defensive mutualists (King & Bonsall, 2017) influence the evolutionary consequences of those protective effects. Competition between parasites and mutualists for resources is expected to in most cases increase virulence; interference competition is expected to decrease virulence, and effects mediated by immune responses may vary significantly in their evolutionary consequences for virulence (Armitage et al., 2022; Vorburger & Perlman, 2018).

Our path analysis also highlights an important consideration for researchers studying interactions between parasites and resource mutualists: is absolute parasite load or parasite density (i.e., body size-adjusted parasite load) a more appropriate metric of infection severity? While absolute parasite load may provide ecologically relevant information about parasite populations, parasite density may be a better indicator of the fitness cost of parasite infection. If larger hosts typically harbor more parasites, resource mutualists may often increase absolute parasite load because they facilitate host growth. However, their effects on parasite density and the net fitness cost of infection may be less predictable. With this in mind we recommend considering carefully how infection-related traits – especially infection intensity – is quantified when in systems in which resource mutualists impact host growth.

Ecological and evolutionary implications of symbionts contributing to heritable variation in infection-related traits

When microbial mutualists contribute to heritable variation for infection-related traits, their evolutionary potential may be greater than what would be expected if these traits were only determined by the host's genome (Henry et al., 2021). For example, the shorter generation time and larger effective population size of microbial mutualists means the potential for much faster evolution (Weller & Wu, 2015). However, if the underlying microbial genes are subject to conflicting selection (for example, divergent selection between the free-living and symbiotic life stages), then joint trait evolution will be difficult to predict based on selection acting on the host alone. Therefore, identifying the genetic basis of strain effects on joint traits, as well as how selection acts on symbiotic extended phenotypes, could be key to understanding the evolution of joint traits, including those related to parasite infection.

Our results highlight the value of considering genetic variance that arises from interactions with other individuals or species (De Lisle et al., 2022; O'Brien et al., 2021). The contribution of rhizobia to genetic variance in its host's response to parasite infection is an example of an indirect genetic effect, whereby the genes in one species influence trait expression in another. This concept was first formalized in the context of social interactions within species, and recently extended to encompass species interactions (De Lisle et al., 2022; Moore et al., 1997; Wolf et al., 1998). If microbial mutualists frequently contribute to genetic variation in host traits, estimates of heritability based only on variance among host genotypes may substantially underestimate evolutionary potential (Henry et al., 2021; Wolf et al., 1998). For example, our experiment found that genetic variance introduced by rhizobia could account for nearly a quarter of genetic variation in virulence (5% and 18% of variation in virulence was attributed to strain and genotype-by-strain interactions, respectively. Furthermore in two infection-related traits – virulence and mutualism robustness – a greater proportion of genetic variance was attributed to rhizobia

403 than to the host. It is important to note, however, that the realized contribution of symbiont indirect
404 genetic effects to genetic variation in a joint trait depends on how hosts and symbionts partner in wild
405 populations. For example, symbionts could actually *decrease* total genetic variation in joint traits if there
406 is a negative genetic correlation between the host's direct genetic effect and the indirect genetic effect of
407 its symbionts.

408 Heritable variation for infection-related traits residing in microbial mutualists is consistent with
409 experiments showing the rapid evolution and coevolution of defensive mutualisms (Ford et al., 2017;
410 King et al., 2016; May & Nelson, 2014; Rafaluk-Mohr et al., 2018, 2022); variation in the protective
411 effect conferred by different strains of defensive mutualist (Martinez et al., 2018); and pervasive effects of
412 microbial mutualists on host trait expression (Friesen et al., 2011). Our study builds on these discoveries
413 in two ways. First, our results add to the growing body of studies showing that symbiont effects on
414 defense are not restricted to defensive mutualisms (Wood et al., n.d., in review). Nitrogen-fixing rhizobia
415 are textbook nutritional mutualists. Yet our study and several others show that they can affect their host's
416 resistance to enemy attack (Irmer et al., 2015; Prévitali et al., 2025; Simonsen & Stinchcombe, 2014;
417 Wood et al., 2018). Second, our experiment joins other recent studies in moving beyond
418 microbe-mediated trait plasticity to microbe-mediated trait evolution. Finally, our study raises several
419 important questions. First, are some antagonists more likely to be impacted by microbial mutualists than
420 others? Understanding the immune- and resource-mediated mechanisms underlying mutualist effects on
421 defense traits might allow us to predict when mutualists will affect interactions with antagonists, and if so,
422 which ones. Microbial mutualists may be especially likely to influence defense against antagonists
423 governed by similar immune pathways, or defenses whose cost overlaps with the resources provided or
424 consumed by the mutualist. For example, in our system, nematodes and rhizobia have significant
425 overlapping pathways by which they interact with their host (Koltai et al., 2001). Finally, our results
426 highlighted the importance of intergenomic epistasis between hosts and symbionts, but we did not
427 consider GxG between the symbionts themselves. Our experiment involved single-strain inoculations, yet
428 plants are can be colonized by multiple rhizobia strains at a time and the benefit that the rhizobia provide

