



Mycorrhizal and rhizobial interactions influence model grassland plant community structure and productivity

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Abstract

Arbuscular mycorrhizal (AM) fungi and rhizobium are likely important drivers of plant coexistence and grassland productivity due to complementary roles in supplying limiting nutrients. However, the interactive effects of mycorrhizal and rhizobial associations on plant community productivity and competitive dynamics remain unclear. To address this, we conducted a greenhouse experiment to determine the influences of these key microbial functional groups on communities comprising three plant species by comparing plant communities grown with or without each symbiont. We also utilized N-fertilization and clipping treatments to explore potential shifts in mycorrhizal and rhizobial benefits across abiotic and biotic conditions. Our research suggests AM fungi and rhizobium co-inoculation was strongly facilitative for plant community productivity and legume (*Medicago sativa*) growth and nodulation. Plant competitiveness shifted in the presence of AM fungi and rhizobium, favoring *M. sativa* over a neighboring C₄ grass (*Andropogon gerardii*) and C₃ forb (*Ratibida pinnata*). This may be due to rhizobial symbiosis as well as the relatively greater mycorrhizal growth response of *M. sativa*, compared to the other model plants. Clipping and N-fertilization altered relative costs and benefits of both symbioses, presumably by altering host-plant nitrogen and carbon dynamics, leading to a relative decrease in mycorrhizal responsiveness and proportional biomass of *M. sativa* relative to the total biomass of the entire plant community, with a concomitant relative increase in *A. gerardii* and *R. pinnata* proportional biomass. Our results demonstrate a strong influence of both microbial symbioses on host-plant competitiveness and community dynamics across clipping and N-fertilization treatments, suggesting the symbiotic rhizosphere community is critical for legume establishment in grasslands.

Keywords Microbial symbioses · *Medicago sativa* · Tripartite interactions · Clipping · N-fertilization · Plant competitiveness

Introduction

Grassland degradation is an extensive global issue, often driven by overgrazing, with subsequent reductions in plant community diversity, grassland productivity, and ecosystem

stability. For example, reduced plant community diversity negatively impacts key physiochemical and biological soil characteristics, such as aggregate stability, nutrient availability, and microbial activity (Bird et al. 2007; Wagg et al. 2014). Therefore, comprehensive regeneration of degraded grassland plant communities is critical for restoration of both above- and belowground ecosystem services (Zhou et al. 2019a). Previous research indicates belowground microbial symbioses are important drivers of individual plant establishment, persistent grassland productivity, and restoration of plant communities in degraded ecosystems (Middleton and Bever 2012; van der Heijden et al. 2008; Zhou et al. 2019b). However, effective strategies to utilize soil microbial partnerships for successful grassland restoration are constrained by numerous knowledge gaps.

An initial step toward improving grassland restoration success is assessment of potential synergies between

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mutualistic belowground associations, such as rhizobium and arbuscular mycorrhizal (AM) fungi. Because leguminous species, such as alfalfa (*Medicago sativa*) form root associations with both N₂-fixing rhizobium and AM fungi, they provide an excellent model for examining multispecies symbiotic dynamics. To acquire growth-limiting resources, most leguminous plants invest in nodule formation to host rhizobium, while also supplying substantial carbon to mycorrhizal partners that build extensive hyphal networks to increase host-plant uptake of soil nutrients, primarily phosphorus (Mickan et al. 2021). Rhizobium and AM fungi play complementary roles for enhancing legume species growth because rhizobia nodules have high P requirements (Sulieman and Tran 2013), and mycorrhizal associations typically provide substantial phosphorus uptake, enabling or increasing root nodulation and nitrogen fixation (Larimer et al. 2014). Additionally, mycorrhizal fungi are N-demanding (Johnson 2010), thus, associating with one symbiont may enhance the ability to invest in, and receive benefit from, the other symbiont.

