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Research

Evolutionary ecology of plant–microbe interactions: soil microbial structure alters selection on plant traits

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Summary

• Below-ground microbial communities influence plant diversity, plant productivity, and plant community composition. Given these strong ecological effects, are interactions with below-ground microbes also important for understanding natural selection on plant traits?

• Here, we manipulated below-ground microbial communities and the soil moisture environment on replicated populations of *Brassica rapa* to examine how microbial community structure influences selection on plant traits and mediates plant responses to abiotic environmental stress.

• In soils with experimentally simplified microbial communities, plants were smaller, had reduced chlorophyll content, produced fewer flowers, and were less fecund when compared with plant populations grown in association with more complex soil microbial communities. Selection on plant growth and phenological traits also was stronger when plants were grown in simplified, less diverse soil microbial communities, and these effects typically were consistent across soil moisture treatments.

• Our results suggest that microbial community structure affects patterns of natural selection on plant traits. Thus, the below-ground microbial community can influence evolutionary processes, just as recent studies have demonstrated that microbial diversity can influence plant community and ecosystem processes.

Introduction

Below-ground microbial communities play a key role in determining the productivity, diversity and composition of plant communities (van der Heijden *et al.*, 2008; Kulmatiski *et al.*, 2008). For example, empirical studies have demonstrated that below-ground microbial diversity influences plant growth and productivity (Marschner & Rumberger, 2004; Hol *et al.*, 2010), nutrient availability (Bonkowski & Roy, 2005), and ecosystem functioning (Degens, 1998; Bradford *et al.*, 2002; Bell *et al.*, 2005). Additionally, many other studies illustrate the importance of plant–soil feedbacks, whereby shifts in microbial community composition feedback to affect plant coexistence and community composition (Bever, 2003; Reynolds *et al.*, 2003). While the effects of plant–soil microbe interactions on plant ecological dynamics appear to be widespread, little research has addressed how below-ground microbial communities influence plant evolutionary processes.

One way in which microbes could alter patterns of natural selection on plant populations is by ameliorating plant responses to abiotic environmental stress. Microbes have relatively short generation times, a high degree of genetic diversity, and the ability to evolve on ecologically relevant timescales. These attributes may allow for rapid changes in microbial community structure, which in turn may shape the way that plant populations respond to novel selective pressures in their environment. Several recent studies have demonstrated that local adaptation in plants is driven by genetic differentiation in closely associated microbes (reviewed in Rodriguez & Redman, 2008). For example, plant tolerance to high temperatures, salinity, and drought has been attributed to colonization by fungal endophytes (Rodriguez *et al.*, 2008), and heavy-metal tolerant strains of mycorrhiza have been shown to facilitate plant colonization of contaminated mine tailings (Adriaensen *et al.*, 2005). Similarly, plant growth-promoting bacteria can increase drought tolerance of their plant hosts by producing

hormones that induce the expression of plant genes associated with drought tolerance (e.g. Timmusk & Wagner, 1999; reviewed in Yang *et al.*, 2009).

These examples highlight the important role that a few key microbial taxa can have in promoting plant adaptation to stress. More often, however, plants form associations with complex, highly diverse, and potentially coevolved microbial communities. The net effect of such plant–microbe interactions can have implications for plant growth and fitness, carbon sequestration, and nutrient cycling (van der Heijden *et al.*, 2006, 2008; Kulmatiski *et al.*, 2008), and may influence plant community responses to global environmental changes (van der Putten *et al.*, 2009). Specifically, diverse microbial communities improve plant growth and vigor (Marschner & Rumberger, 2004) and help to maintain ecosystem functioning in stressful environments (Griffiths *et al.*, 2000). Therefore, diverse microbial communities may protect plants from the negative consequences of stress more effectively than less diverse microbial communities. For example, plant stress tolerance was heightened in the presence of multiple strains of plant growth-promoting bacteria, suggesting potential benefits of diverse microbial communities (Figueiredo *et al.*, 2008). However, soil food webs are notoriously complex, making it difficult to predict how changes in microbial community structure affect ecological processes. As a result, Bradford *et al.* (2002) advocate for empirical approaches for predicting how shifts in soil communities affect ecological functions. For similar reasons, empirical approaches may also be necessary for predicting effects of soil communities on plant evolutionary processes.

Here, we examine how below-ground microbial community structure influences selection on plant traits. We imposed treatments that altered the diversity and composition of the below-ground microbial community associated with replicated *Brassica rapa* populations growing in two soil moisture environments. These experiments allowed us to explore how biotic and abiotic factors influence plant phenotypes and selection on plant traits. We were particularly interested in determining whether below-ground microbes alter selection on plant traits by mitigating the effects of abiotic environmental stress on plant populations. Specifically, we tested whether more complex microbial communities reduce the negative consequences of drought stress on plant growth and reproduction; variation in microbial community complexity alters selection on plant growth and physiological traits; and more complex microbial communities minimize the selective impacts of drought stress on plant traits.

