

Soil microbial communities alter conspecific and congeneric competition consistent with patterns of field coexistence in three *Trifolium* congeners

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Funding information

National Science Foundation, Grant/Award Number: 1342841 and 1342793

Handling Editor: Oscar Godoy

Abstract

1. Coexistence and diversity in plant communities depend upon outcomes of plant competition. Competition and coexistence can be mediated by abiotic soil nutrient differences as well as by soil microbial communities. The latter effects occur through various mechanisms including negative plant–soil feedbacks, when plants foster the build-up of specialized pathogenic microbes, which ultimately reduce conspecific, but not heterospecific, densities. Microbial mutualists can have generalized associations with host plants, and by associating with multiple species might affect coexistence by conferring different levels of benefit to hosts.
2. We examined the effects of abiotic differences and soil microbial communities, including mutualistic nitrogen-fixing rhizobial bacteria, on coexistence processes and asked whether these interactions inform patterns of co-occurrence in natural communities. We measured plant–soil feedbacks in the greenhouse for three native *Trifolium* species that either highly co-occurred or were spatially repulsed at our field site. Using size-fractionated soil microbial inocula prepared from field-collected soils, we explored the effect of soil microbes on nodulation and the outcome of competition (relative interaction intensity). We also examined the effects of soil origin (home vs. away soil) on the outcome of competition between species.
3. Soil microbes had strong positive effects on plant growth and nodulation. Microbes in general reduced the strength of plant competition relative to competition in sterilized soil and altered the relative strength of interactions with conspecific vs. congeneric neighbours, which often occurred in ways predicted to enhance coexistence. In one pair of highly co-occurring *Trifolium*, competition was strong in sterilized soils, but these species facilitated one other in the presence of the full microbial community. The net effect of these microbes in general reduced competition while also increasing performance over that in sterilized soil, suggesting a role for soil microbial mutualists in host coexistence.
4. *Synthesis.* Our results demonstrate that interactions between plants and diverse soil communities can alter plant–plant interactions and plant–soil feedbacks. They may increase niche differences and equalize fitness differences in ways consistent with observed co-occurrence in the field.

KEYWORDS

coexistence, legume-rhizobia symbiosis, mutualism, plant competition, plant–soil (below-ground) interactions, plant–soil feedbacks, stabilizing and equalizing effects, *Trifolium*

1 | INTRODUCTION

Plants engage in a diverse set of biotic interactions, including competition with their conspecific and heterospecific plant neighbours. The outcome of such competition is strongly influenced by both abiotic and biotic components. Many studies have shown how nutrients and water supplied in varying ratios can alter the outcome of plant–plant competition (e.g., Davis, Grime, & Thompson, 2000; MacArthur, 1972; Tilman, 1982, and many more). Species-specific patterns of soil nutrient depletion may reduce the fitness of conspecifics and thus allow species with different nutrient needs to invade. Negative frequency dependence occurs when conspecifics compete more strongly than heterospecifics, either through direct nutrient competition or through indirect interactions mediated by microbes or other species (Adler, HilleRisLambers, & Levine, 2007; Bever, Westover, & Antonovics, 1997; Harpole & Suding, 2007; Petermann, Fergus, Turnbull, & Schmid, 2008). In coexistence theory, negative frequency dependence is necessary for stable species coexistence, since it provides each species an advantage when rare that allows it to recover from low densities (Chesson, 2000; MacArthur, 1972). In modern coexistence theory, negative frequency-dependent dynamics that allow populations to increase when rare are called “niche differences” or “stabilizing effects,” and act in concert with relative differences in fitness (“equalizing effects”) to increase rates of coexistence (HilleRisLambers, Adler, Harpole, Levine, & Mayfield, 2011).

Niche construction is a process through which organisms modify their own and other species' niches. One common source of niche construction is plant–soil feedbacks (PSFs), in which a resident species fosters the accumulation of specialist soil pathogens that reduce the performance of conspecifics more than heterospecifics (e.g., Kardol, Cornips, van Kempen, Tanja Bakx-Schotman, & van der Putten, 2007; Kardol, De Deyn, Laliberté, Mariotte, & Hawkes, 2013; Maron, Smith, Ortega, Pearson, & Callaway, 2016; Petermann et al., 2008; van der Heijden, Bardgett, & van Straalen, 2008; van der Putten et al., 2013). Negative PSFs have been widely documented and are proposed to be an important coexistence mechanism in plant communities (Heinze, Bergmann, Rillig, & Joshi, 2015; Kulmatiski, Beard, Stevens, & Cobbold, 2008; Mangan et al., 2010; Reinhart, 2012; van der Putten, van Dijk, & Peters, 1993), though they have rarely been related explicitly to a coexistence theoretical framework (but see Chung & Rudgers, 2016; Siefert et al., in press). One source of negative frequency dependence in plants arises from PSFs.

In contrast to the specialized pathogens that generate negative frequency dependence, many well-known soil mutualists like arbuscular mycorrhizal fungi (AMF) and rhizobial bacteria are

generalized and associate with a variety of hosts (e.g., Shelby et al., 2016). Negative frequency dependence through PSFs can also arise, somewhat counter-intuitively, from interactions with generalized mutualists (Bever, 1999, 2002). For example, if one species promotes the growth of mutualists that provide greater benefit to another plant species than to conspecifics, this difference in relative benefit can result in negative frequency dependence in the cultivating species (Bever, 2002; Castelli & Casper, 2003; Umbanhowar & McCann, 2005). Less surprisingly, soil mutualists can also generate positive PSFs, which occur when plant species support specialist mutualists that benefit conspecifics more than heterospecifics (Bever, 2003; Bever et al., 1997; Reynolds, Packer, Bever, & Clay, 2003; van der Heijden et al., 2008). Positive feedbacks have been observed in some interactions between plants and AMF (Zhang et al., 2010) and N-fixing rhizobial bacteria (Larson & Siemann, 1998), potentially outweighing the stabilizing effects of negative feedbacks arising from plant–pathogen interactions (Liang, Etienne, Huang, Wang, & Yu, 2015). While positive feedback is predicted to decrease coexistence at the local scale, positive feedbacks may maintain coexistence at broader spatial scales by generating sharp boundaries between species' patches that are stable over long time periods (Molofsky, Bever, & Antonovics, 2001; Parker, 1999). In summary, through their diverse direct and indirect effects, both models and empirical studies indicate that soil microbes may alter the outcomes of plant competition (Bennett & Cahill, 2016; Hartnett, Hetrick, Wilson, & Gibson, 1993; Hodge & Fitter, 2013; Lin, McCormack, & Guo, 2015; Scheublin, van Logtestijn, & van der Heijden, 2007; Umbanhowar & McCann, 2005) and thus coexistence.

Components of the soil microbial communities can also interact and result in different outcomes of plant–plant competition than their effects in isolation. Legumes are particularly likely to experience such interactions because they associate with a large variety of soil microbes, particularly nitrogen-fixing rhizobial bacteria, but also AMF, nematodes, numerous pathogens and others. For example, as generalized mutualists, AMF and rhizobia were able to both colonize seven *Trifolium* species (Shelby et al., 2016). The net effects of these microbes on *Trifolium* hosts were highly variable and depended on the host species and the source (native or introduced range) of the microbes and hosts (Shelby et al., 2016). Moreover, the combined effects of AMF and other microbes have been shown to interact and thus alter both the benefits received by *Trifolium* and PSFs (Alho, Carvalho, Brito, & Goss, 2015; García-Parisi, Lattanzi, Grimoldi, Druille, & Omacini, 2016). Rhizobia and AMF together reduce the competitive suppression of *Trifolium* species by grasses (Hall, 1978; Thompson, Turkington, & Holl, 1990; Wagg, Jansa, & Stadler, 2011). Thus, both AMF and rhizobia are mutualists that have the potential

to alter competitive outcomes among their legume host species through direct and/or indirect effects.