Within mixed plant communities, AM fungal associations can alter competitive relationships between legumes and grasses by favoring legumes over neighboring C₃ grasses, mainly due to the highly positive mycorrhizal responsiveness of legumes (Scheublin et al. 2007). However, opposite outcomes are typically observed in mixed communities of legumes and highly mycorrhizal responsive C₄ grasses (Klabi et al. 2014). This suggests community competition dynamics are dependent on relative mycorrhizal responsiveness of individual plant species, likely driven by competition between AM fungi and rhizobium for host-plant photosynthetic resources (Bauer et al. 2012; Hoeksema et al. 2010; van der Heijden et al. 2008). Rhizobium also can influence plant diversity and community composition by directly supplying plant-available nitrogen, enhancing growth and competitive ability of host plants (van der Heijden et al. 2006; Keller and Lau 2018). Fixed nitrogen can be indirectly transferred and utilized by neighboring non-N₂-fixing species via root exudation and litter decomposition (Li et al. 2015; Paynel et al. 2008), as well as nutrient transfer via common AM hyphal networks (He et al. 2003). Increased plant-available nitrogen can shift community plant composition due to differences in the capacities of plant species to capitalize on soil nitrogen resources (van der Heijden et al. 2006; Xiao et al. 2019). While beneficial effects, such as increased plant production, are well established for independent mycorrhizal and rhizobial associations, interactive effects of these relationships on productivity and structure of plant communities are poorly quantified (Afkhami et al. 2020).

Several studies demonstrate that trade-offs (costs versus benefits) between hosts and multiple microbial symbionts are likely determined by environmental factors (Larimer et al. 2010; Ossler et al. 2015). Context-dependent influences can be abiotic, such as nutrient availability (Larimer et al. 2010), and/or biotic, such as herbivory, mowing, or other ecological interactions (Yang et al. 2018b).

A large body of literature reports that N-fertilization impacts multiple symbiotic interactions, such that benefits derived from AM fungi and rhizobium tend to decline as N-fertilization increases (Hoeksema et al. 2010; Jiang et al. 2018; Keller and Lau 2018; Larimer et al. 2010, 2014). Nitrogen enrichment has been shown to alter soil microbial composition (Treseder et al. 2018), reduce responsiveness to microbial symbionts, and shift AM relationships from mutualism toward parasitism (Yang et al. 2018b). Across soil nitrogen status, there is a lack of data describing the combined influences of mycorrhizal and rhizobial interactions on grassland plant community productivity and structure.

Mowing or defoliation by herbivores represents a vital biotic process that shapes plant and soil communities in most grasslands (Binet et al. 2013; Yang et al. 2018a). Mowing changes plant and soil properties, leading to altered carbon allocation to roots and associated microbes, modifying AM fungal and/or rhizobial community composition and host-plant fitness (Komatsu and Simms 2019; Morris et al. 2013). Symbionts associated with defoliated host plants may shift to less-beneficial taxa that are adapted to reduced host-carbon investments (Morris et al. 2013). Effects of mowing on AM fungal or rhizobial root colonization is neither clear nor consistent (Barto and Rillig 2010), indicating interactions among host plants and associated microbial symbionts likely differ with mowing intensity, vegetation type, and plant community richness (Ballhorn et al. 2016; Yang et al. 2018a).

Recent work highlights how reseeded legumes into degraded grasslands can increase plant community productivity and benefit grassland ecosystem services by providing a sustainable source of soil nitrogen through biological N₂-fixation, alleviating deleterious environmental side effects associated with intensive N-fertilization (Li et al. 2015; Zhou et al. 2019a). Our previous work also indicates that soil microbial functional groups facilitate legume establishment in degraded semi-arid grasslands (Zhou et al. 2019b), supporting restoration of highly diverse and productive grasslands (Klabi et al. 2014). Recent research also indicates loss of rhizobial relationships in disturbed grasslands constrains legume reestablishment and restoration success (Grman et al. 2020). To further clarify the role of mycorrhizal and rhizobial relationships, we conducted a greenhouse study to assess separate and