Materials and Methods

Experimental design

We conducted a factorial experiment to assess how differences in microbial community structure (simplified vs complex) influence plant phenotypes, selection on plant traits, and plant responses to soil moisture (low vs high). We sowed 125 *Brassica rapa* L. (Brassicaceae) seeds into each of 16 mesocosms at 4 cm intervals on 30 May 2008. Seeds used in this experiment were obtained by haphazardly pollinating 200 *B. rapa* Wisconsin Fast Plants™ (standard stock lines, Wisconsin Fast Plants Program, University of Wisconsin, Madison, WI, USA). We used Wisconsin Fast Plants because this study is part of a multigeneration experiment that required plant taxa with short generation times. *B. rapa*, like most members of the Brassicaceae, is non-mycorrhizal; however, *B. rapa* still associates with a diverse rhizosphere community, and prior studies have shown that microbial community composition influences *Brassica oleraceae* growth and nutrient availability (e.g. Hol *et al.*, 2010). The mesocosms consisted of 76 l plastic pots (58 cm diameter, 34 cm height) filled with an autoclaved soil medium (1 part Baccto High Porosity Mix: 1 part perlite: 1 part vermiculate; 121°C, 15 PSI (pounds per square inch), 16 h). Each of the 16 populations was then randomly assigned to one of the replicate ($n = 4$) microbe × soil moisture treatment combinations. In addition, we exposed eight mesocosms without plants to the soil moisture and microbe manipulations (i.e. microbe × soil moisture) to test for the direct effects of moisture on microbial structure. Together, this resulted in a total of 24 experimental units.

We manipulated soil microbial community structure by inoculating each mesocosm with 3 l of either autoclaved (two cycles at 121°C, 15 PSI, 45 min) or unautoclaved field soil. The field soil was collected from an early successional field (Oshtemo Sandy Loam, heavily disturbed *c.* 25 yr ago (Burbank *et al.*, 1992))dominated by many exotic grasses and forbs, including several species in the Brassicaceae, at the Kellogg Biological Station (KBS), Hickory Corners, MI, USA in May 2008. Although autoclaving can alter soil organic matter chemistry and the availability of plant nutrients (McNamara *et al.*, 2003), the inoculum was added to a large quantity of autoclaved potting media and comprised < 5% of the total mesocosm volume. Because the bulk of the soil used in each treatment experienced identical steam-sterilization regimes, we attribute any differences between microbe treatments to the addition of inocula that contained either live or dead soil microorganisms. Owing to the rapid growth rate of many microbial populations, the inoculum size in our study was sufficient to create microbial communities that differed in complexity (primarily diversity) by the end of the experiment (see the 'Results' section). Moreover, when sampled at the end of the experiment, estimates of bacterial richness in the treatments with live inoculum were similar to that observed in natural Michigan field soils (using identical fingerprinting methods) (mean ± 1 SE = 58 ± 3.8 operational taxonomic units (OTUs), this study; 57.5 ± 2.3, field study; JT Lennon, unpublished).

Bacterial and fungal communities in mesocosms receiving autoclaved inocula were less diverse compared with mesocosms receiving nonautoclaved inocula (see the 'Results' section). As such, we refer to the mesocosms with autloclaved inocula as 'simplified' and mesocosms with nonautoclaved inocula as 'complex'. Quantitative PCR (see the 'Microbial Structure' section) indicated that autoclaving the inocula did not reduce the total abundance of bacteria or fungi sampled at the end of the experiment (mean ± 1 SE: $\log_{10}$ bacteria – 'complex' = 6.62 ± 0.06, 'simplified' = 6.50 ± 0.10, $F_{1,17} = 1.14$, $P = 0.30$; $\log_{10}$ fungi – 'complex' = 5.77 ± 0.11, 'simplified' = 5.96 ± 0.18, $F_{1,17} = 0.90$, $P = 0.36$), presumably because weedy, fast-growing microorganisms were able to recolonize the mesocosms. Such effects are probably common, even though investigators rarely characterize the effects of autoclaving on the abundance or composition of microbial communities.

All mesocosms were initially watered with reverse-osmosis (RO) water to stimulate germination of seeds. Shortly after emergence (*c.* 2 d), we ceased watering mesocosms in the low moisture treatment. Mesocosms in the high soil moisture treatment received enough water to ensure that the soil was consistently moist; this resulted in the application of 1000–1500 ml of RO water every other day for mesocosms with plants, and 500–1000 ml of RO water every other day for mesocosms without plants. On a single sampling date halfway through the experiment, we measured gravimetric water content from the surface soils as the loss of water mass after drying soil at 100°C for 24 h. We also measured volumetric soil moisture continuously in one mesocosm per treatment using Decagon (EC-TM) soil moisture probes with accompanying data loggers (EM50 ECH20) (Decagon Devices, Inc., Pullman, WA, USA).

