



Microbial mutualist distribution limits spread of the invasive legume *Medicago polymorpha*

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Abstract The spread of invasive species can be limited or promoted by the distributions of mutualists, which presents an opportunity for managing biological invasions. However, the ways in which the geographical distributions of mutualists shape the range limits and invasion dynamics of introduced species remains unclear. Legumes include noxious invaders whose success is tied to their mutualism with nitrogen-fixing rhizobium bacteria. We examine whether the availability of compatible rhizobia limits the spread of an invasive legume at both small (50 m) and large (United States) spatial scales across natural invasion fronts. Using greenhouse experiments we simulated the dispersal of *Medicago polymorpha* into soils at

increasing distances from a host patch. We find that rhizobial mutualist availability rapidly declines to almost zero within 20 m of established legume patches and legume fitness and the percentage of leaf nitrogen derived from symbiotic nitrogen fixation show a concomitant decline. The decline of fitness due to the lack of rhizobium mutualists differs among *M. polymorpha* genotypes. Our findings support the missed mutualist hypothesis whereby mutualist limitation reduces invasiveness. As *M. polymorpha* colonizes novel areas, seeds following either short- (50 m) or long-range seed dispersal will be mutualist-limited and exhibit low fitness and nitrogen fixation. Without co-introduction via transport of soil containing both mutualists, the patchy distribution of *E. medicae* may limit *M. polymorpha* invasion. Plant lineages exhibit genetic variation upon which selection could act to reduce dependence on rhizobia, thus a scarcity of

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symbionts could cause plant symbiosis traits to evolve during invasion.

Keywords Invasion ecology · Co-invasion · Mutualism · Microbial mutualist · Distribution · Plant–soil interactions

Introduction

Biological invasions, a global problem that can alter habitats and displace native species, can be both impeded and facilitated by mutualism (Kiers et al. 2015; Simberloff et al. 2013). While insufficient mutualism can hinder the spread of exotic species, mutualism can also propel the spread of these species where mutualists are abundant (Traveset and Richardson 2014). During a biological invasion, the positive and negative impacts of mutualism on invasiveness, and ultimately the environment, may shift across regions and geographic scales, yet the dynamic role of mutualism during invasive spread is not well understood (Traveset and Richardson 2014).

When mutualist partners are already available in the novel range, mutualism can promote invasion. Invasiveness may be enhanced through synergistic mutualistic interactions between non-native partner species (reviewed in Roux et al. 2017; Moeller et al. 2015). For example, invasive ants facilitate greater plant recruitment for invasive ant-dispersed plants than native ant-dispersed plants (Prior et al. 2014). Positive feedbacks between invasive mutualists can fuel population growth for both partners and accelerate their spread across invaded landscapes, a process termed ‘invasional meltdown’ (Prior et al. 2014; Hynson et al. 2013; Simberloff and Von Holle 1999). Invasive species can also form novel associations with resident mutualists, via ‘ecological fitting’ (Roux et al. 2017). The enhanced mutualisms hypothesis predicts invasions can be facilitated by ecological fitting when native mutualists favor invasive partner species (Kiers et al. 2015; Reinhart and Callaway 2006).

Conversely, reliance on a mutualist can constrain invasion if that mutualist is limiting or absent in the novel environment, a hypothesis termed “missed mutualists” (Dickie et al. 2017). Invasive host species must either co-invade with their mutualists or encounter compatible mutualists in the new range (Amsellem

et al. 2017; Roux et al. 2017). Dispersal limitation and environmental suitability can constrain the distribution of mutualistic microbial symbionts (Martiny et al. 2006; Mallon et al. 2015). Mutualistic symbiont distributions can in turn limit the distributions of hosts. The spread of insect hosts (Wernegreen 2012), the vertical distributions of coral species (Iglesias-Prieto et al. 2004), the range of brine shrimp (Nougué et al. 2015), the spread of ectomycorrhizal trees (Dickie et al. 2010), and the spread of legumes (Simonsen et al. 2017; Seeds and Bishop 2009) are all constrained by microbial mutualist availability. When mutualists are unavailable, mutualism dependence, the degree to which a host requires a mutualist for growth and reproduction, can slow invasion or limit the realized niche of the invader (Amsellem et al. 2017; Duffy and Johnson 2017; Roux et al. 2017; Simonsen et al. 2017). However, it remains unclear how mutualist partners are geographically distributed, particularly microbial mutualists, which are invisible to the naked eye, and how mutualist distributions impact the invasion dynamics of host species (Petrovskaya et al. 2017).

Microbial mutualists can be limiting to host establishment in novel areas (Simonsen et al. 2017; Dickie et al. 2010), a corollary to the enemy release hypothesis that asserts that invasion success is driven, in part, by fewer co-evolved parasites, pathogens, and predators in non-native habitats (Dickie et al. 2017; Reinhart and Callaway 2006). A lack of available mutualistic symbionts has constrained the range limits of invaders, such as *Pinus contorta*, which is limited by a lack of ectomycorrhizal fungi (Dickie et al. 2010), and *Acacia longifolia* which is constrained by the availability of nitrogen-fixing rhizobium mutualists (Rodríguez-Echeverría 2010). Despite their crucial role in determining plant and animal distributions, little is known about the spatial distributions of microbial symbionts across landscapes (Thakur et al. 2019). A multi-scale perspective of microbial mutualist distributions is critical to understanding the process and impacts of species invasions (Thakur et al. 2019; Mallon et al. 2015) because invaders spread via long- and short-range dispersal (Rodrigues et al. 2015; Wilson et al. 2009; Suarez et al. 2001).

Mutualist limitation may lead to the evolution of reduced mutualist dependence among invasive host genotypes (Dickie et al. 2017). If mutualists are scarce during the spread of an invader, selection could favor

invader genotypes able to survive and reproduce even in the absence of the mutualist. Such selection could result in the evolution of successful invasive genotypes that are less dependent on mutualism, as occurs in *Hypericum perforatum* (St. John's Wort) wherein genotypes from the invaded range receive less benefit from mycorrhizae than do native ones (Seifert et al. 2009). Similarly, terHorst et al. (2018) found invasive genotypes of *Medicago polymorpha* (Burr clover) received less benefit from rhizobia than do native genotypes. To understand whether mutualism will evolve during invasion, it is critical to know whether invasive genotypes harbor genetic variation for mutualism dependence, as this is the raw material upon which selection acts.

The *Medicago polymorpha*—*Ensifer medicae* co-invasion is a powerful system for the investigation of mutualist impacts on host spread because its invasion history is well-studied and these species are experimentally tractable. We examine how the spatial distribution of the nitrogen-fixing rhizobium bacteria, *E. medicae*, impacts the invasion of its specialized host, the legume *M. polymorpha*. *Medicago polymorpha* was introduced to the Americas in the 1700's (Spira and Wagner 1983; Porter et al. 2011), likely from the region near the South Spanish port of Cadiz (Helliwell et al. 2018). It is a prohibited noxious weed in certain areas (e.g. Arizona, USA) (Caravetta 2018) because it is fast-growing, forms monodominant patches that exclude native plants, and its burrs become entangled in the coats of livestock. Prior to European colonization, North American soils lacked rhizobia compatible with legumes such as *M. sativa* (alfalfa) and *M. polymorpha*, both of which engage in nitrogen fixation symbiosis with *E. medicae* (Coburn 1907; Westgate 1908; Wing 1912). Intentional inoculation of fields with *Ensifer* symbionts enabled the North American establishment of these crucial symbionts (Coburn 1907; Westgate 1908; Wing 1912), and *M. polymorpha*'s range now spans the continent, though it is patchily distributed (Porter et al. 2011; Porter and Rice 2013).