interactive influences of AM fungi and rhizobium on competitive relationships between model plant communities of *M. sativa* (legume), *Andropogon gerardii* (C₄ grass) and *Ratibida pinnata* (non-N₂-fixing forb) in mesic conditions. To determine the relative influences of host plants, mycorrhizal, and rhizobial partners, in two-way relationships and in tripartite relationships, we compared experimental plant communities across factorial combinations of symbionts. We examined: (i) separate and interactive effects of AM fungi and rhizobium for communities composed of a single plant species (monoculture) and communities composed of multiple plant species (polyculture); (ii) the effects of N-fertilization and clipping (simulated mowing or herbivory) on plant, mycorrhizal, and rhizobial responses; and (iii) the influences of both microbial functional groups on plant community productivity and individual species competitiveness across treatments. We hypothesized that the relative costs and benefits of both symbioses will be altered by nitrogen and carbon availabilities, leading to a relative decrease in legume competitiveness (proportional community biomass) in fertilized communities and a relative increase in legume competitiveness in clipped communities due to the strong compensatory growth and accelerated N cycling associated with legumes following mowing (Zhou et al. 2019a). Elucidating these tripartite dynamics in a model grassland community is an important step toward leveraging both microbial partners to restore and support grassland plant species coexistence and sustainable biomass production.

Materials and methods

Experiment design

Our greenhouse experiment assessed separate and interactive effects of AM fungi and rhizobium on productivity and competition dynamics of model grassland plant communities. Three plant species (C₃ legume: *Medicago sativa*; C₄ grass: *Andropogon gerardii*; C₃ non-N₂-fixing forb: *Ratibida pinnata*) were selected because of differential mycorrhizal responsiveness (Klabi et al. 2014; Wilson and Hartnett 1998) and differential association with rhizobium. Although *M. sativa* does not occur with *A. gerardii* and *R. pinnata* in native mesic grasslands, *M. sativa* has shown great potential for improving degraded grassland quality in a previous field study (Zhou et al. 2019a). Determining the potential benefits of co-inoculation with AM and rhizobial symbionts is an important first step towards enhancing successful restoration of degraded grasslands and increasing optimization of plant microbiomes for grassland production. In our current study,

we established monoculture microcosms with four microbial treatments (non-mycorrhizal control, soil containing local diverse AM fungi, non-mycorrhizal soil inoculated with commercial rhizobium, or soil containing both local AM fungi and inoculated with commercial rhizobium) for *M. sativa* monoculture. Only two microbial treatments (nonmycorrhizal control or soil containing local diverse AM fungi) were imposed for *A. gerardii* and *R. pinnata* monocultures, as these host plants are not symbiotic with rhizobium. In polycultures of all three plant species, four microbial treatments (nonmycorrhizal control, soil containing local diverse AM fungi, non-mycorrhizal soil inoculated with commercial rhizobium, or soil containing both local AM fungi and inoculated with commercial rhizobium) were imposed. To assess the influence of tripartite interactions among plant, mycorrhizal, and rhizobial partners across resource gradients, a 2 × 2 factorial arrangement (N-fertilization, clipping, N-fertilization + clipping, neither N-fertilization nor clipping) was imposed for polyculture microcosms along with factorial microbial treatments (i.e., 16 combinations of the 4 × 4 microbial and manipulative treatments). Each experimental combination was replicated 6 times, resulting in a total of 144 microcosms (Fig. 1). Microcosms receiving N-fertilization were amended with 15 mg kg⁻¹ NH₄NO₃ following aboveground biomass removal. Aboveground biomass removal was performed by clipping shoot biomass 10 cm above the soil surface at 12 weeks of growth.