Microbial structure

We characterized microbial community structure from mesocosm soils using culture-independent molecular methods. We collected and pooled five subsamples of soil from the top 5 cm of each mesocosm (approximate rooting depth) after plants had set seed. These soil samples were immediately stored at −20°C until later processing. We extracted genomic DNA from 1 g of soil sample using an UltraClean™ Soil DNA Isolation Kit, which was subsequently quantified using a Nanodrop spectrophotometer. We used this DNA to evaluate how microbial structure changed in response to our soil moisture and microbe treatments using quantitative PCR (qPCR) and terminal restriction fragment length polymorphism (T-RFLP).

First, we estimated the abundance of fungi and bacteria in our samples using qPCR. Briefly, qPCR assays consisted of 30 μl reactions containing 1 μl of DNA template, 0.5 μl of each primer (10 μmol l^{-1}), 14.5 μl of DNAse- and RNAse-free H_2O, and 13.5 μl of 5 Prime 2.5x RealMasterMix SYBR ROX (5 Prime, Inc. Gaithersburg, MD, USA). For fungi we used ITS1f (forward) and 5.8S (reverse) primers, and for bacteria we used Eub338 (forward) and Eub518 (reverse) primers (Fierer *et al.*, 2005). qPCR assays were performed with an Eppendorf Mastercycler realplex^2 system using previously reported thermal cycle conditions (Fierer *et al.*, 2005). We generated qPCR standards from a bacterial (*Micrococcus* sp.) and fungal (*Trichosporon* sp.) isolate using the TOPO TA Cloning Kit (Invitrogen). We extracted plasmids from transformed cells (Sambrook & Russell, 2001), and used M13 forward and reverse primers from the cloning kit to generate PCR products that we used for our standard curve, which captured a range of 10^2–10^7 copies μl^{-1}. The coefficients of determination (r^2) for our assays ranged from 0.96 and 0.99, while amplification efficiencies fell between 0.93 and 0.99. Based on melting curve analyses, we found no evidence for primer dimers. We estimated fungal and bacterial abundance based on the estimated gene copy number from their respective standard curves and used these values to calculate fungal to bacteria ratios (F : B).

Secondly, we fingerprinted the soil microbial community using T-RFLP. For fungi, we PCR-amplified DNA using a fluorescently (FAM-6) labeled ITS1-F forward primer, an unlabeled ITS4 reverse primer, and the thermal cycler pattern described by Avis *et al.* (2006). For bacteria, we amplified DNA using a fluorescently (FAM-6) labeled 8F forward primer, an unlabeled 1492R reverse primer, and the thermal cycler pattern described in Lennon & Martiny (2008). We then digested 20–30 ng of the fluorescently labeled product for 24 h with five units of *Hae*III restriction enzyme. Lastly, we used a Qiagen nucleotide removal kit to clean up the enzyme-digested product. The T-RFLP samples were analyzed with an ABI Prism 3100 Genetic Analyzer at the Research Technology Support Facility (RTSF) at Michigan State University. We quantified the size of fluorescently labeled fragments in our samples by comparison to an internal ROX-labeled size standard (50–2000 base-pairs). From the resulting fragment profiles, we differentiated signal peaks from background noise using the methods of Jones & McMahon (2009). We then estimated the OTU richness for bacterial and fungal samples by calculating the sum of peaks that were present in the fragment profiles.

Plant measurements

We censused the central 64 plants in each mesocosm every other day and recorded flowering day and counted the number of leaves at flowering. After most plants had begun to flower, we measured chlorophyll content of the second or third leaf using a chlorophyll meter (SPAD 502; Spectrum Technologies, Inc., Plainfield, IL, USA) as an indicator of leaf nitrogen content and general plant vigor (Bullock & Anderson, 1998; Swiader & Moore, 2002). We estimated

specific leaf area (SLA = leaf area/dry mass) by weighing a leaf disk (0.28 cm^2) collected from the second true leaf produced on each plant (*c.* 3 wk post-germination) with a microbalance after the disk had been dried for 2 d at 65°C. This technique likely overestimated SLA because structural masses (e.g. midveins), which may increase leaf weights, were avoided; however, the SLA estimation technique should be consistent across treatments. This technique was necessary because plants produced less than four leaves on average and removing a whole leaf likely would have had severe effects on plant fitness. All open flowers in each mesocosm were hand-pollinated by other open flowers in that same mesocosm with a soft paintbrush three times per wk (brushes were cleaned with 30% isopropyl alcohol between mesocosms to prevent gene flow). When most plants in each mesocosm had ceased flowering and senesced, we harvested each individual, counted the number of seeds produced, and estimated total flower production by counting the number of flower scars on each plant. After removing seeds, we dried the above-ground biomass at 65°C for 2 d and weighed each individual to estimate above-ground biomass.