depends on the strain(s) they are co-inoculated with (Rahman et al., 2023; Westhoek et al., 2021). This suggests that other symbiotic extended phenotypes, including rhizobia effects on infection-related traits, may also be influenced by GxG between rhizobia strains. These lines of inquiry parallel the emerging focus within parasitology and phytopathology on concepts like the pathobiome (Vayssier-Taussat et al., 2014) and disease tetrahedron (Leveau, 2024), which incorporate ecological realism into host-parasite interactions..

Data and code availability statement

Scripts for data analysis and the generation of figures and datasets are available at <https://github.com/adbmar/StrainVariation2025>.

Author contributions

ADBM and CWW conceived the ideas and designed the methodology; ADBM carried out the experiments, collected data, analyzed data, and wrote the manuscript. Both ADBM and CWW revised manuscript drafts and gave final approval for publication.

Funding

This work was funded by a grant from the National Science Foundation's Division of Environmental Biology (grant: NSF-DEB 2118397) to Corlett Wood.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgements

We would like to thank the many Wood Lab members who assisted in setting up experiments, collecting samples, and collecting data, especially Coumba Diagne, Cameron Zhang, for helping to set up and maintain the experiment; McCall Calvert, Chang-Yu Chang, Eunnuri Yi, Coumba Diagne, Coumba Diagne, Alison Chen, and Anisa Robinson for assistance harvesting samples; Coumba Diagne, Cameron Zhang, Anesu Winston Mugoni, Cameron Kim, and Jazmine Rud for assistance with collecting data; and Chang-Yu Chang and McCall Calvert for assistance analyzing host and rhizobia genomes. We are thankful to Paul Schmidt, Katie Barott, Kim Gallagher, Brian Gregory, and the Plant Seminar group at the University of Pennsylvania for their feedback on the analysis and interpretation of results. We are grateful to Adrienne Gorny at North Carolina State University for nematode lines, Nevin Young and the Medicago HapMap project for seeds, and John Stinchcombe and Tia Harrison for rhizobia strains. We are also grateful to Samara Gray and Kathryn Weber for their management of the greenhouses and growth chambers at the University of Pennsylvania.