While the fitness of annual legumes, which can be approximated using plant biomass (Younginger et al. 2017; terHorst et al. 2018; Jack et al. 2019), is highly dependent upon the nutritional benefit conferred by symbiotic nitrogen fixation (Porter et al. 2011; Porter and Simms 2014), legume seeds do not contain rhizobial mutualists, so seeds must disperse to soils where compatible rhizobia are present to engage in

symbiosis. *M. polymorpha* can be used to quantify the relative abundance of its microbial mutualist because the number of root nodules formed by a plant indicate the quantity of rhizobia in the soil. This is true up to a threshold rhizobial abundance, at which point plants form sufficient nodules to meet their nitrogen requirements and negative regulatory systems of root nodule symbiosis inhibit further nodule formation (Nishida and Suzaki 2018). Previous work found *M. polymorpha* seedling survival and nodulation in field collected soil is positively correlated with the density of conspecifics present at the site of soil collection (terHorst et al. 2018). This suggests that *M. polymorpha* density in the environment is correlated to mutualist abundance, which has reciprocal effects on host fitness. However, further investigation into the distribution of microbial mutualists is required to predict whether small- and large-scale dispersal of seeds from individual host patches will deliver them to compatible mutualists, leading to successful establishment and spread.

To investigate how the spread of invasive legumes across landscapes is influenced by the distribution of beneficial rhizobia, we examine whether *M. polymorpha* seeds dispersed outside of focal host patches are limited by a lack of rhizobial mutualists and if certain plant genotypes are more resilient to mutualist limitation. At small (50 m) scales, we ask: (i), does mutualist abundance decline with distance from host patches? (ii) is host fitness increasingly mutualist limited away from host patches, and (iii) is there genetic variation in *M. polymorpha* on which selection could act to reduce dependence on rhizobia? Additionally, using soil samples collected across *M. polymorpha*'s invaded range we ask, (iv) is the mutualist found in the invaded range beyond the current distribution of its host? Our study connects the fine- and large-scale patterns of mutualist availability in the environment to genetic variation in host fitness outcomes that can shape mutualism evolution during species invasions.

Methods

Experiment 1: Fine-scale mutualist distribution

Soil collection. Collections occurred in March 2015 within the University of California Dawson Los

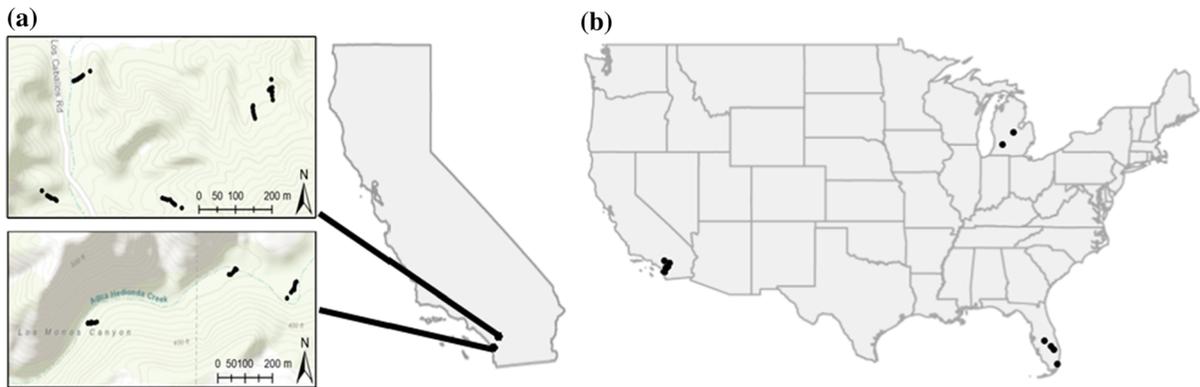


Fig. 1 Soil collection locations. **a** 8 transects through *M. polymorpha* patches from two natural reserves in CA, USA. Black dots show soil collections. Top panel, Emerson Oaks; bottom panel, Dawson Los Monos Canyon. **b** USA soil collection sites. Black dots, 11 sites

Monos Canyon Reserve in Vista, CA (33.148464, -117.249950) and the University of California Emerson Oaks Reserve in Temecula, CA (33.465937, -11.040070) (Fig. 1). At these reserves, *M. polymorpha* occurs in distinct patches. Eight 50 mL soil samples were collected from 1 m² quadrats every 5 m along six 50 m transects and two 30 m transects. Transects started 7.5 m within a wild *M. polymorpha* patch and ended 22.5 m from the patch edge in native vegetation where medics grew no closer to the focal patch than 30 m away. An additional set of samples were collected 42.5 m outside each patch, except at transects 7 and 8 due to dense vegetation. After collecting soil at 42.5 m on transect 2, we discovered a small patch of *M. polymorpha* adjacent to the collection site; therefore these data were excluded from analysis. Soil samples ($n = 496$) were stored at 4 °C for 3 months.

Common garden. *Medicago polymorpha* was grown in field-collected soils within a microbially controlled greenhouse common garden at the University of California, Riverside (33.971601, -117.323300) from June to July 2015 ($n = 537$). By doing our work in the greenhouse, we ensured that none of our *M. polymorpha* lineages would have seed flow or pollen flow into natural populations, and the *E. medicae* they amplified would not ameliorate soil conditions for further *M. polymorpha* expansion. *Medicago polymorpha* native range genotypes originated from Afghanistan (PI250782, USDA) and Spain (PI319029, USDA) and invaded range genotypes originated from California (NM58_12, Porter et al. 2011) and Bolivia (PI478466, USDA). Seeds were

sandpaper scarified and surface-sterilized for 2 min in 5% sodium hypochlorite before imbibing in sterile water for 2 h on a shaker at 150 rpm. Seeds were transferred to 1% agar plates, vernalized at 4 °C for up to 5 days until radicles were 1–2 mm, and were planted into 66-mL cylindrical pots (Stuewe & Sons, Inc., Tangent, OR) containing a 50 mL field soil sample. Half the pots were inoculated with a laboratory strain of *Ensifer medicae* (M58C295B00, Porter and Rice 2013; Porter and Simms 2014). *Ensifer medicae* was cultured for two days in tryptone yeast broth at 700 rpm at room temperature. A week after planting, either 1 mL of *E. medicae* at 10⁹ cells/mL based on OD₆₀₀ in sterile water or 1 mL sterile water was applied to each plant. As methodological controls, 10–11 uninoculated plants per genotype ($n = 41$) were grown in autoclaved Sungro Sunshine Mix #1 potting soil (Sun Gro Horticulture, Agawam, MA). Plants were grown in a customized complete randomized block design, with soil samples from each transect within the same block and randomized. Plants were mist-irrigated for six weeks with tap water that underwent carbon-block filtration to remove chlorine, chloramine and other impurities and fertilized at the appearance of the first trifoliate leaf with 1 mL of 2X Fåhraeus fertilizer (Fåhraeus 1957) containing 7 Atom% ¹⁵N-doubly labeled ammonium nitrate at 14.24 ppm. At harvest, roots were washed and frozen and above ground tissue was dried at 60 °C and weighed. Nodules were counted immediately after thawing roots. From each plant, 1–1.5 mg of dry apical bud and leaf tissue was encapsulated and ¹⁵N:¹⁴N ratios and nitrogen concentrations were

measured on elemental analyzers at Washington State University, Vancouver and the Washington State University, Pullman Northwest Sustainable Agroecosystems Research Unit (NSAR).