Statistical analyses

Microbial structure We used general linear models to determine the significance of main effects (soil moisture, plant presence, and microbe) and interactions (soil moisture × plant presence and soil moisture × microbe) on F : B ratios and OTU richness of the bacterial and fungal communities. When necessary, we used $\log_{10}$ transformations to help meet the assumption of normality and equal variance. To analyze microbial composition data, we used the relative fluorescence output from the fragment profiles to generate separate sample × OTU matrices for fungi and bacteria. We then tested for the main effects and interactions of our experimental manipulations with *adonis* (a permutation-based multivariate analysis of variance) with Bray–Curtis distance matrices using the vegan package in R statistical software (R Development Core Team, 2009).

Plant ecological and evolutionary effects We employed three methods to test how the soil moisture and microbe manipulations influenced plant populations: ANOVA to test for effects of microbe and soil moisture on the expression of plant traits; phenotypic selection analyses (Lande & Arnold, 1983) to estimate selection on each plant treat in each soil moisture × microbe environment; and ANCOVA to test whether microbe and soil moisture treatments altered selection on plant traits.

We tested for the effects of microbe and soil moisture treatments on plant growth, phenological, and fitness traits with mixed-model ANOVA (PROC MIXED, SAS Institute 2001). The microbe treatment, soil moisture treatment, and their interaction (microbe × soil moisture) were included as fixed factors. Mesocosm nested within the microbe × soil moisture interaction was included as a random factor. Response variables included flowering day (d since sowing), leaf number at flowering, chlorophyll content, SLA, above-ground biomass, lifetime flower production, and seed production. All response variables were natural log-transformed to help meet the assumptions of normality and equal variance.

We used phenotypic selection analyses (Lande & Arnold, 1983) to estimate selection differentials and gradients on flowering day, SLA, and biomass in each microbe × soil moisture treatment. Phenotypic selection analyses use regression or multiple regression to quantify the relationship between traits and relative fitness. In all selection analyses, we used seed number as our estimate of lifetime female fitness. Selection differentials, which include both direct selection acting on the trait and indirect selection resulting from selection acting on tightly correlated traits, were estimated by performing separate linear regressions for each plant trait. Selection gradients estimate only direct selection on each trait by accounting for correlations with other traits included in the model and were estimated by performing multiple regressions that included all focal traits (flowering day, SLA and final biomass) as predictor variables and relative fitness (seed number) as the response variable. Traits were standardized within each mesocosm to a mean of zero and a variance of one (results from analyses of nonstandardized traits were qualitatively similar), and relative fitness was calculated based on mean seed number for each mesocosm.

We used analysis of covariance (ANCOVA) to determine whether the direction and/or intensity of selection differed between treatments (Wade & Kalisz, 1990). In the univariate (selection differential) analyses, each model included microbe treatment, soil moisture treatment, the standardized trait value, and all interactions as predictors of relative fitness (seed number). Mesocosm and the mesocosm × standardized trait value interaction were included as random factors. Significant interactions between the microbe or soil moisture treatments and the plant trait indicate that selection differentials differed between treatments. A similar multiple regression analysis that included all traits and all interactions between traits and the microbe and soil moisture treatments as predictor variables was used to test for effects of microbe or soil moisture treatments on selection gradients. Additionally, because the residuals for seed number were not normally distributed (Shapiro–Wilk statistics ranged from 0.86 to 0.92, all $P < 0.0001$), we also used more conservative bootstrapping methods to estimate 95% confidence intervals for each selection differential and gradient in each treatment (%Boot Macro, SAS Institute, 2001).

Results

Soil moisture

Gravimetric water content (θ_d) was more than fourfold higher in the high moisture treatment than the low moisture treatment (1.16 ± 0.070 vs 0.27 ± 0.070 $g\ H_2O/g$ soil; $F_{1,12}$ = 74.5, $P < 0.0001$). Continuous measurements of soil moisture in a subset of mesocosms (n = 1 per treatment) revealed that these patterns were consistent throughout the duration of the experiment (Supporting Information, Fig. S1).

Microbial structure

Our experimental manipulations had strong effects on the structure of soil microbial communities. The mesocosms inoculated with autoclaved field soil ('simplified' microbe treatment) had microbial communities that were less diverse than the mesocosms that were inoculated with intact field soil ('complex' microbe treatment). OTU richness was significantly higher in the complex than in the simplified microbe treatment for both bacteria (ANOVA, $F_{1,16}$ = 17.3, P = 0.0007, 58 ± 3.8 vs 45 ± 4.7 OTUs) and fungi (ANOVA, $F_{1,18}$ = 7.05, P = 0.016, 24 ± 1.4 vs 16 ± 2.4 OTUs). In addition, the microbe manipulation altered bacterial composition (*adonis*, $F_{1,18}$ = 2.55, P = 0.003), but when averaged across the plant presence and soil moisture treatments, we found no difference between fungal composition in the simplified and complex microbe treatments (*adonis*, $F_{1,18}$ = 1.22, P = 0.28). Inoculation with autoclaved field soil tended to reduce the F : B ratio compared with inoculation with intact field soil, although this effect was not statistically significant (ANOVA, $F_{1,17}$ = 3.23, P = 0.09; simplified = 0.16 ± 0.165, complex = 0.34 ± 0.156).