The effects of microbes and abiotic niche differences may play a role in the coexistence of closely related and ecologically similar plant species (e.g., Burns, Brandt, Murphy, Kaczowka, & Burke, 2017; Callaway, Montesinos, Williams, & Maron, 2013). How species respond to AMF may delimit niche differences that can promote coexistence (this feature, Veresoglou, Rilling, & Johnson, 2018). Close relatives may have strong niche overlap, including shared mutualists and pathogens (e.g., Anacker, Klironomos, Maherali, Reinhart, & Strauss, 2014; Callaway et al., 2013), and can also experience strong competition with one another, potentially posing challenges to coexistence (Anacker & Strauss, 2016; Darwin, 1859; reviewed in Weber & Strauss, 2016). On the other hand, close relatives often share niche preferences (Anacker & Strauss, 2016) and are more likely to have similar fitnesses (Godoy, Kraft, & Levine, 2014), thereby increasing their ability to coexist. The role of shared mutualists and other soil components in coexistence of close relatives is underexplored and represents a key knowledge gap.

In this study, we investigated the effects of the soil microbial community on interactions between three native annual *Trifolium* (clover) species that naturally co-occur in northern California coastal grasslands at fine spatial scales (<4 m²). One species has low overlap with the other two, while the other two are highly co-occurring. To understand how soil microbial communities might mediate coexistence of these three species, we grew these species in a greenhouse experiment, factorially manipulating competition, soil origin, and soil microbes, to (a) determine how competition outcomes differ with and without soil microbes, (b) assess the strength and direction of PSFs, (c) determine how microbes of varying size influence the strength and direction of plant interactions with conspecific and congeneric neighbours, and (d) relate these interactions to observed patterns of *Trifolium* species co-occurrence in natural communities. Specifically, microbes could mediate coexistence between highly co-occurring species through several mechanisms. Microbes could promote stronger niche differences, thereby reducing competition between species relative to conspecific competition and generating stabilizing effects that overcome fitness differences. For nonoverlapping species, microbes might also underlie positive PSFs which could generate disjunct species distributions.

2 | MATERIALS AND METHODS

2.1 | Study system background: *Trifolium* co-occurrence at our field site

This study focused on species of native annual *Trifolium* legumes (Fabaceae) in coastal grasslands of northern California at the Bodega Marine Reserve (BMR) in Bodega Bay, CA. *Trifolium* have mutualistic associations with *Rhizobium leguminosarum* biovar *trifolii*, forming nitrogen-fixing nodules on plant roots (Sprent, 2001), and with AMF (e.g., Crush, 1974). *Trifolium* species also interact with various soil antagonists, including bacteria, fungi, and nematodes (e.g., Ayres, Dromph, Cook, Ostle, & Bardgett, 2007) that coexist with these mutualists. To determine the role of below-ground microbes in coexistence, we first needed to document rates of coexistence among *Trifolium* species. These species have been recorded as locally co-existing our field site for many years (Parker & Gilbert, 2007), so our observed dynamics are unlikely to represent a transient community state. In April 2015, we surveyed *Trifolium* species occurrence in coastal grasslands at BMR in northern California (33°18'50.5"N, 123°4'6.3"W) in 415 2×2-m plots along 33 transects to quantify fine-scale co-occurrence patterns of *Trifolium* in nature. Eight annual native *Trifolium* species occurred across this survey. Across these surveys, we took soil cores adjacent to transect plots in areas where each species was abundant to characterize the abiotic soil composition.

We tested for nonrandom co-occurrence of each species pair using a probability-based analysis (Veech, 2006; see Supporting Information Part 1). This analysis compares the observed frequency of co-occurrence with the expected frequency of co-occurrence if each species is distributed independently of the other, and calculates the probability that the species co-occur at a frequency either less than or greater than the observed frequency (detailed methods and R script in Supporting Information).

From these coexistence surveys and analyses, we selected three species with varying degrees of co-occurrence at BMR. Two species, *Trifolium gracilentum* and *Trifolium microdon*, were significantly positively associated in the field, co-occurring 73% more frequently than expected by chance ($p = 0.022$; Table 1). A third species, *Trifolium fucatum* never co-occurred with *T. gracilentum*, giving a significantly negative spatial association ($p = 0.011$), and

TABLE 1 Analysis of co-occurrence of *Trifolium* species pairs in 4-m² plots at Bodega Marine Reserve ($n = 415$), showing the observed frequency of co-occurrence, expected frequency of co-occurrence if each species is distributed independently of the other, and the difference between the observed and expected frequencies (Obs – Exp). Positive values of Obs – Exp indicate the species co-occur more frequently than expected (positive association), and negative values indicate the species co-occur less frequently than expected (negative association). p -values represent the probability of species co-occurring less frequently (P_{lt} , i.e., “less than”) or more frequently (P_{gt} , i.e., “greater than”) than the observed frequency of co-occurrence (methods in Supporting Information Part 1)

Species pair	Observed	Expected	Obs – Exp	P_{lt}	P_{gt}
<i>fucatum</i> – <i>gracilentum</i>	0	0.03	–0.03	0.011	1
<i>fucatum</i> – <i>microdon</i>	0.054	0.088	–0.033	0.039	0.99
<i>gracilentum</i> – <i>microdon</i>	0.12	0.088	0.064	0.99	0.022

rarely co-occurred with *T. microdon*—38% less frequently than expected ($p = 0.039$; Table 1). In the field, on average, *T. fucatum* is the largest species, followed by *T. gracilentum* and then *T. microdon*, though spatial variation in growing conditions can cause great variability in size.

We also took soil samples at the edge of focal plots where we had surveyed *Trifolium* communities (Supporting Information Part 2). Soil analyses revealed similarity of soil elemental composition followed spatial overlap of species distributions; *T. fucatum* soils were most different from *T. gracilentum* soils, with especially higher sodium content (see also Bowsher et al., 2017; Figure S1).

Knowing these rates of co-occurrence, we could then explore the role of soil microbes in generating competition outcomes that might contribute to positive (*T. gracilentum* and *T. microdon*) or negative (*T. fucatum* with *T. gracilentum*/*T. microdon*) patterns of species co-occurrence in the field. Since mutualistic microbes like AMF and rhizobia aid in nutrient acquisition (P and N, respectively), they have the potential to offset or reduce the importance of abiotic soil differences. We predict that if microbes are important to mediating coexistence, they should reduce competition more between species that we find co-occurring (e.g., *T. gracilentum* and *T. microdon*), than between species that are spatially repulsed (*T. fucatum* with *T. gracilentum*, and to a lesser extent, *T. microdon*).

2.2 | Rhizobium strain distribution in the field

If rhizobial strains are localized in particular habitats and confer different benefits across *Trifolium* species, then such patchiness in rhizobial distribution could provide a possible mechanism through which generalized rhizobia might mediate *Trifolium* coexistence. To assess whether rhizobia strains are nonrandomly distributed across our field site, as part of a larger field experiment not presented here (Supporting Information Part 3), we planted *Trifolium* seedlings from the same families into common gardens located across *Trifolium* habitats at BMR. These plants served as “trap plants” for wild rhizobia and were grown until senescence and then collected for rhizobial identification. *Trifolium gracilentum* had very high mortality in this particular field experiment and thus was not included in further analysis. For *T. fucatum* and *T. microdon*, we obtained high-quality *nodC* sequences for 110 nodules across 37 plants, with 2–5 plants per species per site (see Supporting Information Part 1 for precise breakdown, Figure S2). We rehydrated roots, and directly assayed the dominant rhizobial occupant's *nodC* genotype using PCR and Sanger sequencing (methods in Supporting Information Part 3). *nodC* is a rhizobium-specific gene involved in initiating the legume–*Rhizobium* symbiosis for which we have previously found high levels of genetic variation at our field site. We analyzed the presence of rhizobial *nodC* genotypes with respect to host association and common garden. We analyzed OTUs using the sequences from each individual plant as replicates with the jaccard distance metric in adonis permutational ANOVA in the “vegan” package.

2.3 | Greenhouse competition study

To determine how our three *Trifolium* species with different rates of coexistence interacted via their soil microbes, we performed a greenhouse target-neighbour experiment. We grew individuals of our three focal species both alone and pairwise with each hetero-specific and with conspecifics.

We manipulated the soil microbe origin, using field soil collected from replicate areas occupied by either *T. fucatum* only or jointly by *T. gracilentum* and *T. microdon*. As the latter two species co-occur extensively at BMR, using a single “home” soil type for both species is biologically realistic and relevant to understanding the processes driving their patterns of spatial co-occurrence in the field.