Growth conditions

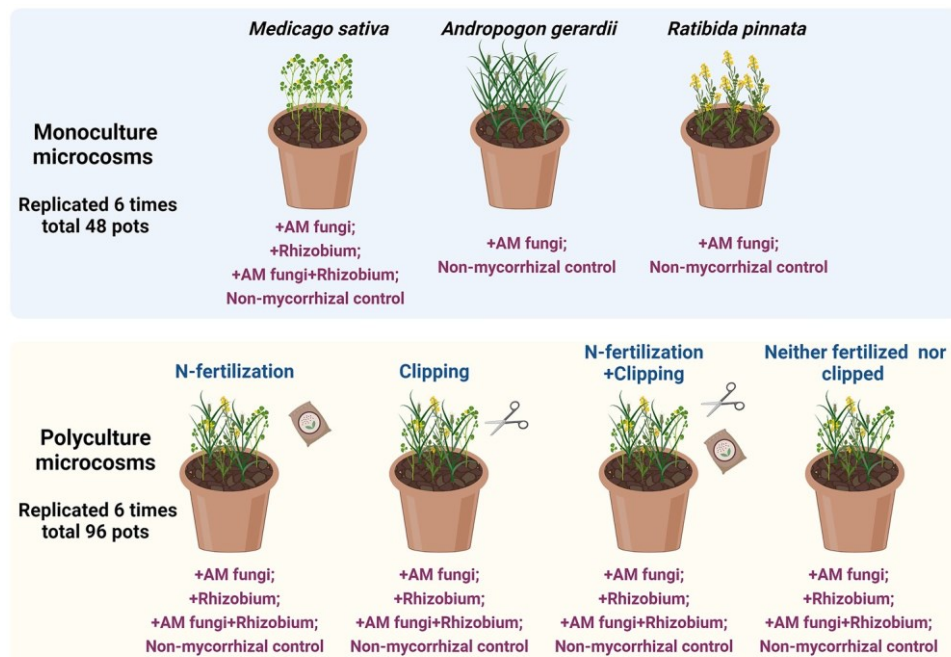
Mesic grassland microcosms were established in 6-L plastic microcosms (21.5 cm diameter × 21.5 cm depth), with a 5.25 kg soil and sand mixture (3:1 soil:sand ratio). Native prairie soil (Chase silty clay loam) was collected from Konza Prairie Biological Station (KPBS; NSF-LTER), located in the Flint Hills of northeastern Kansas, USA (39.1° N, 96.9° W). Konza Prairie is a native tallgrass prairie, dominated by perennial, native warm-season grasses, including *Andropogon gerardii*, *Panicum virgatum*, *Schizachyrium scoparium*, and *Sorghastrum nutans*, and as well as diverse plant species, including cool-season grasses, legumes, and other forbs.

Collected soil was sieved (2-mm mesh) to remove stones, large plant roots, and rhizomes. To improve water infiltration and enhance root recovery at harvest, soil was mixed with autoclaved coarse sand (3:1 soil:sand ratio). Prior to initiating the experiment, the soil-sand substrate was analyzed by the Soil, Water, and Forage Analytical Laboratory (SWAFL) at Oklahoma State University. Soil pH was quantified using a pH electrode in a 1:1 soil to water

suspension. Soil $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ were extracted with 1 M KCl solution and measured by a Lachat Quickchem 8000 Flow Injection Autoanalyzer. Plant-available P and K were extracted using Mehlich 3 solution (Mehlich 1984), and P and K in the extract were measured by inductively coupled plasma emission spectroscopy (ICP). Final substrate had a pH of 7.94, contained $11.75 \text{ mg kg}^{-1} \text{ NO}_3$, $6.81 \text{ mg kg}^{-1} \text{ NH}_4$, 12.6 mg kg^{-1} plant-available P, and 281 mg kg^{-1} plant-available K.

Fig. 1 Experiment design. In monoculture microcosms, four microbial treatments (non-mycorrhizal control, soil containing local diverse AM fungi, non-mycorrhizal soil inoculated with commercial rhizobium, or soil containing both local AM fungi and inoculated with commercial rhizobium) were imposed for *M. sativa* monoculture, and only two microbial treatments (non-mycorrhizal control or soil containing local

diverse AM fungi) were imposed for *A. gerardii* and *R. pinnata* monocultures. In polycultures of all three plant species, four microbial treatments (non-mycorrhizal control, soil containing local diverse AM fungi, non-mycorrhizal soil inoculated with commercial rhizobium, or soil containing both local AM fungi and inoculated with commercial rhizobium) were imposed. To assess the influence of tripartite interactions among plant, mycorrhizal, and rhizobial partners across resource gradients, a 2×2 factorial arrangement of N-fertilization, clipping, N-fertilization + clipping, neither N-fertilization nor clipping treatments were imposed for polyculture microcosms along with factorial microbial treatments (i.e., 16 combinations of the 4×4 microbial and manipulative treatments). N-fertilization microcosms received $15 \text{ mg kg}^{-1} \text{ NH}_4\text{NO}_3$ following aboveground biomass removal at week 12. For clipping microcosms, shoot biomass was clipped 10 cm above the soil surface following 12 weeks of growth. Each experimental combination was replicated 6 times, resulting in a total of 144 microcosms. (Figure created with BioRender.com)