The plant and soil moisture manipulations also affected soil microbial structure, and in some cases interacted with the microbial treatment. For example, fungal OTU richness was significantly greater in the high soil moisture treatments, but only in mesocosms containing *Brassica* (Fig. 1a) and in mesocosms assigned to the complex microbe treatment (Fig. 1b). The soil moisture manipulation also had a strong effect on fungal composition, but this effect was influenced by the presence of *Brassica* (plant presence × soil moisture, *adonis*, $F_{1,18}$ = 3.64, P = 0.008) and by the complexity of the microbial community (soil moisture × microbe, *adonis*, $F_{1,18}$ = 2.45, P = 0.025). By contrast, bacterial communities were less responsive to the soil moisture and plant presence manipulations. Both bacterial OTU richness (Fig. 1c) and bacterial composition were unaffected by plant presence (*adonis*, $F_{1,18}$ = 0.64, P = 0.93) and soil moisture manipulations (*adonis*, $F_{1,18}$ = 1.25, P = 0.18). The F : B ratio was significantly higher under low soil moisture than under high soil moisture (ANOVA, $F_{1,17}$ = 5.80, P = 0.028; low soil moisture = 0.530 ± 0.107, high soil moisture = 0.214 ± 0.115).

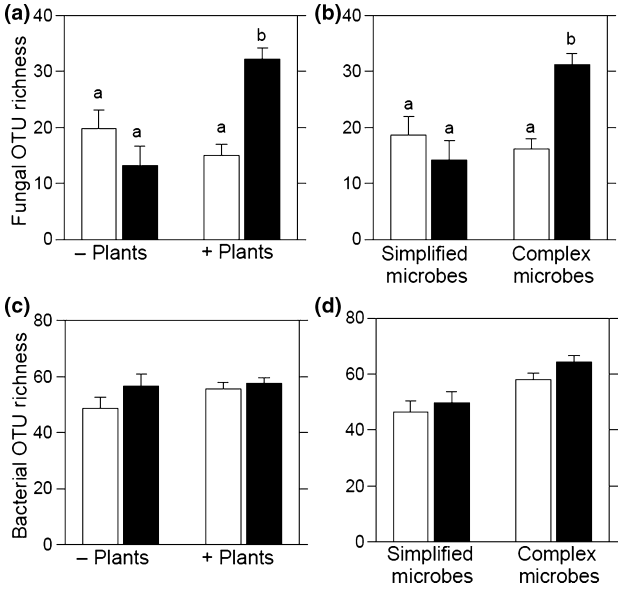

Fig. 1 Interactive effects of soil moisture and plant (a, c) or microbe treatments (b, d) on soil microbial structure. Fungal operational taxonomic unit (OTU) richness in low (open bars) and high (closed bars) soil moisture mesocosms, with and without plants (i.e. *Brassica rapa*) (a) or in the simplified vs complex microbial communities (b). Bacterial OTU richness in mesocosms with or without plants (c) and with simplified vs complex microbial communities (d). Significant interactions between soil moisture and the plant (a, $F_{1,18}$ = 18.19, P = 0.0004) and microbe manipulations (b, $F_{1,18}$ = 12.75, P = 0.0022) were detected on fungal OTU richness. The values are marginal means and standard errors derived from ANOVA models.

Plant ecological effects

The microbe and soil moisture treatments both affected plant traits. On average, plants growing in soils with a complex microbial community were larger (e.g. greater leaf numbers at flowering and above-ground biomass), produced more flowers, and had a higher chlorophyll content than plants growing in the simplified microbe treatment (Table 1). Plants in the low soil moisture treatment had accelerated flowering and marginally reduced chlorophyll concentration (Table 1). Neither the soil moisture nor microbe treatment significantly affected SLA (Table 1).

We did not detect any statistically significant interactions between the soil moisture and microbe treatments on plant traits, but did observe a marginally nonsignificant microbe × soil moisture interaction on seed production ($F_{1,12}$ = 3.50, P = 0.09; Table 1). Specifically, simplification of the microbial community resulted in a 50% reduction in seed production for plants grown in low soil moisture treatments, but the microbe treatment had minimal effects on seed production for plants grown in high soil moisture treatments (Table 1).