We also manipulated the presence and type of soil microbes, growing plants in either (a) sterilized field soil, to account for competitive outcomes due to abiotic differences in the absence of microbes, (b) sterilized field soil inoculated with small microbes added from filtrates (rhizobia, viruses, bacteria, and fungi smaller than 38 μm) only, or (c) soil containing a combination of small and large microbes (including small fraction above plus the remaining large soil fraction containing AMF, nematodes, and other soil microfauna >38 μm). Our experiment thus factorially manipulated *Trifolium* target species (*T. fucatum*, *T. gracilentum*, or *T. microdon*), *Trifolium* neighbour (none or one of the three *Trifolium* species), soil origin (*T. fucatum* or *T. gracilentum*/*T. microdon*), and soil microbes (none, small only, or combined small and large). With 72 treatment combinations and 6 replicates per treatment, this resulted in 432 experimental units.

2.3.1 | Soil origin and microbe treatments

We collected field soils from BMR in April 2015 from two replicate sites occupied by *T. fucatum* and two sites occupied jointly by *T. gracilentum* and *T. microdon*. The purpose of the soil origin treatments was to measure PSFs by comparing performance of target species growing in soil conditioned by conspecifics (home soil) or congeners (away soil). When sterilized, this soil could reflect species-specific patterns of nutrient depletion, or microhabitat variation in abiotic properties. Our design included replicate unpooled soils within species to account for spatial variation in soil nutrients and microorganisms.

To create our experimental inoculations, we isolated small and large soil microbe fractions from field soils using a wet sieving method (Klironomos, 2002). A 75 g subsample of each replicate soil was blended with 250 ml of deionized water for 1 min, and the resulting slurry was washed through an 850 μm sieve followed by a 38 μm sieve (see also methods on INVAM website <https://invam.wvu.edu/methods/spores/spore-extraction>). The small microbe fraction that passed through the 38 μm sieve, including rhizobia and other small organisms, was diluted to 1,500 ml with deionized water. The large microbe fraction collected by the 38 μm sieve, including AM fungal spores, nematode and others, was washed with 0.055% sodium hypochlorite solution, rinsed with deionized water, and also diluted to 1,500 ml. The rinsing and extensive dilution of

filtrate extracted from only 75 g of soil greatly reduced any nutrient addition added through liquid inocula, which themselves were low volume. The large and small microbe fractions were mixed to produce a combined microbe treatment. Previous work using similar filtrate experimental approaches showed either complete or greatly reduced colonization of roots by AM fungi postsieving (e.g., Klironomos, 2002; Lankau, 2010; Pizano, Mangan, Herre, Eom, & Dalling, 2011). Similarly, autoclaving soil from our field site resulted in no colonization by AM fungi of grass species in a separate study (Bennett, Thomsen, & Strauss, 2011); however, the large fraction, like the small fraction, contains more than just AMF, including a mixture of mutualists and enemies.

2.3.2 | Planting and inoculation

Target and neighbour plants originated from seed sources collected at the field site and were subsequently raised in a greenhouse for at least one generation. Experimental plants were germinated in seedling trays in a greenhouse and transplanted into experimental soil cones (3.8 cm diameter at mouth, 14 cm depth; double-coned to prevent cross-contamination) at one-leaf stage in May 2014. Individuals that did not survive transplanting were replaced. Target and neighbour individuals were planted approximately 1 cm apart. Cones were filled with three layers of soil then autoclaved twice at 15 lbs pressure and 120°C temperature for 30 min: 35 ml of potting soil, 35 ml of field soil in the primary rooting zone of the plant, and another 35 ml of potting soil. Each cone was inoculated with 75 ml of extract (sterile deionized water, small microbe fraction, or combined microbe fraction), applied by syringe at the base of the plant, in three doses of 25 ml across three days without watering between applications.

Randomization of treatments in the greenhouse resulted in sterilized soil treatments neighbouring live soil treatments. Despite close proximity, plants in our sterilized soil treatments largely had no nodulation (23% plants with nodules; median = 0, mean = 4.3 nodules/plant). Moreover, the nodule–plant biomass relationship was not significant for any species in sterilized soil, reflecting no significant biomass gain from the few nodules formed; this indicates that cross-contamination rates were low and likely occurred late in the experiment. Removing nodulated individuals from sterilized treatments did not qualitatively alter the results, so all individuals were included in the final analyses.

2.3.3 | Harvesting and phenotypic measurements

Both target and neighbour plants in a given pot were harvested at the onset of flowering of the target plant, from June to September, 2014. We harvested pots at flowering of the target because at flowering legumes start to divest from nodules and put energy into flowers and fruits and we wanted to capture the peak rhizobial mutualism. It then made sense to simultaneously harvest the neighbour, which captured the competition regime until that moment. The *Trifolium* species have different sizes at maturity, and also

varied in flowering phenology, with smaller species flowering earlier than larger ones. The growing period from planting to flowering and harvest depended on species, with mean growing duration ranging from 66 to 102 days by species, with min–max of 32–139 days. We included harvest date (reflecting growing duration) as a covariate in all subsequent analyses to account for differences mediated by phenology. Harvest date was highly significant in most analyses, but did not interact with experimental factors of microbe or neighbour treatments. Across the experiment, as in the field, *T. fucatum* was the largest of the three species (mean target biomass = 1.19 g), followed by *T. gracilentum* (0.08 g), then *T. microdon* (0.028 g).

On target and neighbour plants, we measured total biomass, nodule number, and leaf chlorophyll content (a good proxy for leaf N). We separated roots from shoots, washed the roots, and counted nodules. We then measured biomass on roots and shoots dried at 60°C for 168 hr. We measured chlorophyll content on three randomly selected fresh leaves per plant with a SPAD meter (SPAD-502 plus; Konica Minolta, Osaka, Japan). To determine how well SPAD measurements estimate leaf nitrogen content, we determined total percent nitrogen of dried leaves of 20 individuals per species, selected from across experimental treatments, using Micro-Dumas combustion analysis. The strong positive relationship between dry leaf N content and fresh leaf SPAD values for each species (Pearson $r = 0.72\text{--}0.88$) indicates that SPAD measurements provide a useful proxy for leaf N content.

2.3.4 | Data analysis

We estimated effects of experimental treatments on target plant nodule number, leaf N and biomass using linear models with target species, soil origin (home vs. away), soil treatment (sterilized, small microbes, combined small, and large microbes), neighbour species, and their interactions as predictors and harvest date as a covariate. Preliminary analyses showed that subsite within soil origin had no significant effects on the response variables measured ($p > 0.1$), so we did not include it as a predictor in the analyses presented. Response variables were transformed as necessary to meet assumptions of linear models: biomass was log transformed for *T. fucatum* and *T. microdon* and square root transformed for *T. gracilentum*, and nodule number was log transformed for all species. Our initial analysis produced a large number of significant multiway interactions involving target species, indicating that species varied strongly in their responses to the experimental treatments.

To address whether interactions with rhizobia could determine plant performance, we examined the effect of nodulation on target plant biomass and leaf N using a second set of linear models. In this analysis, target plant biomass or leaf N was the response variable, and nodule number and its interactions with the experimental treatments were included as predictors. We were particularly interested in testing whether the slopes of the nodule–leaf N and nodule–biomass relationships, which indicate the benefit gained by the target plant for each added nodule, varied among soil microbial fraction and neighbour treatments.

These first analyses set the stage for our key analysis: to assess the strength of interactions between *Trifolium* species, and evaluate how these interactions were influenced by soil origin and microbes. To do so, we calculated relative interaction intensity (RII), a robust metric of plant competition (Armas, Ordiales, & Pugnaire, 2004; Díaz-Sierra, Verwijmeren, Rietkerk, Dios, & Baudena, 2017), using the formula:

$$\text{RII} = (\text{biomass}_n - \text{biomass}_a) / (\text{biomass}_n + \text{biomass}_a)$$

where biomass_n is the biomass of a target individual of a given species growing with a conspecific or congener neighbour, and biomass_a is the mean biomass of that target species growing alone in the corresponding soil origin and microbe treatment combination. Negative RII values indicate that the target plant growth is suppressed by its neighbour through competition, and positive values indicate facilitation; the RII metric takes values from -1 to $+1$ and is thus preferable to other traditional competition metrics (Díaz-Sierra et al., 2017). We modelled RII for each species as a function of neighbour identity, soil origin, soil microbe fraction, and their interactions.