Analysis. We used generalized linear mixed effects models (*glmmTMB* package, Brooks et al. 2017) to test for a decline in *E. medicae* abundance (i.e. nodule number) with increasing distance from individual plant patches, and a corresponding decline in *M. polymorpha* fitness (using shoot mass as a fitness proxy (Younginger et al. 2017)) in R 3.6.1 (R Core Team 2019). The impacts of plant genotype, inoculation, reserve, and their pairwise interactions were modelled as fixed effects and transect (i.e. block) was included as a random effect for the response variables nodule number, shoot mass, and nitrogen derived from symbiotic nitrogen fixation. For the fixed effect of genotype, California was used as the reference level as it is the local genotype. Nodule counts were modeled using a zero-inflated negative binomial (NB) mixed model with log links. The NB was used instead of the Poisson because of overdispersion, the presence of which was determined by fitting a NB model with a quadratic parameterization ($V = \mu(1 + \mu/\phi) = \mu + \mu^2/\phi$; 'type 2' NB in *glmmTMB*) and evaluating the dispersion parameter ($\theta > 1$). Plant shoot mass is continuous and positive, so it was modeled using the gamma distribution with a log link. To test if nodulation declines due to soil factors other than rhizobial abundance, inoculated plants (i.e. plants not limited in rhizobial availability) were analyzed as a subset using a zero-inflated NB mixture model with log links and distance from the patch, plant genotype, reserve, and the interaction of distance by host genotype modelled as fixed effects, and transect as a random effect.

We used the ^{15}N stable isotope dilution method to measure rates of symbiotic N-fixation. Fertilizer labeled with the ^{15}N isotope allowed us to measure the quantity of unlabeled atmospheric N_2 legumes acquire via symbiotic N-fixation by comparing ratios of N isotopes in symbiotic plants vs non-symbiotic plants. The percent of nitrogen derived from the atmosphere ($\%N_{\text{dfa}}$) was calculated from symbiotic plant tissue ($\delta^{15}\text{N}_{\text{experimental}}$) and non-fixing plant tissue ($\delta^{15}\text{N}_{\text{reference}}$) (Yelenik et al. 2013). The natural abundance of ^{15}N in seeds was accounted for by subtracting 0.3663 atom% from the values of ^{15}N

atom% measured in all plants (Wolf et al. 2017). $\%N_{\text{dfa}}$ was calculated as:

$$\%N_{\text{dfa}} = \frac{\delta^{15}\text{N}_{\text{reference}} - \delta^{15}\text{N}_{\text{experimental}}}{\delta^{15}\text{N}_{\text{reference}}} * 100. \text{ Since } \%N_{\text{dfa}} \text{ is}$$

constrained to be between 0 and 1, beta regression with a logit link and transect as a random effect was used to analyze whether distance from patch, plant genotype, inoculation, and their interactions predict the percent of nitrogen plants derived from symbiotic nitrogen fixation.

Lastly, nodule number was tested to determine whether it could predict plant shoot mass and $\%N_{\text{dfa}}$. Shoot mass and $\%N_{\text{dfa}}$ were modeled by nodule number, genotype, and the interaction of nodule number and genotype, with transect as a random effect. For shoot mass, a gamma distributed mixed-effects model with a log link was used, and, for $\%N_{\text{dfa}}$, a beta distributed mixed-effects model with a logit link. Wald-Z tests were used to determine significance levels for Experiment 1 models. Pairwise post-hoc tests using t-tests with Tukey's correction for multiple comparisons were conducted for the relationship between shoot mass and nodule number among host genotypes (*emmeans* package, Lenth 2020).

Experiment 2: Large-scale mutualist distribution

Soil collection. Soil was collected from states across the U.S. that harbor populations of *M. polymorpha* but at sites where *M. polymorpha* or other medic species do not occur locally though environmental conditions are suitable for their growth (Lesins and Lesins 1979) (Fig. 1). Soil samples were tested for rhizobia capable of associating with *M. polymorpha*. The USDA Natural Resources Conservation Service was used to verify that *M. polymorpha* occurs in the regions we selected (USDA-NRCS, plants.sc.egov.usda.gov). Each site was searched to ensure no medics or *M. polymorpha* seed pods occurred within 30 m. Sites intersected by roads, streams, and dry streambeds were avoided. From October to December of 2014, 675 50 mL soil samples were collected across 12 sites in California, Michigan, and Florida. Soil was sampled as described above from up to eight haphazardly selected 1 m² subsites, with an average of 6 subsites per site. We collected 6–20 samples per subsite, with an average of 10 samples per subsite. Following the same sampling protocol, soil was collected from an additional site in California where *M. polymorpha* was

present for the purpose of a positive rhizobium control in the common garden experiment.

Common garden. To test for the presence of compatible rhizobia across the U.S., a microbially controlled common garden experiment was used, as described for Experiment 1. Soil samples were planted with nine *M. polymorpha* genotypes including accessions from the native range (PI459130: Turkey, PI493293: Portugal, PI577408: Morocco, PI227025: Iran, and W65559, Belgium) and invaded range accessions (NM58_35: California, PI404795: Uruguay, PI368947: Chile, and W65527: Australia). Accessions were obtained from the USDA except for NM58_35, which was originally collected by S. Porter in California in 2009. Up to 11 seedlings per genotype were planted in the soil from each collection site, with an average of six seedlings per genotype, with one seedling per pot. To test whether soil handling procedures supported rhizobia survival, six additional germinants per plant genotype were planted as a positive rhizobium control in soil collected from beneath *M. polymorpha* at the Emerson Oaks Reserve ($n = 54$). To assess contamination rates, 16 plants from the California genotype were grown in autoclaved Sungro Sunshine Mix #1 potting soil (Sungro Horticulture, MA, USA). A total of 60 additional plants that were grown in field soil samples from each site were inoculated with *E. medicae* as in Experiment 1. However, due to an error in preparation of the inoculum, almost none of these plants formed nodules and all 60 plants were dropped from the experiment. Remaining plants were fertilized three times a week with 1 mL Fåhraeus fertilizer (Fåhraeus 1957) containing 7 Atom% ^{15}N -labeled ammonium nitrate at 14.24 ppm. Plants were arranged in a complete randomized block design and grown for eight weeks as described for Experiment 1, from February 2015 to April 2015 ($n = 552$). At harvest, roots were washed and frozen and above ground tissue was dried at 60 °C and weighed. Nodules were counted immediately after thawing roots.

Analysis. To determine whether plants would form nodules when grown in soil collected in areas that lacked conspecific plants, 95% confidence intervals were calculated for the mean number of nodules formed from soil at each site. The values that fall within these intervals are those most compatible with the data, with the point estimates (i.e. mean number of nodules in this case) and those near it being more

compatible than those near the upper and lower limits. Therefore, the intervals can be used to assess whether a lack in nodules (0) is compatible with our data, given the precision of our estimates.

To compare shoot growth on plants grown in soil collected in areas that contained conspecific plants to plants grown in conspecific absent soils, we used a generalized linear mixed effects model (*glmmTMB* package, Lenth 2020). The impact of site was modeled as a fixed effect and state (California, Michigan, Florida) and plant genotype were included as random effects. For the fixed effect, the California site that contained *M. polymorpha* was used as the reference level as it is the positive control. Plant shoot mass is continuous and positive, so it was modeled using the gamma distribution with a log link.