Table 1 Influence of abiotic (soil moisture) and biotic (microbial complexity) manipulations on *Brassica rapa* plant traits

	Low moisture		High moisture		*F*-statistics			
	Simple microbial community	Complex microbial community	Simple microbial community	Complex microbial community	Soil moisture (S)	Microbe (M)	S × M	Mesocosm
Flowering day	20.70 ± 0.49	20.70 ± 0.49	21.86 ± 0.49	21.80 ± 0.49	**5.37***	0.00	0.00	**χ^2 = 27.2*****
Leaf number	3.30 ± 0.12	3.68 ± 0.12	3.57 ± 0.12	3.71 ± 0.12	0.96	**5.48***	0.85	**χ^2 = 21.6*****
SLA ($cm^2\ g^{-1}$)	0.73 ± 0.03	0.70 ± 0.03	0.76 ± 0.03	0.74 ± 0.03	1.53	0.52	0.02	**χ^2 = 33.1*****
Chlorophyll content	9.68 ± 1.60	16.16 ± 1.53	13.88 ± 1.55	18.51 ± 1.53	3.39$^+$	**12.27****	1.00	**χ^2 = 53.7*****
Above-ground biomass (mg)	59.42 ± 14.00	98.41 ± 14.00	73.97 ± 14.00	113.6 ± 14.00	0.06	**9.68****	0.02	**χ^2 = 76.6*****
Flower number	7.87 ± 1.22	8.60 ± 1.22	11.68 ± 1.22	12.12 ± 1.22	0.01	**11.72****	0.01	**χ^2 = 24.7*****
Seed number	10.66 ± 4.32	23.16 ± 4.32	17.62 ± 4.32	14.82 ± 4.32	0.20	2.61	3.50$^+$	**χ^2 = 52.2*****

SLA, specific leaf area.
Least-squares mean ± 1 SE for each trait in each treatment and *F*-statistics and chi-square values showing the effects of microbe (complex/simple) and soil moisture treatments on plant phenotypes. Values shown are per plant means. Values in bold are statistically significant at $P < 0.05$.
***, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$; $^+$, $P < 0.10$.

Selective effects of microbes and soil moisture

Selection favored plants that flowered earlier and accumulated more biomass in all treatments; however, belowground microbial communities significantly altered the strength of selection on plant traits (Tables 2, 3). Selection for increased biomass was significantly stronger and selection for earlier flowering tended to be stronger for plants grown in simplified than in complex microbial communities (Table 2). We also detected evidence that soil moisture and microbe treatments interacted to affect selection on SLA. Low soil moisture resulted in significant selection for increased SLA, but only in the simplified soil microbe treatment (Table 2). Similar results were observed with the more conservative bootstrapping approach (Table 2). The selection gradient analysis revealed similar patterns (Table 3), although selection gradients on flowering time tended to be weaker than selection differentials, suggesting that a portion of the observed selection on flowering time was likely indirect and the result of selection on correlated traits.

By measuring selection on replicated plant populations in each microbe × soil moisture treatment, we can definitively attribute observed differences in selection to differences in microbial community structure. Selection differentials were similar across mesocosms in a given treatment (coefficient of variation ranged from 0.24 to 0.45), and an analysis in which the selection differentials of each mesocosm were treated as independent response variables also revealed that selection on both flowering day and biomass tended to be weaker on populations grown with complex microbial communities compared with populations grown with simplified microbial communities (e.g. LSMean selection differential ± 1 SE: flowering day – complex −0.26 ± 0.05, simplified −0.39 ± 0.05, $F_{1,12} = 3.01$, $P = 0.11$; biomass – complex 0.59 ± 0.12, simplified 0. 97 ± 0.12, $F_{1,12} = 5.35$, $P = 0.04$).

Table 2 Influence of abiotic (soil moisture) and biotic (microbial complexity) manipulations on selection differentials (univariate analyses) of *Brassica rapa*

	Low moisture		High moisture		*F*-statistics			
	Simple microbial community	Complex microbial community	Simple microbial community	Complex microbial community	Soil moisture (S)	Microbe (M)	S × M	Mesocosm
Flowering day	**−0.33** (−0.43, −0.22)	**−0.20** (−0.28, −0.11)	**−0.44** (−0.59, −0.32)	**−0.32** (−0.44, −0.21)	2.16	2.57	0.00	$\chi^2 = 0.1$
SLA	**0.19** (0.04, 0.33)	−0.05 (−0.16, 0.07)	−0.08 (−0.27, 0.12)	0.15 (−0.02, 0.40)	0.16	0.00	**5.83***	$\chi^2 = 0.5$
Above-ground biomass	**0.83** (0.62, 0.97)	**0.63** (0.50, 0.74)	**1.08** (0.92, 1.27)	**0.56** (0.36, 0.78)	0.44	**7.58***	1.25	**χ^2 = 33.2*****

SLA, specific leaf area.
Values in brackets are bootstrapped 95% confidence intervals. Selection differentials that are significantly different from zero are shown in bold. *F*-statistics from the ANCOVA analyses testing the effects of soil moisture, microbe and microbe × soil moisture interactions on selection differentials also are shown.
***, $P < 0.0001$; *, $P < 0.05$.