We assessed the significance of main effects and interaction terms in the linear models using ANCOVAs, with harvest date as a covariate. Because the data were unbalanced resulting from uneven mortality across treatment groups, and strong interactions between experimental factors were present, we performed ANCOVAs with type III sums of squares, using the “ANOVA” function in the “car” package (Fox & Weisberg, 2011) in R (R Core Team, 2015). We tested for differences in means (and slopes for nodule relationships) among treatment levels by performing general linear hypothesis tests, using the “glht” function in the “multcomp” package (Hothorn, Bretz, & Westfall, 2008) in R. We tested for differences in RII in interactions using *a posteriori* means tests (JMP Pro 13).

3 | RESULTS

3.1 | Distribution of rhizobial strains across five common gardens at BMR

We identified 13 distinct dominant *nodC* genotypes in the nodules of our experimentally planted *T. fucatum* and *T. microdon* plants across five common garden sites at the reserve (Figure 1). These rhizobial genotypes were nonrandomly distributed across sites (adonis $R^2 = 0.196$, $p = 0.003$), but not across host species (adonis $R^2 = 0.0212$, $p = 0.579$; Supporting Information Part 3; Table S1). Thus, rhizobia may mediate *Trifolium* coexistence through patchy distributions combined with conferring differential benefits to different *Trifolium* species.

3.2 | Effects of microbial fraction and plant neighbours on *Trifolium* spp. nodulation, leaf N, and plant performance

Adding soil microbes drastically increased nodulation in all species ($p < 0.001$; Figure 2; few plants in sterilized soil nodulated, see

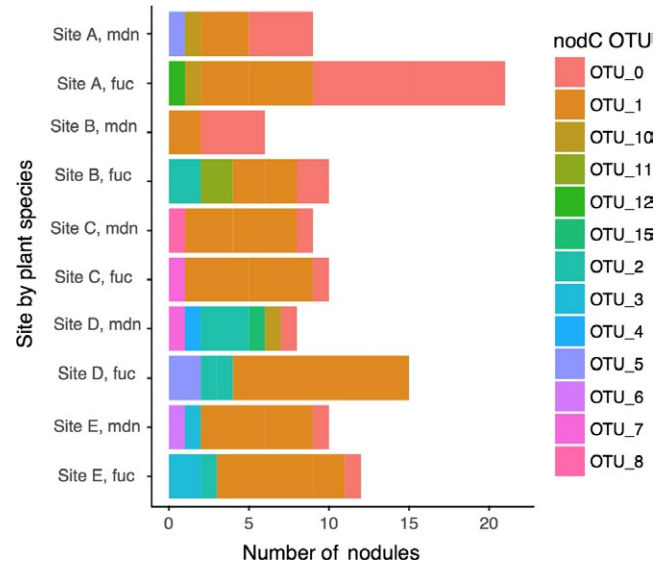


FIGURE 1 Rhizobia associating with experimental plants in the field are diverse, generalized across species, and structured by site. *Trifolium* species: fuc = fucatum, mdn = microdon. Five sites were considered in this analysis, see Supporting Information Part 3 for more details. Each sub-bar represents the number of nodules from experimental plants of a given species at a given field garden (sites A–E) whose *nodC* OTU belonged to a given cluster [Colour figure can be viewed at wileyonlinelibrary.com]

Methods), and was accompanied by an overall increase in both leaf nitrogen and plant biomass (Figure 2; Tables 2 and 3).

The effects of soil origin, soil microbe fraction, and neighbour identity on nodulation were species-specific (Table 2). For *T. fucatum*, the effect of soil origin on nodulation depended on neighbour treatment. *Trifolium fucatum* produced more nodules in home than away soil when growing with a conspecific neighbour, but there was no difference between home and away nodulation in alone or congeneric neighbour treatments. For *T. microdon*, nodulation depended on soil origin, soil microbe, and neighbour treatments. In the small microbe fraction treatment, *T. microdon* produced fewer nodules in home than away soil ($t = 3.08$, $p = 0.004$), and this effect was strongest when *T. microdon* was growing with a conspecific neighbour. Nodule number of *T. gracilentum* was not affected by neighbour, soil origin, or their interactions with soil microbe treatment.

We used the relationship between nodulation and plant performance as a measure of the strength of the *Trifolium*-*Rhizobium* mutualism; these were strongly correlated for all species when growing in the presence of soil microbes ($p < 0.03$; Figure 3). We then asked whether the mutualism was impacted by our experimental manipulations of soil microbes, soil origin, and plant competition. For *T. fucatum*, the slope of the nodule–biomass relationship was steeper in home than away soil ($t = 2.554$, $p = 0.012$; Figure 3a), suggesting that *T. fucatum* benefits most on a per-nodule basis from rhizobial strains in home soil. The relationship was also steeper in the small microbe treatment compared to the combined microbe treatment ($t = 2.57$, $p = 0.03$), suggesting that interactions with large microbes reduce the per-nodule host benefit. The nodule–biomass relationship for *T. fucatum* was also

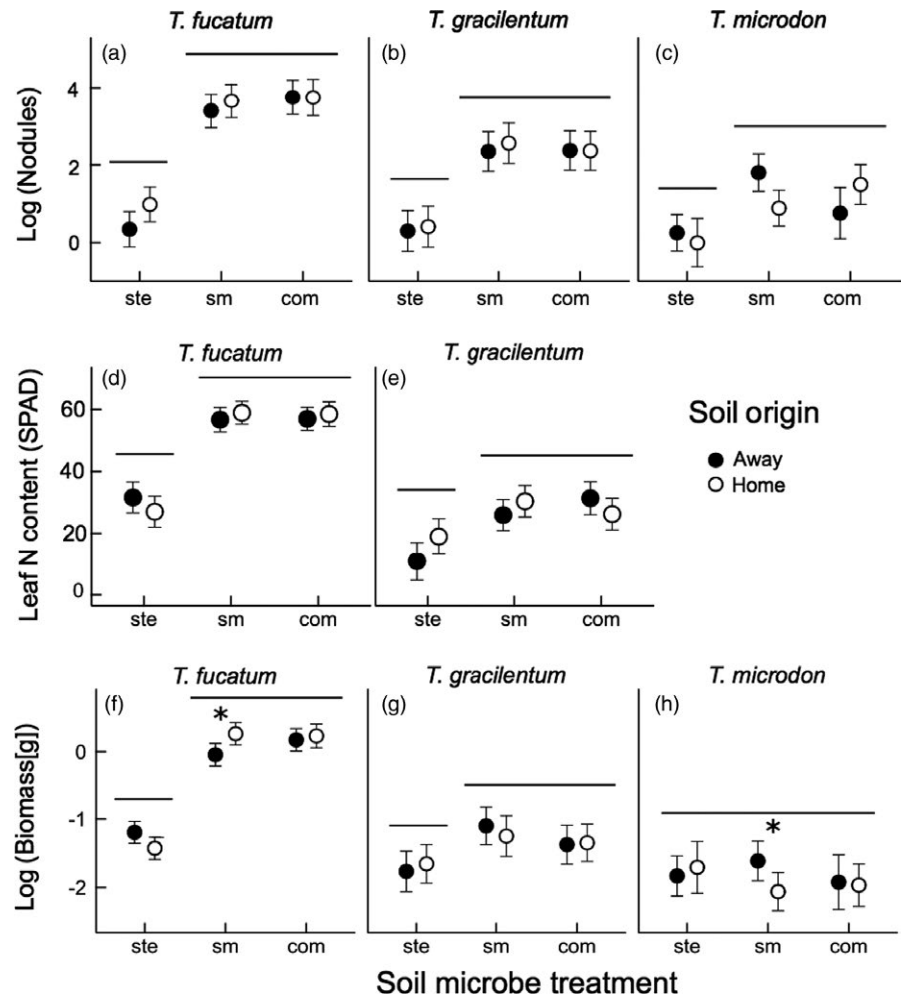


FIGURE 2 Soil microbes are often mutualistic and can be important relative to abiotic plant–soil feedbacks. Effects of soil microbe treatment (ste–sterilized [abiotic feedbacks only], sm–small microbes, com–combined large and small microbes) and soil origin (home vs. away) on target plant nodulation, leaf nitrogen content, and growth. Nonoverlapping horizontal lines above points indicate significant differences between soil microbe treatments. Stars in (f–h) indicate significant differences ($p < 0.05$) between home and away soil origin within soil microbe treatments, i.e., plant–soil feedback