Results

Experiment 1: Fine-scale mutualist distribution

(i) *Decline of mutualist abundance with distance from plant patches.* Nodule number is negatively correlated with distance from a plant patch (*Distance from patch*, estimate = -1.221 , SE = 0.132, $Z = -9.24$, $P < 0.0001$, Fig. 2a, Table S1). Across patches, plants grown in soil collected closest to the center of wild *M. polymorpha* patches, 7.5 m within the patch, produce 78% more nodules (all percent changes based on raw means) than plants grown in soil collected 7.5 m outside the patch. This pattern is indistinguishable across transects (Fig. S1a–S1h) and reserves (Table S1). Two exceptions to this pattern occur where dry streambeds run through transects (transect 5 at 17.5 m and transect 7 at 12.5 m, Fig. S1e and S1g). Plants grown in soil from 42.5 m on transect 2 also form nodules, but this soil is adjacent to a *M. polymorpha* population discovered after soil collection and these data were excluded from analysis (Fig. S1b).

Inoculation of field-collected soil with *E. medicae* increases nodule number by 44%, compared to plants grown with naturally occurring rhizobia (*Inoculation*, estimate = 1.049, SE = 0.196, $Z = 5.364$, $P < 0.0001$, Table S1). The effect of inoculation on nodule number depends on distance from the patch (*Distance:Inoculation*, estimate = 0.829, SE = 0.106, $Z = 7.85$, $P < 0.0001$, Table S1). For soil collected

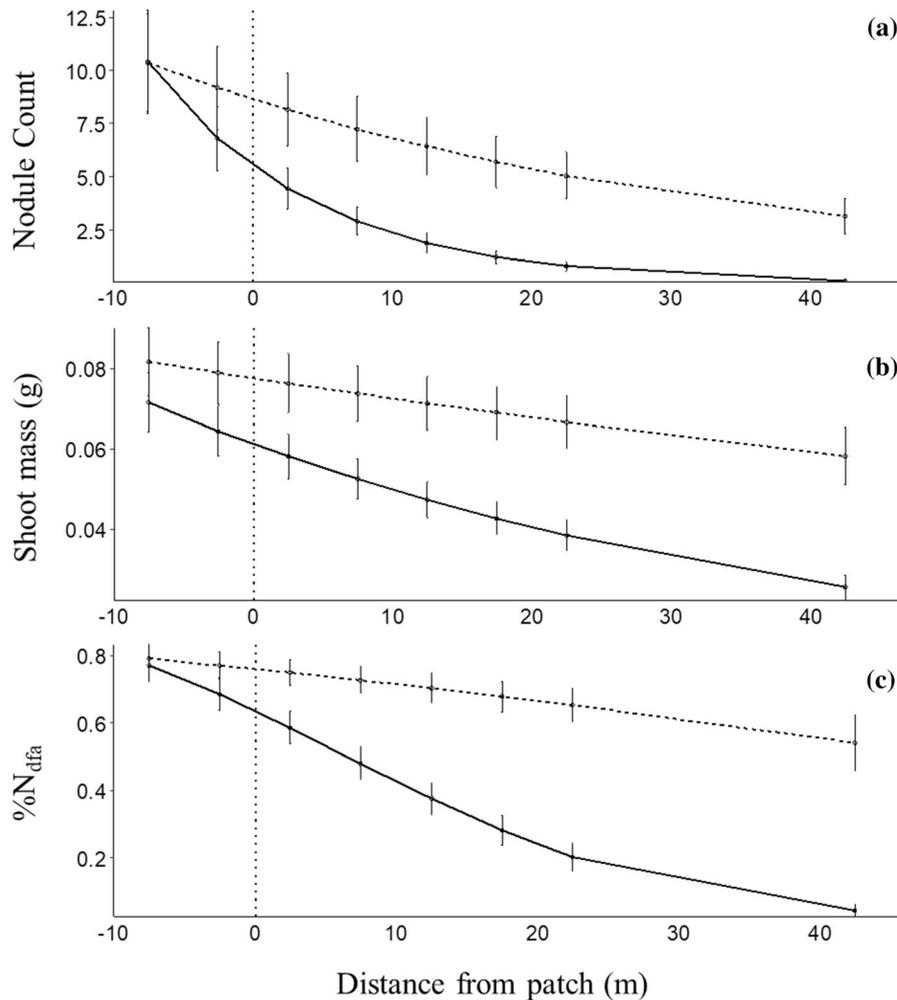


Fig. 2 Nodule count (a), shoot mass (b), and symbiotic nitrogen fixation (percent nitrogen derived from the atmosphere; $\%N_{dfa}$, c decrease with distance from patches of *M. polymorpha*. Dotted vertical lines, edge of patches. Solid lines; plants grown without

inoculation. Dashed lines; plants inoculated with *E. medicae*. Points are estimated marginal means for plants grown in soil collected from each point along the transects. Error bars are standard errors

within patches, inoculated plants produce 2% more nodules than uninoculated plants, whereas in soil collected 7.5 m outside of patches, inoculated plants produce 56% more nodules than uninoculated plants.

We ran an analogous analysis of nodule number on a subset of the data that includes only inoculated plants, with 10^9 rhizobia added, to determine whether the number of nodules *M. polymorpha* forms varies along a transect due to soil factors other than rhizobial abundance. Despite the high number of rhizobial cells added to inoculated plants, nodule number still declines away from the plant patch (*Distance from patch*, estimate = -0.358 , SE = 0.112, $Z = -3.20$,

$P = 0.0014$, Table S2). We did not detect a difference between reserves (*Reserve*, estimate = 0.444, SE = 0.313, $Z = 1.42$, $P = 0.157$, Table S2).

ii) *Plant fitness is increasingly mutualist limited away from plant patches.* Plant shoot mass decreases in soil collected at increasing distances away from patches (*Distance from patch*, estimate = -0.244 , SE = 0.055, $Z = -4.44$, $P < 0.0001$, Table S1, Fig. 2b). Inoculation has a greater fitness benefit for plants grown in soils far from established patches than plants within the patch (*Distance:Inoculation*, estimate = 0.188, SE = 0.047, $Z = 4.01$, $P < 0.0001$, Table S1). Inoculated plants are 2% larger than

uninoculated plants grown in soil collected 7.5 m within patches, but 30% larger in soils 7.5 m outside of patches. These patterns are generally consistent across transects (Fig. S1i-S1p) and reserves.

The percent of nitrogen plants receive from symbiont fixation decreases with distance from the patch (*Distance from patch*, estimate = -1.319 , SE = 0.218 , $Z = -6.041$, $P < 0.0001$, Table S1). At and beyond 10 m from the patch, %N_{dfa} is close to zero (Fig. 2c). The positive effect of inoculation on nitrogen fixation is stronger for plants in soils collected farther from patches (*Inoculation:Distance*, estimate = 0.847 , SE = 0.180 , $Z = 4.705$, $P < 0.0001$, Table S1).

(iii) *Genetic variation in M. polymorpha biomass per nodule. Medicago polymorpha* genotypes differ in their growth responsiveness to rhizobial abundance (Fig. 3). Nodule number is positively correlated with