Plant and microbial materials

Seeds of *A. gerardii* and *R. pinnata* were obtained from the USDA Natural Resources Conservation Service Plant Materials Center, Manhattan, Kansas. Seeds of *M. sativa* ('OK 49' cultivar) (Caddel et al. 1992, 2002) were obtained from the Oklahoma Agricultural Experiment Station, Stillwater, Oklahoma. Seeds were surface sterilized in 50% ethanol for 2 min, then soaked in 5% sodium hypochlorite for 1 min, and thoroughly rinsed with sterile water two times. Surface-sterilized seeds were germinated in vermiculite for 4 weeks; seedlings were then transplanted into microcosms. For monoculture microcosms, 6 seedlings of each species were evenly spaced in a ring. For polyculture microcosms (containing all three species), 2 seedlings of each species were planted in a uniform pattern so conspecifics were always opposite one another across the

ring, and the nearest neighbors always were heterospecific (planting pattern: *M. sativa*, *A. gerardii*, *R. pinnata*, *M. sativa*, *A. gerardii*, *R. pinnata*). Each seedling was planted ~4 cm distant from the nearest neighbors (Fig. 1).

Live soil-sand substrate was used in mycorrhizal $\times$ rhizobial treatments, and pasteurized soil-sand substrate (heated at 90°C for 120 min and allowed to cool and equilibrate for 48 h) was used in non-mycorrhizal controls and nonrhizobial treatments. Pasteurized soil-sand substrate had a pH of 7.95, contained $5.25 \text{ mg kg}^{-1} \text{ NO}_3$, $19.85 \text{ mg kg}^{-1} \text{ NH}_4$, 14.7 mg kg^{-1} plant-available P, and 328.8 mg kg^{-1} plant-available K (determined by SWAFL at Oklahoma State University). While the soil chemistry of the pasteurized soil was lower in NO_3 and higher in NH_4 and plantavailable P and K, these differences were not significant ($n = 8$; samples randomly collected throughout pasteurized and non-pasteurized soils). We utilized a microbial wash (see Koide and Li 1989) to add the majority of native microbes,

except AM fungi, back to steamed soil treatments (non-mycorrhizal soil with or without rhizobial inoculation). This experimental method was previously utilized in Larimer et al. (2014) to establish rhizobial and mycorrhizal factorial combinations. Based on plant responses in our study, we found no evidence the native grassland soils we utilized contained alfalfa-specific rhizobia, and no nodules were observed in alfalfa grown in pasteurized soil with the rhizobia-free microbial wash. To obtain our microbial wash, we blended soil and water in a 1:2 ratio for 10 s and passed the slurry through a 25- μm sieve, trapping AM fungal spores that are larger than 25- μm but passing smaller nonmycorrhizal biota (Johnson et al. 2010). All experimental microcosms were amended with 100 mL of microbial wash.

A commercial rhizobial inoculum containing cultures attached to granular peat (*Sinorhizobium Meliloti* $> 3 \times 10^8$ CFU per g; pH ~ 7.94), manufactured by Visjonbiologics (4385 Seymour Highway Wichita Falls, TX 76,309), was applied at transplant, in proximity to seedling roots (Hacisalihoglu et al. 2020; McKenna et al. 2020). To control for potential nutrients contained in the rhizobial inoculum, 1.25 g of live inoculum was applied to each *M. sativa* seedling in rhizobial treatments, and 1.25 g of autoclaved inoculum (autoclaved for 90 min at 121 °C) was added to all non-legume seedlings and non-rhizobial *M. sativa* plants. Any seedlings that did not survive within 2 weeks of transplanting were replaced by similar-age seedlings.