Table 3 Influence of abiotic (soil moisture) and biotic (microbial complexity) manipulations on selection gradients (multivariate analysis) of *Brassica rapa*

	Low soil moisture		High soil moisture		*F*-statistics			
	Simple microbial community	Complex microbial community	Simple microbial community	Complex microbial community	Soil moisture (S)	Microbe (M)	S × M	Mesocosm
Flowering day	**−0.13** (−0.25, −0.01)	**−0.13** (−0.22, −0.04)	−0.14 (−0.33, 0.04)	**−0.26** (−0.40, −0.10)	0.38	0.48	0.84	$\chi^2 = 0.3$
SLA	**0.18** (0.07, 0.28)	0.01 (−0.09, 0.12)	**0.13** (0.00, 0.26)	**0.21** (0.05, 0.48)	1.26	0.56	3.95^{+}	$\chi^2 = 0.0$
Above-ground biomass	**0.78** (0.52, 0.95)	**0.60** (0.47, 0.74)	**1.06** (0.84, 1.30)	**0.56** (0.38, 0.77)	0.80	**5.92***	1.77	$\chi^2 = \mathbf{21.9}^{***}$

SLA, specific leaf area.

Values in parentheses are bootstrapped 95% confidence intervals. Selection gradients that are significantly different from zero are shown in bold. *F*-statistics from the ANCOVA analyses testing the effects of soil moisture, microbe and microbe × soil moisture interactions on selection gradients also are shown.

***, $P < 0.0001$; *, $P < 0.05$; $^{+}$, $P < 0.10$.

Discussion

In this study, we quantified how below-ground microbial structure influenced plant growth and fitness traits; plant responses to abiotic stress (soil moisture); and selection on plant traits. Microbes did not modify plant phenotypic responses to soil moisture; however, microbial community structure altered plant growth and influenced the strength of selection on plant traits (above-ground biomass and SLA) and tended to alter selection on flowering day. Selection for increased above-ground biomass was 50% stronger in mesocosms containing a simplified microbial community compared with a more complex microbial community. Contrary to our predictions, we detected little evidence that microbes influenced selection by ameliorating drought stress. Instead, the influence of our microbial manipulation on selection on plant growth and phenological traits typically was consistent across soil moisture treatments, suggesting that soil communities can alter the strength of selection on plant traits independent of abiotic environmental conditions. Just as recent findings have shown that microbial diversity is important to plant productivity and ecosystem functioning (Marschner & Rumberger, 2004; Bell *et al.*, 2005), our study suggests that aspects of microbial diversity affect plant evolutionary processes. If our system is typical, effects of variation in below-ground microbial community composition on natural selection may be common and strong. Furthermore, although few studies manipulate both abiotic and biotic environmental variables, our study suggests that biotic selection agents can be equally or more important drivers of natural selection than abiotic selection agents.

Ecological consequences of plant–microbe interactions

Simplification of the microbial community led to reductions in below-ground bacterial and fungal richness, which ultimately reduced plant growth, chlorophyll content (a trait correlated with plant nutrient status), and seed production. While several recent studies have shown how the presence, abundance, or diversity of specific classes of microbes, such as mycorrhizal mutualists or rhizobium symbionts, may influence plant productivity (van der Heijden *et al.*, 2006; Maherali & Klironomos, 2007; reviewed in van der Heijden *et al.*, 2008), very few studies have examined how the complexity of microbial communities influences above-ground productivity (but see Marschner & Rumberger, 2004; Hol *et al.*, 2010). In our study, the positive effects of complex microbial communities on plant performance may be the result of effects of microbial diversity on resource availability (Griffiths *et al.*, 2000; Loreau, 2001) or through the suppression of pathogenic microbes (Garbeva *et al.*, 2004). For example, in subsequent plant generations, we noted high incidences of disease in the simplified mesocosms and were able to recover viable populations of *Fusarium* sp. (a commonly pathogenic fungus) from mesocosms in the simplified microbe treatment, but not from the more diverse microbial communities in the complex microbe treatment. Interestingly, recent studies in other systems have convincingly shown how manipulation of the soil environment in ways that favor bacterial growth can limit the growth and abundance of *Fusarium*, presumably because of increases in *Fusarium*-antagonists, including antibiotic-producing bacteria (Perez *et al.*, 2008).

Compared with the microbe manipulation, soil moisture had rather limited effects on plant phenotypes. The only significant effect we detected was that plants in the low soil moisture treatment flowered *c.* 1 d earlier than plants in the high soil moisture mesocosms, which is consistent with other studies showing accelerated phenologies in a variety of plant taxa under drought stress (Aronson *et al.*, 1992; Wu *et al.*, 2010). Thus, both abiotic and biotic factors can influence the expression of plant phenoypes, but, perhaps not surprisingly, abiotic and biotic factors affected different

traits. In addition, we detected limited evidence that microbial community composition influenced plant responses to soil moisture; no statistically significant interactions between soil moisture and microbe treatments on any plant traits were detected.