TABLE 2 F statistics for analysis of covariance of target plant biomass, nodule number, and leaf nitrogen (N) for *Trifolium fucatum*, *Trifolium gracilentum*, and *Trifolium microdon*. Soil microbe treatments: sterilized, small fraction, or combined small and large fractions. Plant neighbour treatments: alone, conspecific, or congener species (two species treated as separate levels). Soil origin treatments: home or away

Model term	df	Nodules			Leaf N	
		<i>fucatum</i>	<i>gracilentum</i>	<i>microdon</i>	<i>fucatum</i>	<i>gracilentum</i>
Harvest date	1	1	0.89	0	7.23**	1.05
Microbial extract (M)	2	108.38***	35.81***	13.37***	100.59***	13.55***
Plant neighbour (N)	3	1.6	0.33	1.5	0.06	0.84
Soil origin (O)	1	2.47	0.47	0.75	0.12	1.63
M×N	6	0.84	1.2	0.73	0.29	1.01
M×O	2	0.46	0.42	5.32**	1.33	3.45*
N×O	3	1	0.81	4.27*	2.1	0.34
M×N×O	6	2.89*	1.73	2.27†	0.38	0.62
Residual df		98	95	41	79	60

† $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

influenced by neighbour treatment ($F_{3,108} = 4.10$, $p = 0.008$), with the slope being shallowest for plants grown alone and steepest for plants grown with *T. gracilentum*, suggesting that rhizobia provide an extra benefit to *T. fucatum* in competition with that species, with which it competes strongly (see Results on plant competition below).

For *T. gracilentum* and *T. microdon*, the relationship between nodule number and biomass did not vary among soil origin, soil microbe, or neighbour treatments, suggesting these species'

relationships with rhizobia are more generalized (Figure 3b,c). In addition, given that *T. microdon* nodulated least in home soils, there may be negative feedbacks with rhizobia or other soil microbes for *T. microdon* in home soils.

Another metric of how beneficial rhizobial partners are is leaf nitrogen, since symbiotic nitrogen-fixation is one of the key innovations in the legume-rhizobium relationship. Soil microbe addition had a strong positive effect on leaf N content of *T. fucatum* and

Model term	df	Plant biomass			Leaf N	
		<i>fucatum</i>	<i>gracilentum</i>	<i>microdon</i>	<i>fucatum</i>	<i>gracilentum</i>
Harvest date	1	13.27***	7.19**	3.26	4.98*	1.41
log(nodules)	1	5.01*	24.79***	8.88**	1.52	1.92
Microbial extract	2	20.26***	5.15**	6.10**	13.18***	0.12
Plant neighbour	3	8.00***	1.73	0.65	0.16	0.47
Soil origin	1	4.13*	1.14	0.04	0.37	6.37*
Nodule×microbe	2	3.87*	5.43**	1.69	0.10	1.88
Nodule×neighbour	3	4.10**	0.31	0.93	0.21	0.30
Nodule×origin	1	6.52*	2.29	0.11	0.72	6.02*
Residual df		108	102	50	89	70

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

TABLE 3 F statistics for analysis of covariance of target plant biomass and leaf nitrogen (N) with target plant nodule number as a covariate

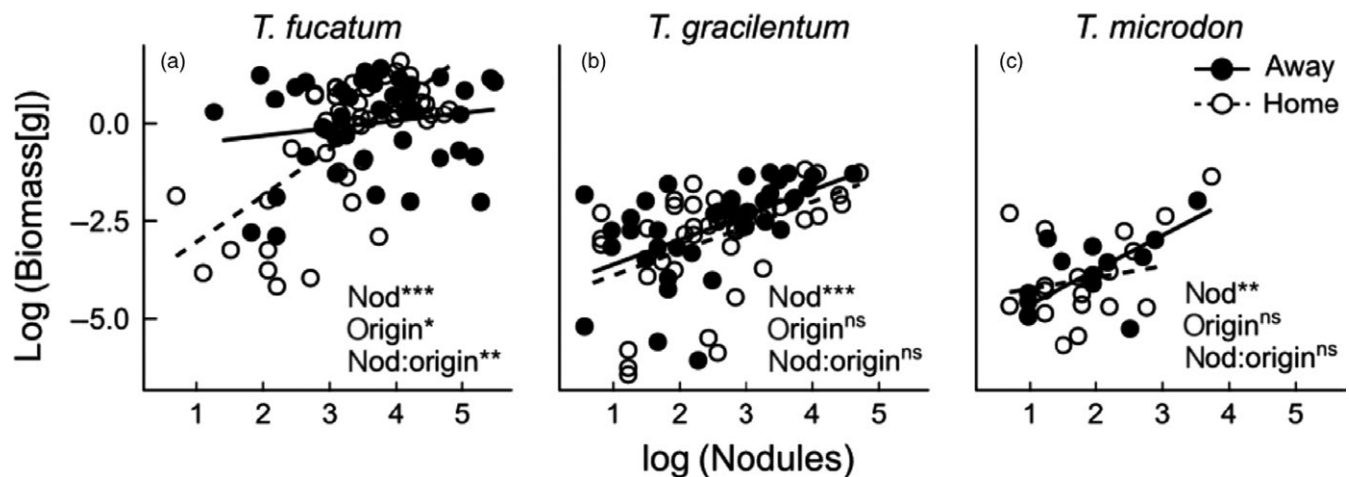


FIGURE 3 The strength of the *Trifolium*-*Rhizobium* mutualism varies by species and soil origin. Relationship between nodule number and biomass of target plants in home and away soil. Data are shown for target plants grown in the presence of soil microbes (small microbe or combined small and large microbe treatments). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

T. gracilentum ($p < 0.001$); *T. microdon* leaves were too small to be measured, precluding comparison. Leaf N content was not affected by neighbour treatment or its interactions with other experimental factors for any species (Tables 2 and 3).

In summary, our three species had very different relationships with rhizobia. *Trifolium fucatum* relationships with rhizobia were more specialized, with greater biomass accrued per nodule, especially in home soil, and greater leaf N content with increased nodulation regardless of soil type. In contrast, for *T. gracilentum* and *T. microdon*, biomass increased at the same rate with nodulation in any soil. These results suggest that *T. microdon* and *T. gracilentum* have more generalized relationships with rhizobia than *T. fucatum*.

3.3 | Soil microbes tend to enhance *Trifolium* performance and lead to species-specific plant-soil feedbacks

Soil microbes enhanced growth of *T. fucatum* (sterilized vs. small and combined microbe treatments: Figure 2c; Table 3) and *T. gracilentum*, Figure 2; Tables 3 and 4; Table S2); interestingly *T. microdon*

biomass was unaffected by microbial treatment. This positive effect on the other species can likely be attributed to the effects of small microbes, as there was no difference in biomass between the small and combined microbe treatments for either species (Figure 2; Table S2). Two species showed significant PSFs in home vs. away soils, but these occurred in opposite directions and also depended upon the microbe fraction. *Trifolium fucatum* in the small microbe treatment performed better in home than away soil (Table S2; Figure 2f), indicating a positive microbially mediated feedback, likely mediated by bacteria (Figure 2f-h; Table 3; Table S2). Small microbes significantly decreased growth of *T. microdon* in home soil relative to away soil, consistent with negative PSFs and frequency dependence (Figure 2h; Table 3; Table S2).