Greenhouse conditions and maintenance

Microcosms were arranged in a randomized complete block design, watered daily, and maintained at 20–25 °C during day, night, and photoperiod hours 14/10 (day/night), with 50%–70% relative humidity. To ensure micronutrient sufficiency, all microcosms were amended with 100 mL (P-free, 2.0 ppm NH_4NO_3) nutrient solution adapted from McKnight's solution every 2 weeks throughout the 18-week experiment (McKnight 1949). Resulting N-additions represent an N supply typically encountered in tallgrass prairie ecosystems over a growing season, including N mineralization and N from dry deposition sources (Seastedt et al. 1991). For N-fertilization treatments, 15 mg kg^{-1} NH_4NO_3 (as a dry powder) was added to the McKnight's solution for a liquid fertilizer amendment that was applied following aboveground biomass removal at week 12 of our study, using rate recommendations for alfalfa production in the Southern Great Plains (Caddel et al. 2001). For clipped plant communities, shoot biomass was removed to a 10-cm stubble height.

Sample collection and analysis

After 18 weeks, representing an approximate growing season, plant shoots and roots were harvested and sorted by species. Plant shoots were dried at 60 °C for 48 h and then weighed to determine aboveground biomass. While we assessed aboveground production of all three plant species, only *M. sativa* tissue quality is reported because plant biomass for *A. gerardii* and *R. pinnata* was extremely small (≤ 0.05 g), and plant-tissue N and P could not be accurately determined. Furthermore, our primary focus was the separate and additive effects of mycorrhizal and rhizobial partnerships on host-plant production, and these effects only could be assessed for *M. sativa*, as neither *A. gerardii* nor *R. pinnata* nodulates. Shoot biomass samples of *M. sativa* were ground and analyzed for shoot protein with a N-to-protein conversion factor of 6.25 via N combustion using a 2300 Kjeltex Analyzer Unit (FOSS, Sweden) (Page et al. 1982). Subsamples of shoot tissue were used to determine concentrations of P, Ca, K, Na, S, Cu, Fe, Mg, Mn, and Zn by the methods of Farnham et al. (2011) using inductively coupled plasma optical emission spectroscopy (CIROS ICP Model FCE12).

The roots of each plant species were washed free of soil over a 2-mm screen and subsampled to measure root colonization by AM fungi. The roots were not intertwined and could easily be separated for each individual species. Six subsamples of roots were pooled for each plant species (2–3 cm root fragments) for every replicate monoculture or polyculture microcosm and cleared with 10% KOH and stained with trypan blue in lacto-glycerol (modified from Phillips and Hayman 1970) and measured under a digital microscope (Hirox KH 7700) using the magnified gridline intersect method (McGonigle et al. 1990). To determine AM fungal root colonization, all observed AM fungal structures (intra-radical hyphae, arbuscules, vesicles, and coils observed at 50 root intersections per subsample) were combined for total colonization (Reinhardt and Miller 1990). To evaluate the potential N_2 fixing capability of *M. sativa*, nodules were removed from live roots, and pink nodules were identified as effective. All effective nodules were counted, dried, and weighed. The remaining roots were dried at 60 °C and weighed to determine belowground biomass.

The biomass of microbial functional groups (gram-positive and gram-negative bacteria; saprophytic and AM fungi), and total microbial biomass were assessed using phospholipid fatty acid (PLFA) biomarkers. As constituents of biological membranes, PLFA is widely applied to estimate the active biomass of fungi and bacteria, as biovolume and cell surface area are well correlated (Frostegård et al. 2011). Soil samples in close proximity to the plant roots were