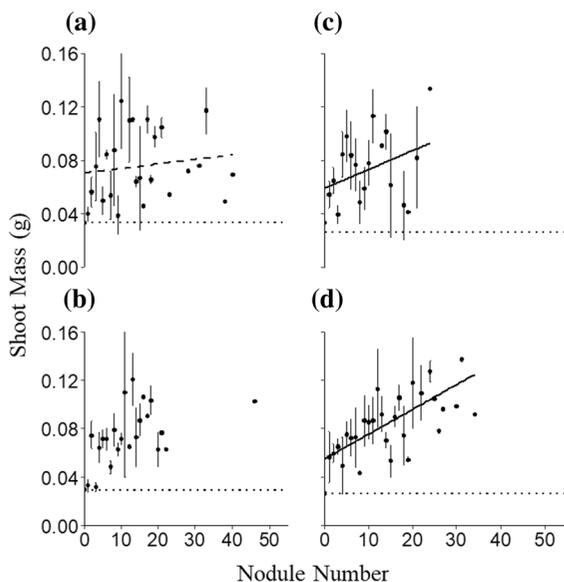


Fig. 3 *Medicago polymorpha* genotypes differ in responsiveness to rhizobium abundance. Mean shoot mass (points) increases with the number of nodules. The relationship between nodule number and shoot mass varies by genotype. Invaded range genotypes from **a** California and **b** Bolivia. Native range genotypes from **c** Afghanistan and **d** Spain. Solid trend lines illustrate the genotypes where the relationship between nodule number and shoot mass were found by Wald-Z tests to be significantly different than the reference genotype (California, dashed line). Also shown, mean standard error (vertical lines) and mean shoot mass of each genotype in the absence of rhizobia (horizontal dotted line). Points represent raw mean shoot mass for plants with the indicated number of nodules for each plant genotype

shoot mass (*Nodules*, estimate = 0.033 , SE = 0.006 , $Z = 5.38$, $P < 0.0001$, Table S3), but the relationship between shoot mass and nodule number differs among plant genotypes (*Nodules:Genotype* [*California-Bolivia*, *California-Afghanistan*, *California-Spain*], estimate = [0.019 , 0.022 , 0.018], SE = [0.010 , 0.011 , 0.009], $Z = [1.89, 2.00, 2.15]$, $P = [0.059, 0.046, 0.032]$, Table S3). Pairwise post-hoc tests did not find that any two genotypes have significantly different relationships between nodule number and shoot mass (*Plant genotype* [*California-Bolivia*, *California-Afghanistan*, *California-Spain*, *Bolivia-Afghanistan*, *Bolivia-Spain*, *Afghanistan-Spain*], estimate = [-0.019 , -0.022 , -0.018 , -0.003 , 0.001 , 0.004], SE = [0.010 , 0.011 , 0.009 , 0.012 , 0.010 , 0.011], t.ratio = [-1.891 , -1.997 , -2.145 , -0.253 , 0.076 , 0.350], $P = [0.234, 0.190, 0.141, 0.994, 0.100, 0.985]$, Table S4). Nitrogen fixation, measured as %N_{dfa}, does not differ among plant genotypes (*Plant genotype* [*California-Bolivia*, *California-Afghanistan*, *California-Spain*], estimate = [0.010 , 0.046 , 0.027], SE = [0.032 , 0.038 , 0.028], $Z = [0.318, 1.204, 0.974]$, $P = [0.750, 0.229, 0.330]$, Table S1).

Experiment 2: Large-scale mutualist distribution

(iv) *Compatible rhizobia are not widely distributed across M. polymorpha's invaded range.* Plants grown in medic-free soil from different locations in the U.S. do not form nodules. For all sites, except the positive control which contained *M. polymorpha*, 95% confidence intervals indicate a mean of zero nodules per plant is consistent with our data (Fig. 4).

Plant fitness suffers in medic-free soil from different locations in the U.S. For all medic-free sites, shoots for the same genotypes were bigger at the site (CA_{pos}) where *M. polymorpha* was present than at any other site (*Site* [CA_{pos}-CA1, CA_{pos}-CA2, CA_{pos}-CA3, CA_{pos}-FL1, CA_{pos}-FL2, CA_{pos}-FL3, CA_{pos}-FL4, CA_{pos}-FL5, CA_{pos}-FL6, CA_{pos}-MI1, CA_{pos}-MI2], estimate = [-0.950 , -0.184 , -0.968 , -1.371 , -1.454 , -2.331 , -2.008 , -1.592 , -2.034 , -0.901 , -0.825], SE = [0.081 , 0.080 , 0.082 , 0.080 , 0.080 , 0.082 , 0.075 , 0.093 , 0.114 , 0.081 , 0.081], $Z = [-11.76, -2.29, -11.81, -17.04, -18.09, -28.57, -26.69, -17.14, -17.88, -11.12, -10.21]$, $P = [< 0.0001, 0.022, < 0.0001, < 0.0001, < 0.0001, < 0.0001, < 0.0001, < 0.0001, < 0.0001, < 0.0001, < 0.0001]$) (Fig. 4).

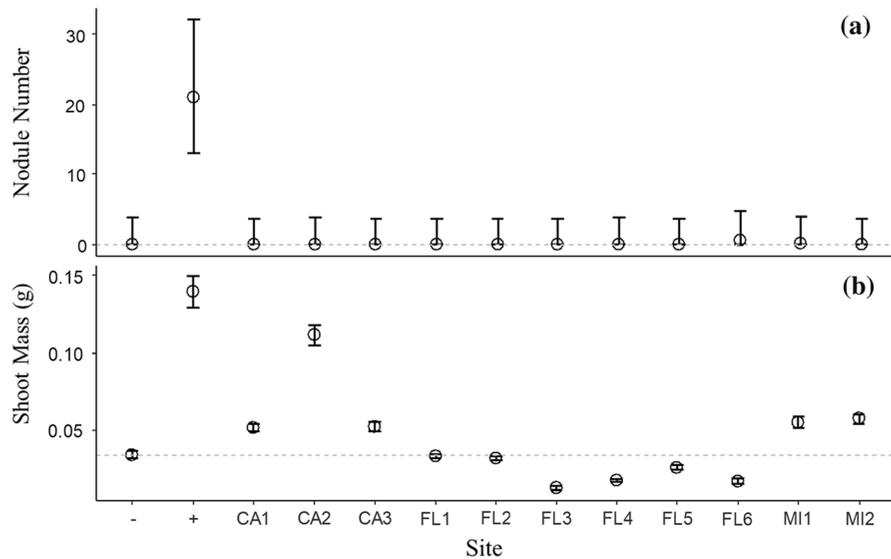


Fig. 4 Compatible mutualists are absent from sites where *M. polymorpha* does not already occur within its invaded range across the United States. Shown are **a** the exact Poisson 95% confidence intervals (vertical lines) for mean nodule number (unfilled circles) and **b** standard errors (vertical lines) for mean

shoot mass (unfilled circles) for plants grown in autoclaved potting soil (–), in Californian soil (Emerson Oaks Reserve) known to contain compatible rhizobia (+), and in soil from three sites in California (CA1–3), from six sites in Florida (FL1–6), and from two sites in Michigan (MI1–2)

Discussion

Our results elucidate the distribution of a microbial mutualist (*Ensifer medicae*) at multiple geographic scales across its invaded range. Rhizobia distributions are strongly aligned with the distributions of their hosts. If the nitrogen requirements of invasive plants are not met beyond established populations due to a lack of compatible rhizobia, then the missed mutualists hypothesis predicts a slowed or constrained invasion front. We find a rhizobium mutualist is absent in soils that lack its host (*Medicago polymorpha*) even meters outside established host patches. This suggests that successful host establishment following either long- or short-range seed dispersal is likely to require co-introduction with soil containing its mutualist. Though many factors may prevent the establishment of invasive plants, including soil characteristics, climate, and herbivores, the patchy distribution of *E. medicae* may also limit or slow *M. polymorpha* invasion, and vice versa. However, *M. polymorpha* genotypes may differ in the rate at which additional nodules increase shoot mass and natural selection could act upon such variation to favor hosts whose growth is less constrained by low mutualist abundance.