3.4 | Effects of soil origin and microbes on plant-plant interactions

We evaluated the strength of competition among species relative to both soil origin and microbial fraction treatment using RII, which measures the degree to which species are suppressed by neighbours

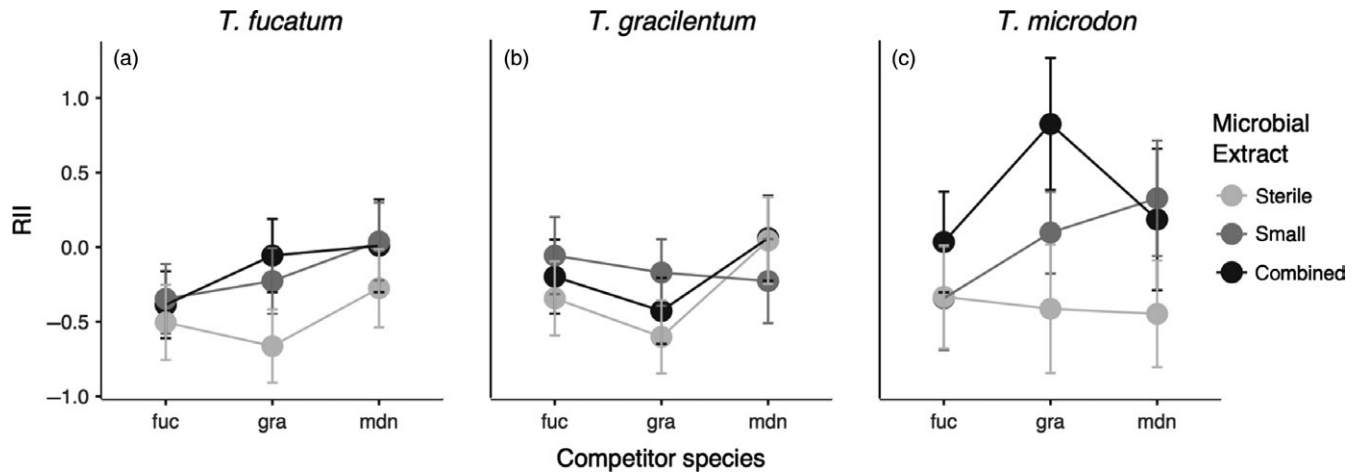


FIGURE 4 Effects of soil microbes on plant–plant interactions vary depending on microbial community complexity and plant identities. Each panel depicts the relative interaction intensity (RII) of a single focal species (fuc–*Trifolium fucatum*, gra–*Trifolium gracilentum*, mdn–*Trifolium microdon*) growing with each of the other three species in our experiment, compared to growth alone under the matching microbial treatment. Point and standard error bars are coloured according to microbial treatment: ste–sterile, sm–small, com–combined large and small. Points less than zero with nonoverlapping SEs depict competition while those above the line depict facilitation. Differences between least-squared means reported in Table S4 and text

relative to the performance of that species grown alone in the same soil origin and soil microbe fraction.

A general pattern in our experiment was that suppression by neighbours was greatest in sterilized soils, with the presence of microbes reducing plant competition ($p < 0.001$; Figure 4; Table 5; Table S3). This is a clear effect of microbes reducing the strength of competition, which can thus increase fitness. *Trifolium fucatum*, which coexisted least with the other two species in the field, was most suppressed by competition as measured in the greenhouse, followed by *T. gracilentum*; *T. microdon* was least suppressed, and was sometimes facilitated by neighbours (Figure 4; Table S4). There were, however, strong interactions between target species, neighbour and microbial treatment on the strength of competition (Figure 4; Table 5; Tables S3–S7), suggesting that soil microbes play a role in coexistence.

Trifolium fucatum interactions with neighbours were more negative in sterilized soils than in the small or combined microbe treatments (Figure 4a; Tables S3 and S7). In sterilized soil, *T. fucatum* was strongly and equally suppressed by conspecifics and *T. gracilentum* but significantly less so by *T. microdon*. With both small and combined soil microbes, *T. fucatum* was suppressed more by conspecifics than it was suppressed by its congeners (Table S3). Thus, despite being beneficial in terms of overall performance, and despite the fact that the strength of conspecific competition was less with small microbes than in sterilized soils, microbes caused far greater relative suppression of *T. fucatum* by conspecifics than congeners (Table 6, Table S7), consistent with self-limitation and negative frequency dependence. This result is also consistent with microbes increasing niche differences for *T. fucatum* and *T. gracilentum*.

Trifolium gracilentum was significantly suppressed by conspecifics, somewhat less by *T. fucatum*, and not affected by *T. microdon*, with the outcome of competition dependent upon soil microbe treatment (Figure 4b). *Trifolium gracilentum* suffered strongest

TABLE 4 ANCOVA results on target plant biomass (ln-transformed) with full factorial of Target species, Neighbour, Microbial extract, Soil Origin main effects with harvest date as a covariate

Model term	df	Sum of squares	F ratio	Prob > F
Microbial extract (M)	2	106.112	36.556	<0.0001
Target sp (T)	2	206.668	71.1975	<0.0001
Soil origin (O)	1	0.299	0.2057	0.651
M×O	2	0.320	0.1101	0.900
T×O	2	2.105	0.7251	0.485
M×T×O	4	17.885	3.0806	0.017
Neighbour sp. (N)	3	12.842	2.9493	0.034
M×N	6	18.133	2.0823	0.056
T×N	6	16.144	1.8539	0.090
O×N	3	2.731	0.6273	0.600
M×T	4	127.169	21.905	<0.0001
M×T×N	12	25.689	1.475	0.134
M×O×N	6	9.997	1.148	0.336
T×O×N	6	4.743	0.5447	0.773
M×T×O×N	12	18.972	1.0894	0.370
Harvest date	1	24.771	17.0672	<0.0001
Residual	237			
Total df	307			

Note. Adjusted R -squared: 0.68, $p < 0.0001$.

competition from conspecifics in sterilized soil, but no competition from conspecifics in the small microbe treatment, and modest competition in the presence of combined microbes. In contrast to

TABLE 5 *F* statistics for analysis of covariance of relative interaction intensity (RII) for *Trifolium fucatum*, *Trifolium gracilentum*, and *Trifolium microdon*

Model term	df	Sum of squares	F-value	Pr(>F)
(Intercept)	1	2.061	13.946	0.000
Harvest time	1	0.656	4.436	0.037
Target species (T)	2	1.270	4.296	0.015
Neighbour species (N)	2	1.639	5.545	0.005
Microbial extract (M)	2	4.512	15.265	0.000
Soil origin (O)	1	0.159	1.075	0.301
T×N	4	1.997	3.379	0.011
T×M	4	1.594	2.697	0.033
N×M	4	1.155	1.955	0.104
T×O	2	0.578	1.954	0.145
N×O	2	0.397	1.345	0.263
M×O	2	1.289	4.360	0.014
T×N×M	8	2.438	2.062	0.042
T×N×O	4	0.402	0.680	0.607
T×M×O	4	0.600	1.016	0.401
N×M×O	4	0.168	0.284	0.888
T×N×M×O	8	1.390	1.176	0.316
Residual	168	24.828		

Note. Adjusted *R*-squared: 0.26, *p* < 0.001.

conspecifics, interactions with congeners were not altered by soil microbes.

Soil microbes alleviated *T. microdon*'s response to competition with all species (Figure 4c). In sterilized soil, *T. microdon* was equally suppressed by conspecifics and congeners (Figure 4c; Table S7). Despite its small size, *T. microdon* was not suppressed by any species in the presence of small soil microbes and was facilitated by *T. gracilentum* in the presence of combined microbes (Table S7).

3.5 | Results summary: Relating competition outcomes, microbial communities, feedbacks, and coexistence

Given the complexity of our results, we attempt to summarize the salient patterns and relate them to observed coexistence in the field by these species. RII averaged over target species showed that *T. fucatum* was the most suppressed by neighbours, the strongest competitor of the three species, and our field observations found it to occur the most spatially isolated from other species. Averaged over all extract treatments measured in our greenhouse experiment, we find the strongest overall RII between non-co-occurring *T. fucatum* and *T. gracilentum* (RII = -0.26), which have high reciprocal competitive effects on each other. *Trifolium fucatum* and *T. microdon* have lower competitive effects on each other (RII = -0.145)

and some overlap in the field, with highly co-occurring *T. microdon* and *T. gracilentum* having either neutral or facilitative effects on each other with a net positive interaction (RII = 0.0645). We note that the combined microbe treatment had the most positive RII values, followed by the small microbe fraction, followed by sterile (Table 6).