collected, homogenized, and freeze-dried. Fatty acids were extracted using a modification of the Bligh and Dyer extraction (White et al. 1998). Qualitative and quantitative PLFA analyses were performed using gas chromatography with a GCMS unit Agilent MS 5975C/GC 7890A. Biomarker c:19 was utilized as an internal standard. Biomarkers i-15:0, a-15:0, i-17:0, and i-16:0 were selected to represent the functional group of gram-positive bacteria; biomarkers 16:1 ω 7, cy19:0, cy17:0 ω 9, 2-OH 14:0, 2-OH 16:0, 3-OH 14:0, and 18:1 ω 9 trans were selected to indicate gram-negative bacteria; biomarkers selected for extra-radical AM fungal biomass consisted of 16:1 ω 5c, 20:1 ω 9, and 22:1 ω 13; biomarkers selected for saprophytic fungi were 18:2 ω 9, 12, and 18:1 ω 9c. The composition of soil microbial communities was summarized using a correspondence analysis on the relative mole abundances of PLFAs in each sample (Allison et al. 2005). Concentration of each individual functional group was calculated by summing corresponding selected biomarkers of each group. Ratios of fungi to bacteria (F/B) were calculated by the sum of relative mole abundances of saprophytic fungi and AM fungi divided by relative mole abundances of all bacterial microbial group signatures. Gram-positive to gram-negative bacterial ratio (GP/GN) was calculated by dividing all gram-positive microbial group signatures by all gram-negative microbial group signatures. Total microbial biomass was calculated by summing all functional groups including non-specific microbial biomarkers (14:0, 15:0, 16:0, 17:0, 18:0, and 20:0) (Allison et al. 2005).

Statistical analyses

Because the biomasses of individual plants within species differed by < 1.0%, the biomass of each species was determined by averaging the biomass of all individuals grown in the same microcosm. Total plant community above- and belowground productivity was determined by summing all the biomasses of plant species grown together in an individual microcosm (van der Heijden 2004). Mycorrhizal growth response (MGR), as determined by the aboveground biomass was calculated using: $MGR = \log(e)(Myc/Nonmyc)$, where Myc is the aboveground biomass for all plants in the mycorrhizal microcosm, and Nonmyc is the mean value of the aboveground biomass for corresponding non-mycorrhizal plants (Johnson 2010, Liu et al. 2021).

The effects of competition between plant species were calculated using the relative crowding coefficient (K values). This index can evaluate and compare the

competitive ability of one plant species to the others in a community (De Wit 1960). K was calculated as:

$$KM_s = \frac{(Y_{pa} \times F_{o2})}{((Y_{ma} - Y_{pa}) \times F_{pa})}$$

$$KA_G = \frac{(Y_{pb} \times F_{o2})}{((Y_{mb} - Y_{pb}) \times F_{pb})}$$

$$KR_p = \frac{(Y_{pc} \times F_{o2})}{((Y_{mc} - Y_{pc}) \times F_{pc})}$$

where KM_s , KA_G , and KR_p are the relative crowding coefficient of *M. sativa*, *A. gerardii*, and *R. pinnata* in the polyculture microcosms, respectively. Y_{pa} , Y_{pb} and Y_{pc} are the aboveground biomasses of *M. sativa*, *A. gerardii*, and *R. pinnata* in polyculture; Y_{ma} , Y_{mb} and Y_{mc} are the aboveground biomasses of *M. sativa*, *A. gerardii*, and *R. pinnata* in monoculture; and F_{pa} , F_{pb} and F_{pc} are the sown proportions of *M. sativa*, *A. gerardii*, and *R. pinnata* in polyculture. F_{o2} in each formula represents the total corresponding proportions of the other two plant species in polyculture. The greater the K value of one species, the more competitive and dominant that species was in polyculture (Bi et al. 2019). The proportional biomass also was used to measure the effect of N-fertilization and clipping on the competitive ability of each species in polyculture, and the relative contribution by each species to the biomass of the entire plant community in polyculture (Klabi et al. 2014).

Data were analyzed using the GLM procedure of SAS version 9.2. In generalized linear mixed models for biomass and AM fungal root colonization of *A. gerardii* and *R. pinnata*, the mycorrhizal treatment was utilized as a fixed effect, and block was employed as a random effect. For biomass, tissue nutrients, AM fungal root colonization, and *M. sativa* root nodule mass, as well as individual and model plant community biomass, and PLFA assessments, the rhizobium treatment additionally was included as a fixed effect.