(i) *Mutualist abundance declines with distance from host patches.* We find the distribution of a microbial mutualist in its invaded range is nearly the same as the distribution of its invasive host plant. The abundance of mutualistic rhizobia declines to nearly undetectable levels within meters of individual host patches. Plants grown in soil collected only 7.5 m outside patches produce 78% fewer nodules than plants grown in soil collected from within patches. Experimentally inoculating field soils with *E. medicae* increases nodulation by plants grown in soil collected outside patches by 56%. Our results bolster previous studies showing that microbes are not ubiquitous (Stanton-Geddes and Anderson 2011; Rout and Callaway 2012; Wandrag et al. 2013; Mallon et al. 2015); by demonstrating symbiont limitation at multiple spatial scales. Our study thus contributes to our understanding of the process of species invasions (Thakur et al. 2019; Mallon et al. 2015) by quantifying the extent of symbiont limitation on host performance across the landscape.

Despite the fine-scale symbiont limitation that we observed, paths and dry streambeds appear to transfer rhizobia across the landscape. On two transects, soils were unavoidably collected across dry streambeds and fitness and nodule number for plants grown in these

soils were equivalent to inoculated plants. This shows these areas can harbor compatible rhizobia even in the absence of the host. Little is known about how legumes and rhizobia are co-introduced (Roux et al. 2017), but future research could investigate the impacts of paths and streams as conduits for mutualist dispersal and co-invasions; personal observation of pods being dispersed by rainfall events suggests that hosts can travel in this way as well. Predictions of invasion dynamics may require the incorporation of potential dispersal corridors for microbial mutualists into invasion models.

(ii) *Host fitness is increasingly mutualist limited away from conspecifics.* In soils far from conspecifics, *M. polymorpha* is mutualist-limited. Declines in rhizobium abundance away from host populations are correlated to declines in host fitness. The benefit of artificial inoculation with rhizobia depends on the natural availability of compatible rhizobia: inoculation increases shoot biomass by 2% for plants grown in soil collected 7.5 m within patches but by 30% for plants grown in soils just 7.5 m outside of patches. The presence of compatible *E. medicae* can ameliorate the negative effects of antagonistic soil microbes on *M. polymorpha* fitness (Jack et al. 2019), suggesting that host fitness can benefit from the presence of mutualistic microbes via multiple mechanisms.

Concordant with the decline in plant fitness with rhizobium limitation, rhizobium limitation also reduces nitrogen fixation. Experimental plants receive almost no nitrogen from rhizobial fixation at and beyond 10 m from patch margins. As *M. polymorpha* can fix nitrogen at a rate of $120 \text{ kg ha}^{-1} \text{ year}^{-1}$ (Sulas et al. 2016), the limited availability of compatible symbionts substantially decreases the potential for nitrogen inputs into the soil. Because legumes are a primary contributor of biological nitrogen fixation (Vitousek et al. 2013; Galloway et al. 2004), understanding the distributions and availability of rhizobial mutualists can shed light on geographic patterns of nitrogen inputs. Our findings are consistent with previous work that indicates rhizobium limitation can reduce host growth at a regional scale (Stanton-Geddes and Anderson 2011) and in soils where the host is either present or absent (Wandrag et al. 2013). Our work is novel because it quantifies how variation in mutualist availability impacts host fitness in local environments while providing support for large-scale mutualist limitation.

At a larger scale, at sites across the U.S. invaded range, *M. polymorpha* plants form no nodules in soils collected from sites where conspecifics are absent, despite the fact that *M. polymorpha* is present in the region. This suggests that our finding that compatible symbionts are rare in the absence of host patches is consistent across the areas of the US we examined. We infer that *M. polymorpha*'s failure to nodulate in these soils is consistent with an absence of mutualistic rhizobia in the soils. Though factors such as high soil nitrogen can inhibit nodulation, we find that *M. polymorpha* grown in different U.S. soils where conspecifics are absent are not only non-nodulating, but are also consistently diminutive compared to plants grown in soils where nodulation does occur, consistent with inadequate nitrogen nutrition. If soil nitrates in these soils were sufficient to inhibit nodulation (Nishida and Suzaki 2018), we would expect plants to be similar in size to nodulated plants due to soil nitrate fertilization (Regus et al. 2017). The absence of nodulation in concert with low plant biomass suggests compatible rhizobia were not present at diverse U.S. sites that lacked the host, similar to our findings at small spatial scales at natural reserves in California. These findings are consistent with predictions from the missed mutualists hypothesis and suggest host and microbial mutualist distributions can be tightly linked.

Because microbial mutualist availability, host fitness, and symbiotic nitrogen fixation decrease in concert away from conspecific hosts, a patchy mutualist distribution is predicted to slow the rate at which invasive hosts spread. This parallels the influence microbial mutualists have on the range limits of native species, as seen for grass and fungal endophytes (Afkhani et al. 2014), leafcutter ants and fungal mutualists (Mueller et al. 2011), brine shrimp and their gut microbiota (Nougué et al. 2015), coral and dinoflagellates (Iglesias-Prieto et al. 2004), and partridge pea and rhizobia (Stanton-Geddes and Anderson 2011). However, the impact of microbial mutualist distributions on the distributions of invasive species has been less clear. While Nuñez et al. (2009) demonstrated that pine invasion is constrained by the lack of mycorrhizal mutualists, invasion by *Acacia* species has been reported to be unaffected by rhizobium limitation (Wandrag et al. 2013, 2020). We find rhizobium limitation in the invaded range reduces *M. polymorpha* growth and therefore predict that

symbiont limitation will slow host invasion. Mutualistic cooperation is critical to the growth of invasive *M. polymorpha*, and short- (50 m) or long-range seed dispersal away from established patches will likely result in growth limitation due to an absence of symbiotic rhizobia.

Understanding the distribution and abundance of mutualists within an invaded region will be critical to predict the establishment of newly introduced host populations. Mutualist limitation reduces invasiveness, known as the “missed mutualists” hypothesis, at multiple spatial scales (Dickie et al. 2017). Our finding that mutualist-dependent plants may not encounter critical mutualists following dispersal supports the “missed mutualists” hypothesis as biologically important in this co-invasion, even within a region that is widely invaded by conspecifics. Though *M. polymorpha* invasion likely relies on co-dispersal with its rhizobium mutualist, it is possible hosts can amplify low, experimentally undetectable levels of compatible rhizobia (Denison and Kiers 2011). The accumulation of microbial mutualists beneath host populations may shape heterogeneous mutualist distributions within the invaded range, which would constrain the rate of spread of invasive species (Lau and Suwa 2016; Denison and Kiers 2011). This elucidates a biological mechanism by which accumulation of mutualists by conspecifics may drive spatial autocorrelation in models of invasive species spread, whereby proximity to conspecific hosts predicts the probability of establishment (Guélat and Kéry 2018).

Mutualist limitation drives declines in host fitness and nitrogen fixation; inoculation rescues both for host plants grown in soil collected outside of patches, and the benefit of adding rhizobium increases farther away from the host. However, even among plants inoculated with abundant rhizobial mutualists, nodulation declines farther from established patches. This pattern could result if nodulation is inhibited by biotic or abiotic environmental factors (Mallon et al. 2015; Martiny et al. 2006), such as a lack of other mutualists such as arbuscular mycorrhizal fungi, farther from established patches, or if pathogens inhibit nodulation farther from established patches. It is possible that variation in strain quality could result in plants exposed to low quality strains forming many low-quality nodules and plants exposed to high quality strains to form few high-quality nodules (Pahua et al. 2018; Simonsen and Stinchcombe 2014). However,

Porter and Simms (2014) found no evidence of *M. polymorpha* associating with non-beneficial strains; all sampled rhizobium isolates from host populations in California were beneficial to host growth. Furthermore, when grown with *E. medicae*, nodule number is positively correlated with *M. polymorpha* biomass (Jack et al. 2019).