Much of the overall RII was influenced by high competition in sterilized soils, but microbes clearly moderated and modified these effects (Figures 4 and 5; Table 6). For example, *T. microdon* exhibited strong competition in sterilized soil with *T. gracilentum*, but was significantly facilitated by *T. gracilentum* in the presence of the whole microbial community, and had neutral interactions with this species in the presence of small microbes. In the field, these species coexist together 78% more often than expected by chance alone, so the interaction outcomes we found with microbes were most consistent with observed patterns of coexistence for these species. Similarly, *T. fucatum* and *T. microdon* had lower competitive effects with one another in the presence of microbes than did nonoverlapping *T. fucatum* and *T. gracilentum*. With live soil microbial fractions, the whole community of soil microbes resulted in conspecific competition being much stronger than heterospecific competition for both *T. fucatum* and *T. gracilentum*, a pattern that was not as strong in sterilized soils (Table 6).

Soil feedbacks, differences between home and away soils, were found primarily in conspecific competition with *T. fucatum* and *T. microdon*, and mostly with small microbes. Coexistence depends on the degree to which conspecifics suppress themselves relative to heterospecifics, in conjunction with relative fitness differences. *Trifolium microdon* facilitated itself under conspecific competition with small soil microbes in away soils; however, it was facilitated more by *T. gracilentum* in the same microbe/soil combination. *Trifolium gracilentum* experienced neutral interactions from *T. microdon* in the same treatments.

In sum, it appears that abiotic differences in soil nutrients, as measured in our sterilized soil treatment, may be important to the outcomes of competition. Furthermore, these abiotic properties appear to differ in the microhabitats occupied by at least *T. fucatum* and *T. gracilentum* (Figure S1). However, because many soil microbes can increase accessibility of limiting nutrients, they may be able to compensate for some of these abiotic stressors. Moreover, different microbial components also include fitness-affecting pathogens, or mutualists that do not operate via nutrients (Friesen et al., 2011). Ultimately, our data suggest that changes in the composition of the microbial community may have large effects on competitive outcomes, as they often reduced niche differences in abiotic conditions as observed in sterilized soil.

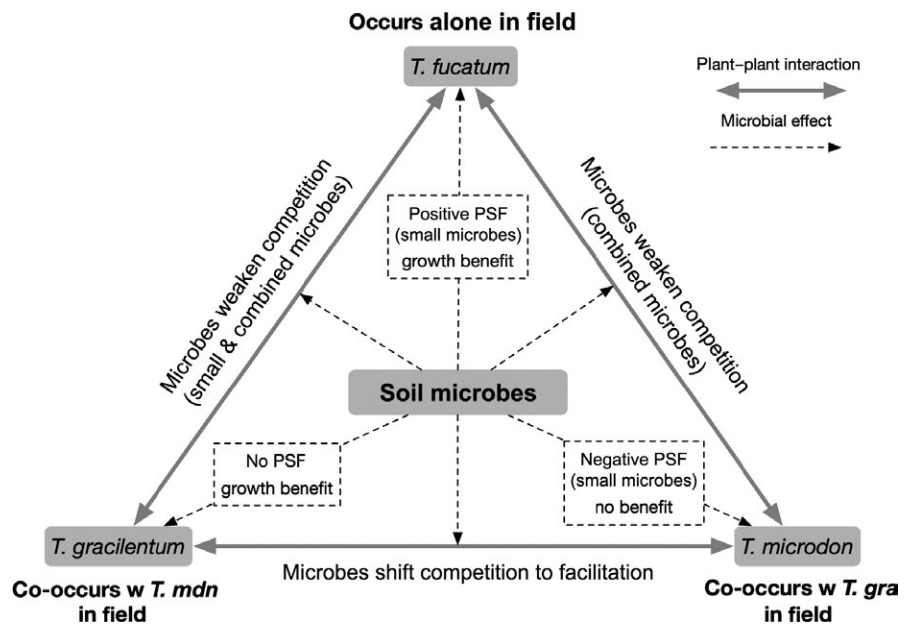
4 | DISCUSSION

Stable coexistence of species ultimately requires that species suppress conspecifics more than congeners, and that these niche differences are strong enough to overcome average fitness differences between species (Chesson, 2000). Closely related species may share

TABLE 6 Relative interaction intensity (RII) by microbial extract under microbial treatments and in relation to coexistence theory. Whether heterospecific (hetero) RII is significantly less than conspecific (consp) RII is based upon least-square means test, as reported in Table S7. Field co-occurrence values are fold changes calculated from Table 1. RII values that are positive are bolded

Microbial extract	Neighbour spp	Target spp	Field co-occurrence	RII consp.>hetero?	lsmean RII	<i>A posteriori</i> difference
combined	gra	mdn	+0.73	Y	0.825	A
combined	mdn	gra	+0.73	Y	0.061	BCD
combined	fuc	mdn	-0.38	N	0.032	BCDE
combined	mdn	fuc	-0.38	Y	0.012	BCDEF
combined	gra	fuc	-1	Y	-0.057	BCDEF
combined	fuc	gra	-1	N	-0.198	CDEFGH
small	gra	mdn	+0.73	N	0.096	BC
small	mdn	fuc	-0.38	Y	0.040	BCD
small	fuc	gra	-1	N	-0.056	BCDEFG
small	gra	fuc	-1	N	-0.228	CDEFGH
small	mdn	gra	+0.73	N	-0.231	CDEFGH
small	fuc	mdn	-0.38	N	-0.346	DEFGHIJ
sterile	mdn	gra	+0.73	Y	0.046	BCD
sterile	mdn	fuc	-0.38	N	-0.271	CDEFGHI
sterile	fuc	mdn	-0.38	N	-0.329	CDEFGHIJ
sterile	fuc	gra	-1	N	-0.345	EFGHIJ
sterile	gra	mdn	+0.73	N	-0.408	DEFGHIJ
sterile	gra	fuc	-1	N	-0.662	J

FIGURE 5 Soil microbes can influence coexistence in multiple ways simultaneously. Summary of overall effects of soil microbes on plant-soil feedbacks and plant-plant interactions in relation to observations of field co-occurrence



more similar fitnesses (Godoy et al., 2014), increasing the likelihood of coexistence; but they could also compete more strongly (Anacker & Strauss, 2016; Weber & Strauss, 2016), decreasing that same likelihood if interspecific competition approaches the strength of intraspecific competition. Close relatives are also more likely to share biotic interactors (e.g., Anacker et al., 2014), and these shared associates can indirectly influence coexistence outcomes. Plant

species may also construct niches via a build-up of specialized pathogens, which, in turn, can result in negative PSFs that reduce fitness of residents (Parker & Gilbert, 2018; Petermann et al., 2008), and result in negative frequency dependence. Plants may also amplify soil mutualists, many of which, like AMF and rhizobia, appear to be more generalized in their ability to associate with hosts, especially closely related hosts. Generalized mutualists can cause complex

coexistence dynamics through the relative benefits provided to hosts that share them (e.g., Umbanhowar & McCann, 2005; Runquist & Stanton, 2013). Niche construction can also result from species-specific patterns of nutrient depletion, thereby affecting coexistence (e.g., MacArthur, 1972; Tilman, 1982).

Here, we explored aspects of niche construction important to the coexistence of closely related *Trifolium* species that we show share generalized rhizobial mutualists (Figure 1). These three *Trifolium* species differed in their fine-scale co-occurrence: *T. fucatum* never co-occurred with *T. gracilentum*, and did so less than expected with *T. microdon*. The latter two species (*T. microdon* and *T. gracilentum*) show high levels of co-occurrence, much greater than expected by chance (Table 1). We then separated size fractions of field-collected soil that had only microbes <38 μm , which would include rhizobia, from larger fractions that had AM fungi, nematodes, collembola and other large microbes, to understand their role in *Trifolium* coexistence. We found that, overall, soil microbes generally reduced the strength of competition between *Trifolium* relative to their interactions in sterilized soil (Figure 4). Microbial community effects on neighbour interactions were more consistent with the degree of spatial overlap in the field than competitive outcomes in sterilized soil (Table 6). We also found varying degrees of specialization to “home” rhizobial strains across *Trifolium* species. Plant performance with only small microbes was the most responsive to home and away soils, and thus we infer that PSFs in our system are mediated by microbes in the small size range (Figure 2).