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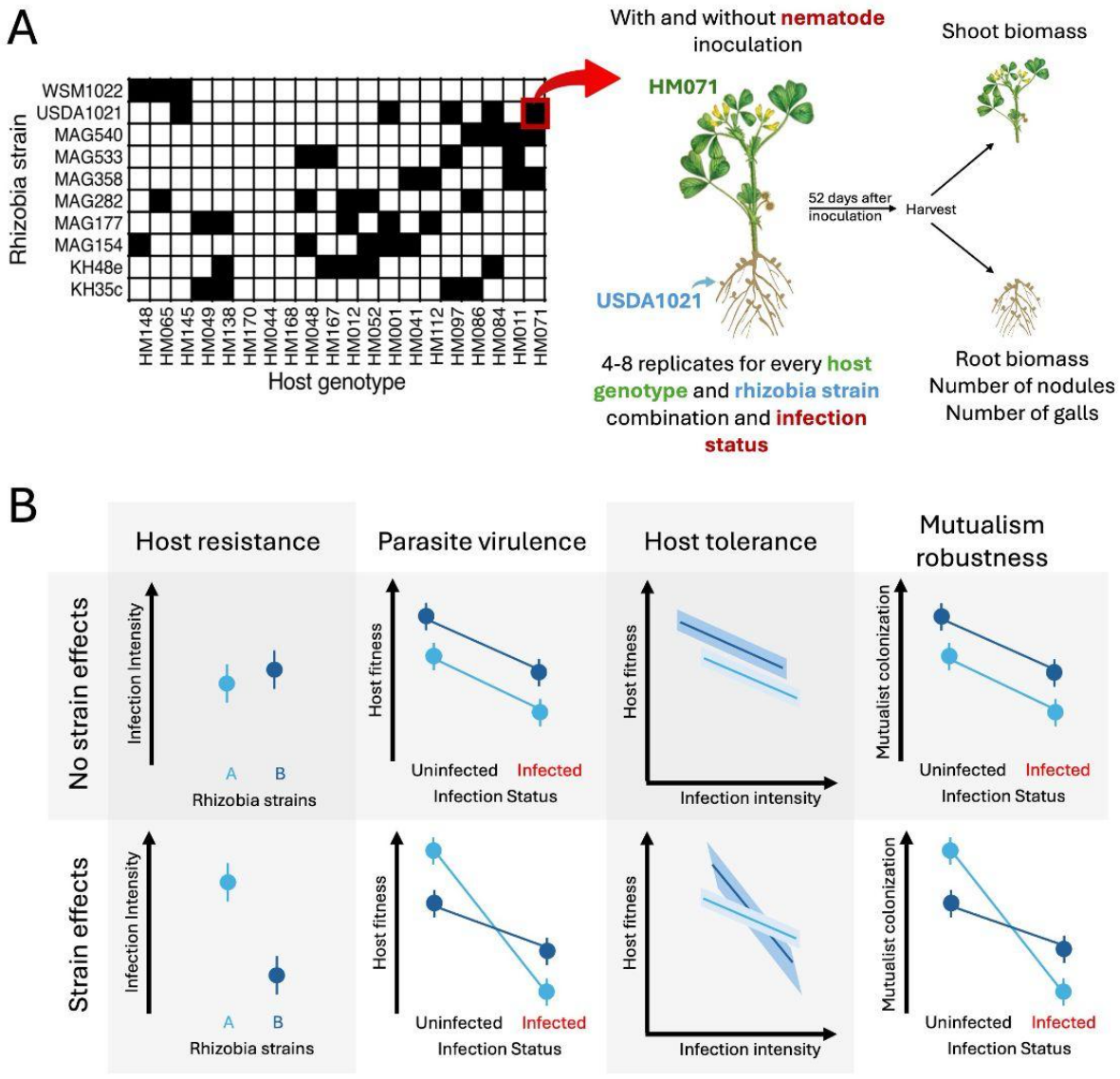
A
Rhizobia strain
WSM1022
USDA1021
MAG540
MAG533
MAG358
MAG282
MAG177
MAG154
KH48e
KH35c
HM148
HM065
HM145
HM049
HM138
HM170
HM044
HM168
HM048
HM167
HM012
HM052
HM001
HM041
HM112
HM097
HM086
HM084
HM011
HM071
Host genotype
With and without nematode inoculation
HM071
USDA1021
52 days after inoculation
Harvest
Shoot biomass
Root biomass
Number of nodules
Number of galls
4-8 replicates for every host genotype and rhizobia strain combination and infection status
B
Host resistance
Parasite virulence
Host tolerance
Mutualism robustness
No strain effects
Strain effects
Infection Intensity
Rhizobia strains
A
B
Host fitness
Uninfected
Infected
Infection Status
Infection intensity
Mutualist colonization

Figure 1: A) A conceptual diagram showing our experimental design. See materials and methods for details. B) A conceptual diagram showing how infection-related traits were quantified and statistically analyzed for a rhizobial strain effect. Strain effects on host resistance is indicated by differences in infection intensity across treatments with different rhizobia strains. Stain effects on parasite virulence is indicated by different rhizobia strain slopes in the reaction norm of host fitness proxy across uninfected and infected treatments. Strain effects on tolerance is indicated by differences in the slopes of the relationship between host fitness and infection intensity for different rhizobia strains. Strain effects on mutualism robustness is indicated by differences in the slopes in the reaction norm of mutualist colonization across uninfected and infected treatments.