While plant phenotypes showed minimal responses to the soil moisture treatments, the below-ground fungal community responded strongly to this manipulation. Culture-independent PCR-based techniques indicated that high soil moisture treatments decreased F : B ratios, increased fungal richness, and altered fungal community composition (relative abundances of OTUs). These results are consistent with several studies demonstrating that soil moisture can alter both total microbial biomass and microbial community structure (Schimel *et al.*, 1999, 2007; Gleeson *et al.*, 2008; see Blankinship *et al.*, 2011 for review). Bacteria community composition was less responsive to our soil moisture treatments, similar to findings from glasshouse experiments (Kassem *et al.*, 2008) and natural surveys (Clark *et al.*, 2009) but inconsistent with a recent field experiment documenting large negative effects of precipitation reductions on bacterial communities, but limited effects on fungi (Yuste *et al.*, 2011).

Natural selection and plant–microbe interactions

Microbial community structure altered selection on plant traits. The general trend was for the simplified microbial community to increase selection on plant growth and phenological traits compared with the complex microbial community (Tables 2, 3). This finding may be driven by reductions in fungal diversity in the simplified microbial treatments. Fungal OTU richness was typically negatively correlated with the strength of selection (selection differentials for flowering time, $r = -0.55$, $P < 0.03$; SLA, $r = -0.30$, $P = 0.26$; and biomass, $r = -0.47$, $P < 0.07$). Although it is possible that microbial diversity directly affects patterns of natural selection, given that plants were significantly larger when grown in the presence of a more complex microbial community, the observed microbe effects on selection may result from indirect effects that occur because microbial diversity influences nutrient availability. Regardless of the proximate mechanism, however, our results suggest that microbial diversity can affect selection on plant traits, just as recent studies have demonstrated that fungal diversity can affect plant productivity, plant diversity, and ecosystem functions (van der Heijden *et al.*, 1998; Jonsson *et al.*, 2001; Maherali & Klironomos, 2007; reviewed in van der Heijden *et al.*, 2008).

Given the lack of strong evidence that variation in microbial community composition mediates plant fitness responses to soil moisture, it is perhaps not surprising, that the effects of microbial communities on selection on plant growth and phenological traits typically were consistent across soil moisture treatments. In this system, the effects of microbes on patterns of selection do not appear to result from microbes ameliorating selection imposed by drought stress.

The relative importance of biotic vs abiotic factors

Biotic agents are recognized as important evolutionary forces that contribute to the generation and maintenance of biodiversity (Ehrlich & Raven, 1964; Kursar *et al.*, 2009; see also Benton, 2010). Our results revealed that biotic agents (i.e. soil microbes) affected the expression of plant traits and patterns of selection more so than abiotic agents (i.e. soil moisture). Relatively few studies of natural selection simultaneously manipulate abiotic and biotic variables, so it is difficult to ascertain whether our results are part of a more general pattern. In fact, two recent studies show contrasting results: Stanton *et al.* (2004) found that patterns of selection on *Sinapsis arvensis* differed more between two light environments (abiotic variable) than between two competition treatments (biotic), while Lau *et al.* (2010) detected virtually no effect of elevated atmospheric CO_2 concentrations (abiotic variable), but strong effects of competitors (biotic) on selection on *Arabidopsis thaliana.* We suspect that the relative importance of abiotic vs biotic factors on selection varies from system to system and likely depends on both the intensity of the biological interaction or abiotic stressor and the amount of genetic variation in traits mediating the interaction or the abiotic stress response.

Indirect effects and the potential for co-adaptation to a common abiotic environment

Given that both plants and below-ground microbial communities responded to the soil moisture treatments in this experiment and given that variation in microbial community composition can alter selection on plant traits, it is interesting to consider how plants and their below-ground microbes might respond in a tightly linked manner to variation in the abiotic environment. Feedbacks between above-ground plant and below-ground microbial communities are well documented (Bever, 2003; Kulmatiski *et al.*, 2008), and because multiple generations of both plants and microbes may face similar environmental conditions, plants and below-ground microbes could potentially co-adapt to soil moisture environments or other stressors. Although few studies have investigated evolutionary interactions between plants and their total below-ground community, Pregitzer *et al.* (2010) observed plant local adaptation to soils that differed in microbial structure and soil effects on the heritability of plant traits. By contrast, Wagner *et al.* (2011) found no evidence that plant populations were locally adapted to soil biota. In more tightly linked, pairwise symbiotic relationships, microbially mediated adaptive changes

have been documented for a wide variety of systems from aphids and their *Buchnera* endosymbionts (Zilber-Rosenberg & Rosenberg, 2008) to plants and their mycorrhiza mutualists (Johnson *et al.*, 2010). Moreover, recent studies on plant–rhizobia mutualisms suggest that specific plant–mutualist combinations are necessary for high fitness (Heath & Tiffin, 2007) and that genome × genome epistatic interactions (*sensu* Wade, 2007) may be common. As a result, changes in both plants and their associated microbes may be necessary for strong adaptive responses to environmental change. Understanding how a focal plant population changes in response to abiotic environmental variation, therefore, also requires understanding how closely associated community members, such as below-ground microbes, respond to the same environmental variable and how these biotic interactors influence plant evolutionary processes.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Time series showing volumetric water content in one representative mesocosm of each soil moisture treatment over the duration of the experiment.

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