(iii) *Host genetic variation in rhizobial dependence.*

Though the patchy distribution of a microbial mutualist across a species’ invaded range may limit invasion, genetic differences in the benefit hosts obtain from their mutualist such as we observed in our study could lead to the evolution of reduced mutualism dependence. Invasive plants can undergo rapid evolutionary change following introduction (Buswell et al. 2011; Bossdorf et al. 2005), and mutualist limitation can act as an agent of selection. Invaded range genotypes can be less responsive to soil mutualists than native range genotypes (Seifert et al. 2009), and this genetic variation in responsiveness, or the degree to which hosts benefit from their mutualist, could lead to the evolution of reduced mutualism dependency. Our results indicate that genotypes of invasive *M. polymorpha* have similar fitness (i.e. biomass) when mutualists are absent but differ in fitness as the availability of mutualists increases. A genotype with a relatively strong positive growth response to fewer nodules would be more likely to establish in mutualist-limited soils than other genotypes. Wald-Z tests indicate the relationship between host biomass and nodule number is significant when comparing the California genotype to those originating from Afghanistan and Spain; however, pairwise post-hoc tests using *t*-tests with Tukey’s correction for multiple comparisons did not find that any two genotypes have significantly different relationships between nodule number and shoot mass (Table S4). This is consistent with the conclusion that the genotypic differences we detect are weak, given the weak evidence for the difference between slopes in the regression model coupled with the fact that the hypothesis tests for the slopes do not account for a family-wise error rate, whereas the pairwise post-hoc tests do so, leading to higher adjusted *p*-values. Though the differences are weak, the genetic variation we detect in mutualism responsiveness corroborates the finding that invasive genotypes of *M. polymorpha* benefit less from rhizobia than native genotypes (terHorst et al. 2018). Further study of how genotypes

of an invasive host plant respond to natural mutualist availability are needed to better predict the outcomes of host dispersal in the invaded range. Seeds dispersed from a host patch are likely mutualist limited and selection for reduced mutualist dependence is predicted to reduce the constraint that mutualist limitation would otherwise impose on host invasions.

Conclusion

Biological invasions are predicted to accelerate with global change (Dickie et al. 2017), so it is increasingly important to understand how mutualistic microbes impact host invasions. We find host plants and their microbial symbionts occur in the environment together at both fine and broad spatial scales. The absence of microbial symbionts restricts host fitness and nitrogen fixation, but there is genetic variation in host growth response to mutualist abundance. Therefore, invasive plants are predicted to evolve to be less dependent on mutualists. This study adds to growing knowledge of the ecological linkage between host and microbe distributions and gives insight into how plant–microbe mutualisms could evolve over the course of a biological invasion.

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Author contributions ZL, SP, and MF conceived the ideas and designed methodology; ZL, SP, MF, and EVW collected the soil and seeds; ZL and SP collected data; ZL, LN and SP analysed the data; ZL and SP led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Availability of data and material All data underlying the analyses in this paper will be made publicly available on Dryad at the time of publication.

Code availability All code to recreate analyses in the paper is available as a supplemental document.

References

- Afkhami M, McIntyre PJ, Strauss SY (2014) Mutualist-mediated effects on species' range limits across large geographic scales. *Ecol Lett* 17(10):1265–1273.
- Amsellem L, Brouat C, Duron O, Porter SS, Vilcinskas A, Facon B (2017) Importance of microorganisms to macroorganisms invasions. *Adv Ecol Res* 57:99–146
- Bossdorf O, Auge H, Lafuma L, Rogers WE, Siemann E, Prati D (2005) Phenotypic and genetic differentiation between native and introduced plant populations. *Oecologia* 144(1):1–11
- Brooks ME, Kristensen K, van Benthem KJ, Magnusson A, Berg CW, Nielsen A, Skaug HJ, Maechler M, Bolker BM (2017) glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R J* 9(2):378–700
- Buswell J, Moles AT, Hartley S (2011) Is rapid evolution common in introduced plant species?: rapid evolution in introduced plants. *J Ecol* 99(1):214–224
- Caravetta GJ (2018) Arizona Administrative Code. Title 3: Agriculture, vol. 3 A.A.C. 4.
- Coburn FD (1907) The book of Alfalfa. Orange Judd Company, USA.
- Denison RF, Kiers ET (2011) Life histories of symbiotic Rhizobia and Mycorrhizal fungi. *Curr Biol* 21(18):R775–R785
- Dickie IA, Bolstridge N, Cooper JA, Peltzer DA (2010) Co-invasion by Pinus and Its Mycorrhizal fungi. *New Phytol* 187(2):475–484
- Dickie IA, Bufford JL, Cobb RC, Desprez-Loustau M, Grelet G, Hulme PE, Klironomos J et al (2017) The emerging science of linked plant-fungal invasions. *New Phytol* 215(4):1314–1332
- Duffy KJ, Johnson SD (2017) Specialized mutualisms may constrain the Geographical Distribution of Flowering Plants. *Proc Roy Soc B Biol Sci* 284 (1866).
- Fähræus G (1957) The infection of Clover root hairs by Nodule bacteria studied by a simple glass slide technique. *Microbiology* 16(2):374–381
- Galloway J, Dentener F, Capone D, Boyer E, Howarth R, Seitzinger S, Asner G et al (2004) Nitrogen cycles: past, present, and future. *Biogeochemistry* 70(2):153–226
- Guélat J, Kéry M (2018) Effects of spatial autocorrelation and imperfect detection on species distribution models. *Methods Ecol Evolut* 9(6):1614–25.