Host resistance

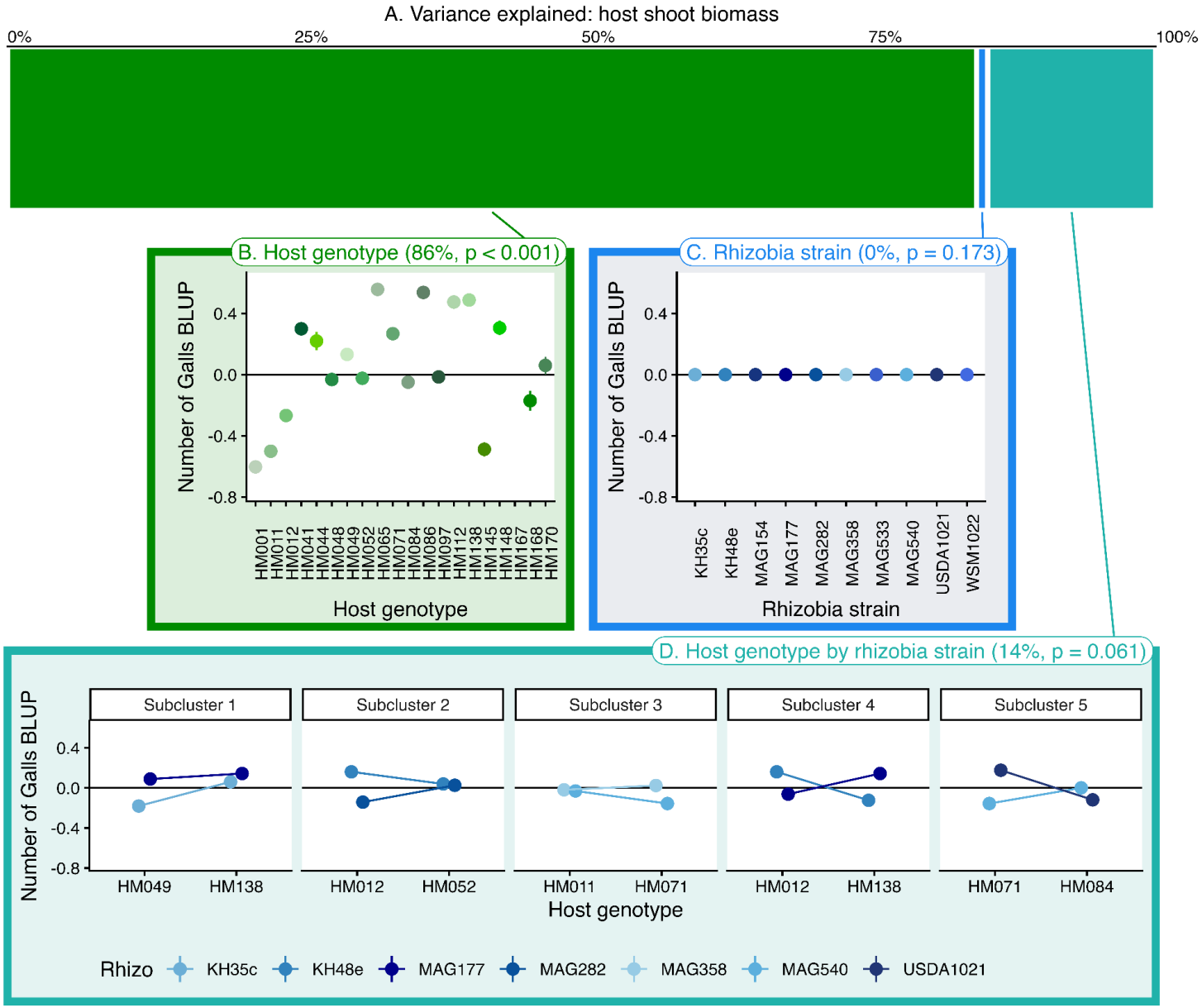

Figure 2: Visualizing variation in number of galls and host resistance. A) Variance component analysis of variation in host resistance data broken into variance attributed to host genotype, rhizobia strain, and the genotype-by-strain interaction. B) Visualizing G_{Host} in host resistance by showing the best linear unbiased predictor of host genotype effects on number of galls (host resistance). C) Visualizing $G_{Rhizobia}$ in host resistance by showing the best linear unbiased predictor of rhizobia strain effects on number of galls. D) Visualizing $G_{Host \times Rhizobia}$ in host resistance by showing the best linear unbiased predictor of the genotype-by-strain interaction on the number of galls grouped by host genotype and rhizobia strain (see SFigure 2 for subclusters).