4.1 | A specialized *Trifolium* species that coexists less shows positive soil feedbacks

Interactions with mutualists can generate positive PSFs that are predicted to destabilize local coexistence by increasing the performance of the locally common species relative to rare species (Bever, 1999; Bever et al., 1997). However, positive feedbacks may maintain coexistence at broader spatial scales by generating sharp boundaries between species patches that are stable over long time periods (Molofsky et al., 2001; Parker, 1999). Positive PSFs may explain why *T. fucatum* and its congeners have coexisted through time within our study site but rarely co-occur at fine scales (i.e., within 4-m² plots). We find the steepest positive relationship between nodule number and plant biomass in the home, small microbe treatment for *T. fucatum*, suggesting that there is an advantage of associating with familiar rhizobial strains (Figure 3). This result is consistent with previous work showing that some *Trifolium* species and genotypes perform better when inoculated with rhizobial strains isolated from their own nodules (Mytton, 1975; Chanway, Holl, & Turkington, 1989; Yates et al., 2005, 2008; C. A. Friel, M. L. Friesen, & S. Y. Strauss, unpubl. data for *T. fucatum*). Our surveys of *Rhizobium* communities at BMR found that rhizobial genotypes were strongly spatially structured (Figure 1). Spatial clustering of rhizobial strains coupled with strong strain specificity may explain why *T. fucatum* has a limited distribution, occupying a restricted

habitat along coastal bluffs, and low spatial overlap with other annual *Trifolium* in our study site. Thus, because *T. fucatum* fitness is highly dependent on its interactions with another trophic level, soil microbes, the spatial distribution of these microbes will be key to influencing fitness and *T. fucatum* coexistence with other *Trifolium* species. Indeed, experimentally imposed spatial heterogeneity greatly increased fitness differences in two *Rumex* congeners (Burns et al., 2017), potentially strongly altering coexistence dynamics.

Positive feedbacks with bacterial mutualists may be opposed by interactions with more complex communities, however. Indeed, the positive feedback observed for *T. fucatum* in the presence of small soil microbes disappeared when large soil microbes were also present, possibly due to negative feedbacks with antagonists in the large soil biota fraction. Previous work has shown that interactions with soil pathogens and mutualists may generate conflicting feedbacks. Fitzsimons and Miller (2010), studying five temperate grassland species, found that negative feedbacks were common in the presence of the complete soil microbial community, but feedbacks were primarily positive when plants were grown with AM fungal mutualists only. Similarly, Liang et al. (2015), examining PSFs in subtropical trees, found that positive feedbacks mediated by AM fungi largely outweighed negative feedbacks mediated by bacterial pathogens, resulting in neutral feedbacks in the presence of the complete soil community. The extent to which microbial community composition fluctuates across years will be important data to collect in the future, and could play a major role of the coexistence dynamics of these species.

4.2 | Soil microbes may also promote coexistence by equalizing fitness differences

Species that typically grow together may do so through a variety of mechanisms that either equalize fitness differences or increase niche differences. In contrast to patterns for *T. fucatum*, our two highly co-occurring study species, *T. gracilentum* and *T. microdon*, showed no differences in nodulation or nodule effectiveness between home and away soils. *Trifolium gracilentum* did not exhibit PSFs, while *T. microdon* showed a negative soil feedback as evidenced by lower biomass in home soil than away soil when only small microbes were present, suggesting a role for small microbial pathogens. However, we also find evidence that soil microbes as a community may promote coexistence between *T. gracilentum* and *T. microdon*—species that have both a broad spatial extent and a positive spatial association in our field site that has been maintained over more than 10 years (see also Parker & Gilbert, 2007). Overall, live soil microbial extracts greatly reduced competition relative to competition in sterilized soil, and even caused facilitation in some cases. In this case, because these species are widespread and strongly positively associated spatially, and occupy areas with similar soil chemistries (Figure S1), microbes may increase coexistence in these species by equalizing fitness differences rather than by increasing niche differences.

4.3 | Caveats and future opportunities

There are a number of caveats to our results. Most importantly, we do not know the identities of most of the microbes in these fractions. Previous work using similar filtrate methods showed little AMF colonization of roots inoculated with small filtrates (Klironomos, 2002; Lankau, 2010), and the INVAM website suggests <38 μm will remove AMF. In previous studies, we also have shown no detected colonization by AMF in two grass species when we used the same sterilization protocols of soil collected from the same field site at BMR (Bennett et al., 2011). Thus, we think AMF are largely absent or greatly reduced in both our small and sterilized treatments. That said, our large fraction soils also likely contained nematodes and other players that contribute to the net combined fraction effect. We did look at nodulation rates across treatments; nodulation occurred in our small and combined fractions, as expected, and nodulation conferred benefits under most species and contexts. Nodulation in sterilized soil treatments was nonexistent in most samples, and very low with no detected benefits to hosts when it occurred. Again, however, small fractions contain both enemies and mutualists. Thus, we do not know which organisms, other than rhizobia, might be responsible for the effects we found. That said, our fractions do represent the whole field soil communities, and separating the small and large players has given us insights into their roles in *Trifolium* coexistence; molecular methods characterizing these communities and their functionality represent a clear next step in further dissecting and documenting these interactions.

In addition, soil sterilization likely altered soil nutrients; however, since all the microbe treatments were added back into sterilized soil as well, those changes owing to sterilization would be held constant across extract treatments. We thus are confident that our results indicate an important potential role for soil microbial communities in modulating plant–plant interactions, and based on our nodulation data believe that mutualistic rhizobia play a critical role in this system.

Our study adds to the growing literature, highlighted by the present Special Issue, recognizing the importance of biotic context on determining the outcomes of plant interactions and coexistence (Chung & Rudgers, 2016; Lanuza, Bartomeus, & Godoy, 2018; Pendergast, Burke, & Carson, 2013; Siefert et al., in press), specifically in the framework of modern coexistence theory. We show that microbes, another trophic level, can influence both fitness and the niche components of coexistence in plant species in a manner that is consistent with long-term records of their fine-scale coexistence.

5 | CONCLUSIONS

Plant–soil feedbacks have been proposed as an important driver of plant community structure and coexistence, both by directly influencing plant performance and by altering plant–plant interactions. These are typically geared towards specialized pathogens that act to reduce fitness under high conspecific density, allowing other

species to invade. By measuring nodulation and nodulation benefits, we showed that relative *Trifolium* performance and N-fixation were affected by the soil microbial community. Moreover, the measured interactions of three congeneric legumes in the greenhouse with live microbial communities best accorded with patterns of fine-scale co-occurrence in natural communities. Soil microbes also, in most cases, altered the relative strength of conspecific vs. congeneric competition in a way that would promote long-term species coexistence. Our data emphasize the need to understand how much microbial communities change across years, and to expand our consideration of both mutualistic and antagonistic microbial communities as players in processes that modulate the coexistence of closely related plant species.

ACKNOWLEDGEMENTS

We are grateful to Greg Gilbert and Ingrid Parker for starting *Trifolium* coexistence data collections and initiating us into their field plot surveys; Caprice Lee, Carissa Zielinski, and Emily McLachlan for assisting with field, greenhouse, and laboratory work and data collection; and Jackie Sones for coordinating access to field sites at Bodega Marine Reserve. This work was supported by National Science Foundation grants 1342841 to S.Y.S. and 1342793 to M.L.F. The authors have no conflicts of interest to declare.

AUTHORS' CONTRIBUTIONS

A.S., M.L.F., and S.Y.S. conceived the ideas and designed methodology; A.S., K.W.Z., collected the greenhouse data; all authors collected field data; M.L.F. led nodule data collections; A.S., M.L.F. and S.Y.S. analysed the data; A.S., M.L.F., and S.Y.S. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data available from the Dryad Digital Repository <https://doi.org/10.5061/dryad.8gg5mk2> (Siefert, Zillig, Friesen, & Strauss, 2018).

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SUPPORTING INFORMATION

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How to cite this article: Siefert A, Zillig KW, Friesen ML, Strauss SY, . Soil microbial communities alter conspecific and congeneric competition consistent with patterns of field coexistence in three *Trifolium* congeners. *J Ecol.* 2018;106:1876-1891. <https://doi.org/10.1111/1365-2745.13042>