- Helliwell EE, Faber-Hammond J, Lopez ZC, Garoutte A, Wettberg E, Friesen ML, Porter SS (2018) Rapid establishment of a flowering Cline in *Medicago Polymorpha* after Invasion of North America. *Mol Ecol*.
- Hynson NA, Merckx VS, Perry BA, Treseder KK (2013) Identities and distributions of the Co-invading Ectomycorrhizal Fungal symbionts of exotic Pines in the Hawaiian Islands. *Biol Invasions* 15(11):2373–2385
- Iglesias-Prieto R, Beltrán VH, LaJeunesse TC, Reyes-Bonilla H, Thomé PE (2004) Different algal symbionts explain the vertical distribution of dominant Reef Corals in the Eastern Pacific. *Proc Roy Soc B Biol Sci* 271(1549):1757–1763
- Jack CN, Wozniak KJ, Porter SS, Friesen ML (2019) Rhizobia protect their legume hosts against soil-borne microbial antagonists in a host-genotype-dependent manner. *Rhizosphere* 9:47–55. <https://doi.org/10.1016/j.rhisph.2018.11.005>
- Kiers ET, Ives AR, Kawakita A (2015) Global change and mutualisms. Oxford University Press, Oxford.
- Lau JA, Suwa T (2016) The changing nature of plant-microbe interactions during a biological invasion. *Biol Invasions* 18(12):3527–3534
- Lenth R (2020) emmeans: Estimated marginal means, aka Least-Squares Means. R Package version 1.4.8. <https://CRAN.R-project.org/package=emmeans>.
- Lesins KA, Lesins I (1979) Genus *Medicago* (Leguminosae): a taxogenic study. Kluwer Boston, Hingha
- Mallon CA, van Elsas JD, Falcão Salles J (2015) Microbial invasions: the process, patterns, and mechanisms. *Trends Microbiol* 23(11):719–729
- Martiny JB, Bohannan BJ, Brown JH, Colwell RK, Fuhrman JA, Green JL, Horner-Devine MC et al (2006) Microbial biogeography: putting microorganisms on the map. *Nat Rev Microbiol* 4(2):102–112
- Moeller HV, Dickie IA, Peltzer DA, Fukami T (2015) Mycorrhizal co-invasion and novel interactions depend on neighborhood context. *Ecology* 96(9):2336–2347
- Mueller UG, Mikheyev AS, Hong E, Sen R, Warren DL, Solomon SE, Ishak HD et al (2011) Evolution of cold-tolerant fungal symbionts permits winter fungiculture by leafcutter ants at the Northern Frontier of a Tropical Ant-Fungus Symbiosis. *Proc Natl Acad Sci* 108(10):4053–4056
- Nishida H, Suzuki T (2018) Nitrate-mediated control of root nodule symbiosis. *Curr Opin Plant Biol* 44(August):129–136
- Nougué O, Gallet R, Chevin L, Lenormand T (2015) Niche limits of symbiotic gut microbiota constrain the salinity tolerance of Brine Shrimp. *Am Nat* 186(3):390–403
- Núñez MA, Horton TR, Simberloff D (2009) Lack of below-ground mutualisms Hinders Pinaceae invasions. *Ecology* 90(9):2352–2359
- Pahua VJ, Stokes PJ, Hollowell AC, Regus JU, Gano-Cohen KA, Wendlandt CE, Quides KW, Lyu JY, Sachs JL (2018) Fitness variation among host species and the Paradox of ineffective Rhizobia. *J Evol Biol* 31(4):599–610
- Petrovskaya N, Petrovskii S, Zhang W (2017) Patchy, not patchy, or how much patchy? Classification of spatial patterns appearing in a model of biological invasion. *Math Modell Nat Phenomena* 12(6):208–225
- Porter SS, Rice KJ (2013) Trade-offs, spatial heterogeneity, and the maintenance of microbial diversity. *Evolution* 67(2):599–608
- Porter SS, Simms EL (2014) Selection for cheating across disparate environments in the legume-rhizobium mutualism. *Ecol Lett* 17(9):1121–1129. <https://doi.org/10.1111/ele.12318>
- Porter SS, Stanton ML, Rice KJ (2011) Mutualism and adaptive divergence: co-invasion of a heterogeneous grassland by an exotic legume-rhizobium symbiosis. *PLoS ONE* 6 (12).
- Prior KM, Robinson JM, Meadley Dunphy SA, Frederickson ME (2014) Mutualism between co-introduced species facilitates invasion and alters plant community structure. *Proc Roy Soc B Biol Sci* 282 (1800).
- Regus JU, Wendlandt CE, Bantay RM, Gano-Cohen KA, Gleason NJ, Hollowell AC, O'Neill MR, Shahin KK, Sachs JL (2017) Nitrogen deposition decreases the benefits of symbiosis in a native legume. *Plant Soil* 414:159–170
- Reinhart KO, Callaway RM (2006) Soil biota and invasive plants. *New Phytol* 170(3):445–457
- Rodrigues LA, Mistro DC, Cara ER, Petrovskaya N, Petrovskii S (2015) Patchy invasion of stage-structured Alien species with short-distance and long-distance dispersal. *Bull Math Biol* 77(8):1583–1619
- Rodríguez-Echeverría S (2010) Rhizobial Hitchhikers from down under: invasional meltdown in a plant-bacteria mutualism?" *J Biogeography*.
- Rout ME, Callaway RM (2012) Interactions between exotic invasive plants and soil microbes in the Rhizosphere suggest that 'Everything Is Not Everywhere'. *Ann Bot* 110(2):213–222
- Roux JJ, Hui C, Keet J, Ellis AG (2017) Co-introduction vs ecological fitting as pathways to the establishment of effective mutualisms during biological invasions. *New Phytol* 215(4):1354–1360
- Seeds JD, Bishop J (2009) Low Frankia inoculation potentials in primary successional Sites at Mount St. Helens, Washington, USA. *Plant Soil* 323(1–2):225–233
- Seifert EK, Bever JD, Maron JL (2009) Evidence for the evolution of reduced mycorrhizal dependence during plant invasion. *Ecology* 90(4):1055–1062
- Simberloff D, Martin J, Genovesi P, Maris V, Wardle DA, Aronson J, Courchamp F et al (2013) Impacts of biological invasions: What's What and the way forward. *Trends Ecol Evol* 28(1):58–66
- Simberloff D, Von Holle B (1999) Positive interactions of nonindigenous species: invasional meltdown?
- Simonsen AK, Stinchcombe JR (2014) Standing genetic variation in host preference for mutualist microbial symbionts. *Proc Roy Soc B Biol Sci* 281(1797):20142036–20142036
- Simonsen AK, Dinnage R, Barrett LG, Prober SM, Thrall PH (2017) Symbiosis limits establishment of legumes outside their native range at a global scale. *Nat Commun* 8(April):14790
- Spira TP, Wagner LK (1983) Viability of seeds up to 211 years old extracted from adobe brick buildings of California and Northern Mexico. *Am J Bot* 70(2):303–307. <https://doi.org/10.2307/2443276>
- Stanton-Geddes J, Anderson CG (2011) Does a facultative mutualism limit species range expansion? *Oecologia* 167(1):149–155

- Suarez AV, Holway DA, Case TJ (2001) Patterns of spread in biological invasions dominated by long-distance jump dispersal: insights from Argentine Ants. *Proc Natl Acad Sci* 98(3):1095–1100
- Sulas L, Campesi G, Mercenaro L (2016) Quantification of N₂-fixation from Medicago Polymorpha L. Grown as Cover Crop in Vineyard, no. 114: 5.
- terHorst CP, Wirth C, Lau JA (2018) Genetic variation in mutualistic and antagonistic interactions in an invasive legume. *Oecologia* 188(1):159–171
- Thakur MP, van der Putten WH, Cobben MMP, van Kleunen M, Geisen S (2019) Microbial invasions in terrestrial ecosystems. *Nat Rev Microbiol* 17:621–631. <http://www.nature.com/articles/s41579-019-0236-z>
- Traveset A, Richardson DM (2014) Mutualistic interactions and biological invasions. *Annu Rev Ecol Evol Syst* 45(1):89–113
- Vitousek PM, Menge DN, Reed S, Cleveland C (2013) Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. *Philos Trans Roy Soc B Biol Sci* 368(1621):20130119
- Wandrag EM, Sheppard A, Duncan RP, Hulme PE (2013) Reduced availability of Rhizobia limits the performance but not invasiveness of introduced *Acacia*. *J Ecol* 101(5):1103–1113.
- Wandrag EM, Birnbaum C, Klock M, Barrett LG, Thrall PH (2020) Availability of soil mutualists may not limit non-native *Acacia* invasion but could increase their impact on native soil communities. *J Appl Ecol* 57(4):786–793
- Wernegreen JJ (2012) Mutualism meltdown in insects: bacteria constrain thermal adaptation. *Curr Opin Microbiol* 15(3):255–262
- Westgate JM (1908) Alfalfa. *Farmers' Bulletin* 339. U.S. Department of Agriculture.
- Wilson JR, Dormontt EE, Prentis PJ, Lowe AJ, Richardson DM (2009) Something in the way you move: dispersal pathways affect invasion success. *Trends Ecol Evol* 24(3):136–144
- Wing JE (1912) *Alfalfa Farming in America*. Sanders Publishing Company, Chicago, IL
- Wolf AA, Funk JL, Menge DN (2017) The symbionts made me do it: legumes are not hardwired for high nitrogen concentrations but incorporate more nitrogen when inoculated. *New Phytol* 213(2):690–699
- Yelenik S, Perakis S, Hibbs D (2013) Regional constraints to biological nitrogen fixation in post-fire forest communities. *Ecology* 94(3):739–750
- Younginger BS, Sirová D, Cruzan MB, Ballhorn DJ (2017) Is biomass a reliable estimate of plant fitness? *Appl Plant Sci* 5(2).

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