Parasite virulence

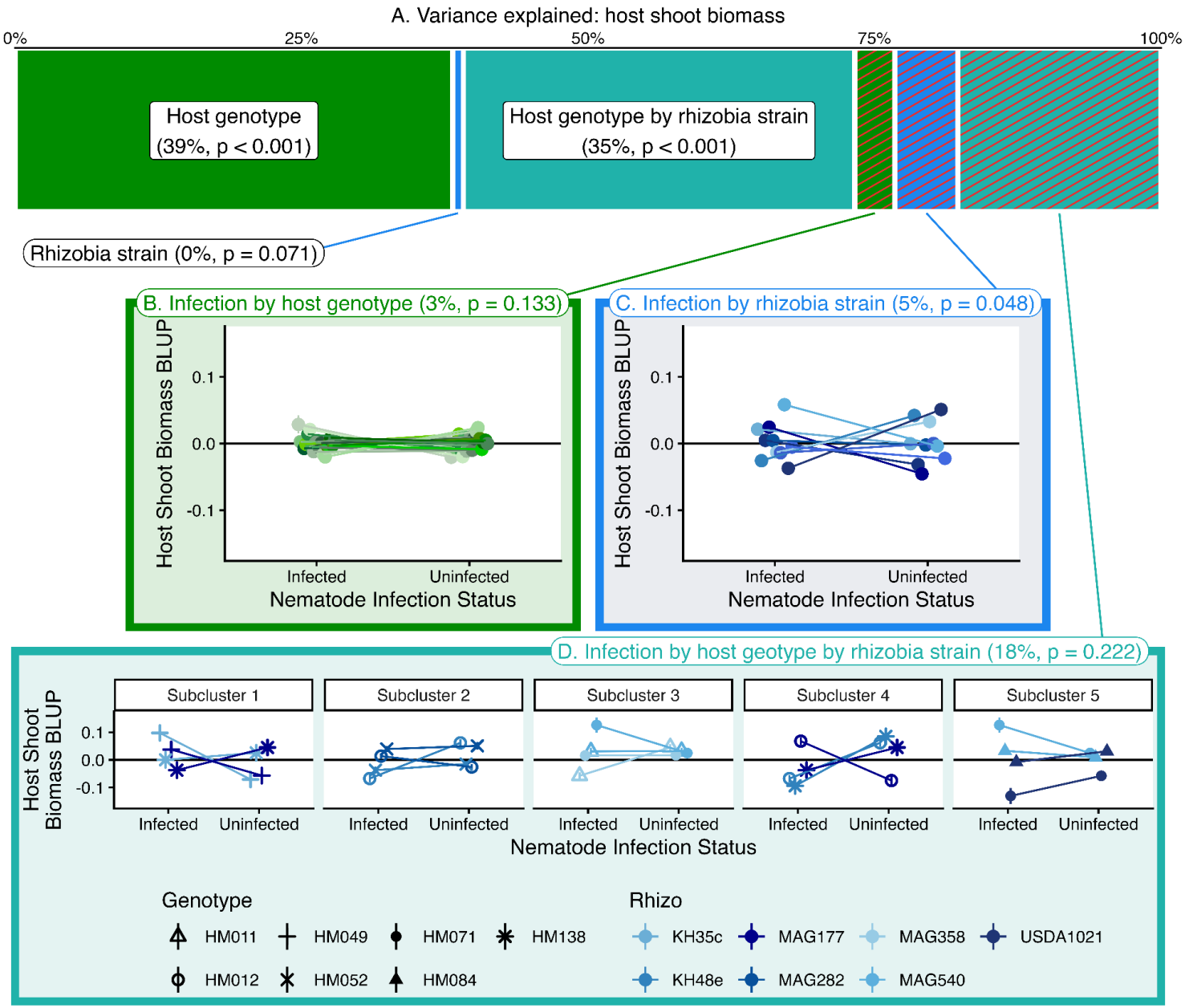

Figure 3: Visualizing variation in host fitness and parasite virulence. A) Variance component analysis of variation in shoot biomass (host fitness) data broken into variance attributed to host genotype, rhizobia strain, and genotype-by-strain, infection-by-genotype, and infection-by-rhizobia interactions. As parasite virulence is the decrease in host fitness due to infection, variation in parasite virulence is captured by effects interacting with infection status. B) Visualizing G_{Host} in parasite virulence by showing the best linear unbiased predictor of infection-by-genotype interactions on shoot biomass grouped by host genotypes. C) Visualizing $G_{Rhizobia}$ in parasite virulence by showing the best linear unbiased predictor of infection-by-strain interactions on shoot biomass grouped by rhizobia strain. D) Visualizing $G_{Host \times Rhizobia}$ in parasite virulence by showing the best linear unbiased predictor of the infection-by-genotype-by-strain interactions on the number of galls grouped by rhizobia strain and host genotype (see SFigure 2 for subclusters).

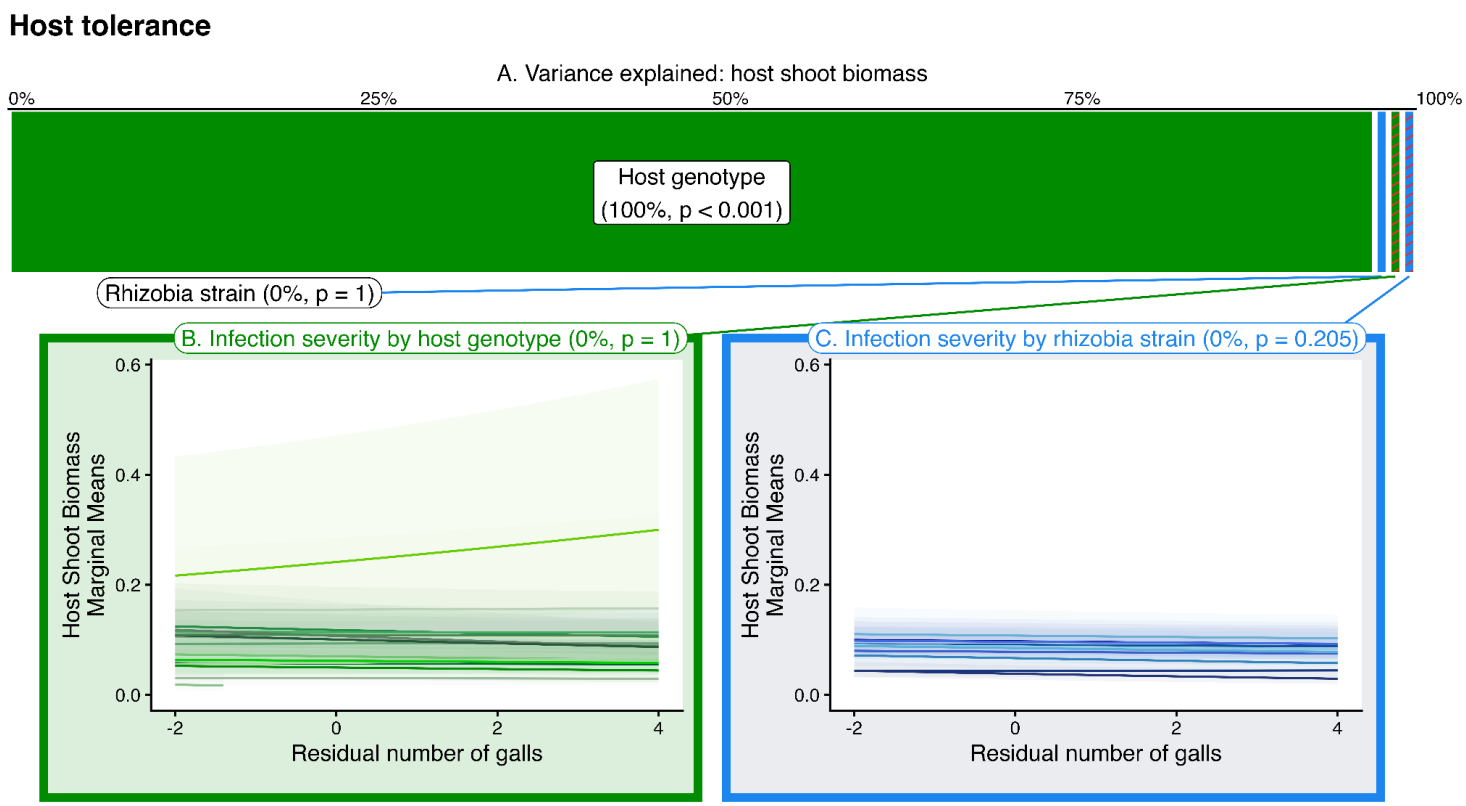

Figure 4: Visualizing variation in host fitness and host tolerance. A) Variance component analysis of variation in shoot biomass (host fitness) data broken into variance attributed to host genotype, rhizobia strain, and galls-by-strain, galls-by-genotype, and galls-by-genotype-by-strain interactions. As host tolerance is the relationship between host fitness and infection severity, variation in host tolerance is captured by effects interacting with the number of galls. B) Visualizing G_{Host} in host tolerance by showing the best linear unbiased predictor of the galls-by-genotype interactions on shoot biomass grouped by host genotype. C) Visualizing $G_{Rhizobia}$ in host tolerance by showing the best linear unbiased predictor of the galls-by-strain interactions on shoot biomass grouped by rhizobia strain. No significant infection-by-strain interactions were observed, indicating no effect of rhizobia strain on host tolerance.

Mutualism robustness

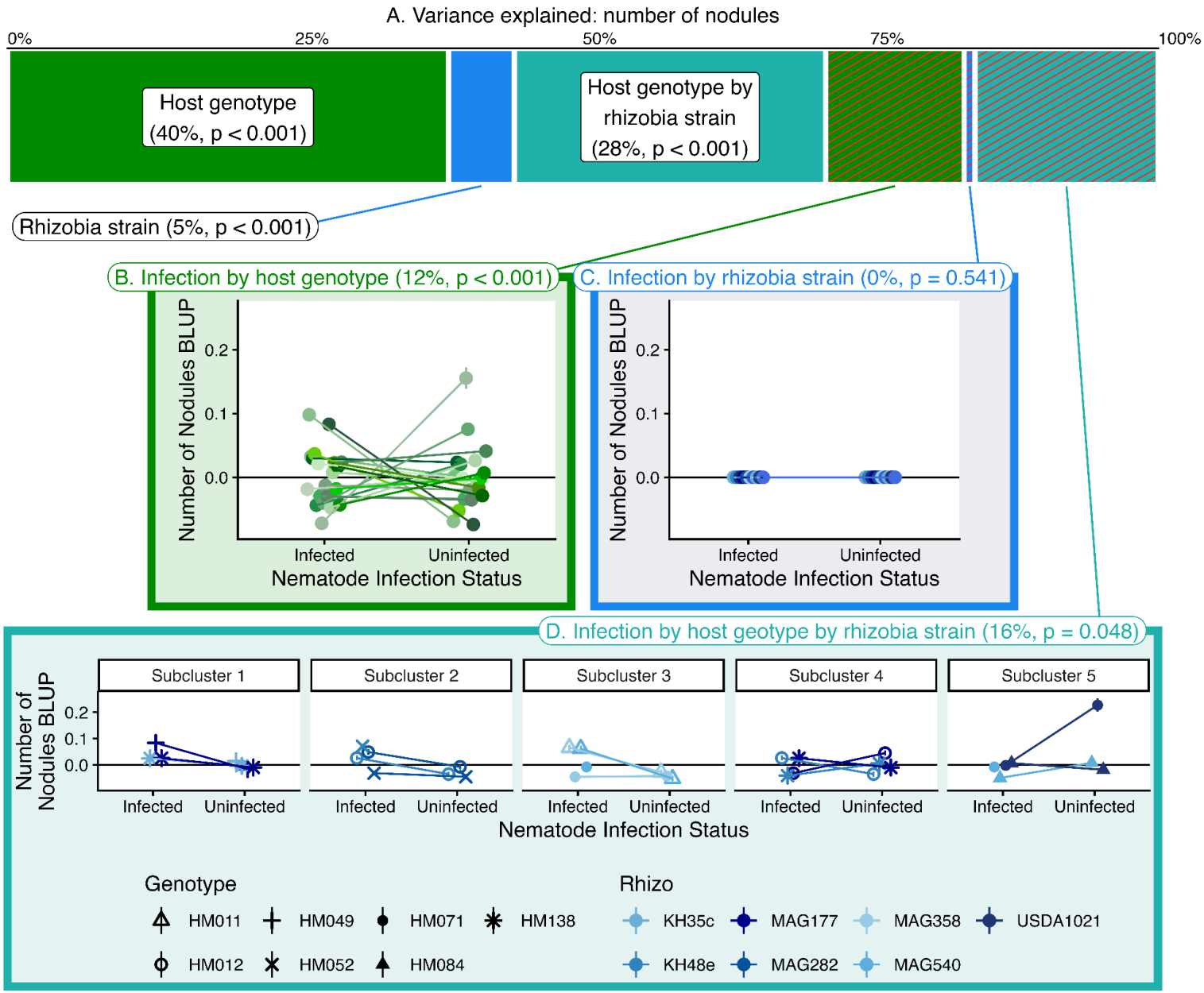

Figure 5: Visualizing variation in number of nodules and mutualism robustness. A) Variance component analysis of variation in number of rhizobia nodules data broken into variance attributed to host genotype, rhizobia strain, and infection-by-strain, infection-by-genotype, and infection-by-genotype-by-strain interactions. As mutualism robustness is the decrease in number of nodules due to infection, variation in mutualism robustness is captured by effects interacting with infection status. B) Visualizing G_{Host} in mutualism robustness by showing the best linear unbiased predictor of the galls-by-genotype interactions on shoot biomass grouped by host genotype. C) Visualizing $G_{Rhizobia}$ in mutualism robustness by showing the best linear unbiased predictor of the galls-by-strain interactions on shoot biomass grouped by rhizobia strain. D) Visualizing $G_{Host \times Rhizobia}$ in mutualism robustness by showing the best linear unbiased predictor of the infection-by-genotype-by-strain interactions on the number of nodules grouped by rhizobia strain and host genotype (see SFigure 5 for subclusters).

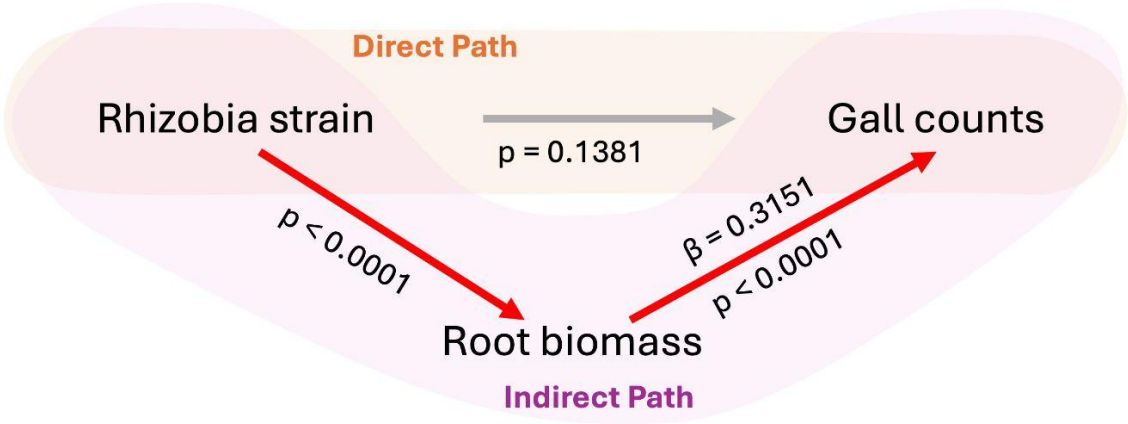

Figure 6: Path analysis results showing significant effect of rhizobia strain on root biomass ($p < 0.0001$) and a significant effect of root biomass on the number of galls ($\beta=0.3151$, $p < 0.0001$) but no direct significant effect of rhizobia strain on gall counts without root biomass included ($p = 0.1381$) indicating that the effect of rhizobia strain on host resistance is likely largely or in part mediated by their effect on root biomass. Our path analysis corrected for host genotype effects.

Summary table of significant sources of genetic variances for infection-related traits

	G_{Host}	$G_{Rhizobia}$	$G_{Host \times Rhizobia}$
Host resistance	✓	N.S.	(✓)
Parasite virulence	N.S.	✓	N.S.
Host tolerance	✓	N.S.	Not tested
Mutualism robustness	✓	N.S.	✓

Table 1: A summative table showing whether genetic variances attributed to host genotype, rhizobia strain, or genotype-by-strain were found for each infection related traits tested. Check marks indicate a significant effect ($p<0.05$), check marks within parentheses indicate a marginally significant ($p<0.1$) effect, and N.S. indicates no significant effect ($p>0.1$).