We further analyzed the data without including non-mycorrhizal plants to assess plant responses to N-fertilization and clipping in polyculture microcosms because the necessity of AM fungi for growth of all three plant species had been observed. There were significant growth benefits for all mycorrhizal treatments, compared to corresponding nonmycorrhizal treatments, and non-mycorrhizal plants were not colonized by AM fungal

structures (Table S1). In the absence of AM fungi, all plant species failed to grow beyond the seedling stage. All non-mycorrhizal plants produced extremely little biomass, especially the non-legumes (*A. gerardii* and *R. pinnata*), which weighed between 0.01 and 0.05 g. Therefore, we only analyzed the effects of rhizobium, N fertilization, and clipping on growth of the three plant species grown in polyculture and inoculated with AM fungi. Generalized linear mixed models also were utilized to analyze biomass and AM fungal root colonization of *A. gerardii* and *R. pinnata*, with N-fertilization and clipping as fixed effects and block as a random effect. To measure response variables of *M. sativa* and the overall plant community in polyculture, the effect of rhizobium was included as a fixed effect. Within models, post hoc comparisons of means were calculated using Tukey's HSD ($p \leq 0.05$).

Regression modeling was employed to analyze the relationships of root colonization and effective root nodulation (independent variables) on biomass production of each plant species (dependent variables). Relationships between AM fungal root colonization and tissue quality of *M. sativa* were analyzed using Spearman's correlations. A total of 66 possible pairwise correlations were examined. All tests of significance were determined at $p \leq 0.05$.

Redundancy analysis (RDA) was applied to examine the variation in soil PLFA biomarkers in relation to treatments by using CANOCO 4.5 (Lepš and Šmilauer 2003). Soil PLFA biomarkers were used as response variables and treatments used as environmental variables for RDA. Monte Carlo tests were employed in the RDA with restricted random permutations of samples reflecting the experimental design. Log-transformations were carried out to standardize the variables of different scales and magnitudes.

Results

Plant productivity

We found a synergistic benefit of dual inoculation with AM fungi and rhizobium for *M. sativa* grown in polyculture, as *M. sativa* derived additional benefits from associating with both symbionts (Fig. 2a); growth exceeded additive expectations based on growth with the individual symbiont types. Specifically, for *M. sativa* inoculated with mycorrhizal fungi, rhizobial inoculation had a positive main effect on above- and belowground biomass of *M. sativa* (Table 1), as well as the total plant community biomass (Table 1). The biomass (above- and belowground) of *M. sativa* in polyculture treatments was greater than in corresponding monoculture microcosms, whereas *A.*

gerardii and *R. pinnata* produced less above- and belowground biomass in polyculture microcosms than monoculture microcosms (Fig. 2c–f). Mycorrhizal fungi significantly influenced above- and belowground productivity of all three plant species, with biomass increasing regardless of rhizobium inoculation (Fig. 2). In fact, in the absence of AM fungi, all plant species failed to grow beyond the seedling stage, with a total biomass of every species less than 0.05 g.

In polyculture microcosms inoculated with AM fungi, aboveground biomass following clipping increased significantly for all plant species, compared to non-clipped plants (Table 1). In clipped microcosms, *M. sativa* inoculated with AM fungi produced the greatest aboveground biomass, regardless of N-fertilization or rhizobium inoculation (Fig. 3c). In the absence of clipping, *M. sativa* inoculated with AM fungi and fertilized with N produced the least aboveground biomass (Fig. 3c). *A. gerardii* produced greater aboveground biomass in polyculture microcosms with rhizobium inoculation, compared to plants without rhizobium and without clipping or N-fertilization (Fig. 3e). Across all mycorrhizal treatments, *M. sativa* accounted for more than 80% of the biomass of polyculture microcosms (Fig. S1).

Clipping significantly reduced proportional biomass of *M. sativa* and increased *A. gerardii* and *R. pinnata* in all microcosms (Fig. S1). In contrast to aboveground biomass, belowground biomass of *M. sativa* and the total plant community decreased significantly when plants were clipped. In polyculture microcosms, N-fertilization significantly promoted belowground biomass of *R. pinnata*, compared to non-fertilized microcosms (Table 1). However, there were no significant interactions among rhizobium, clipping, and N-fertilization (Table 1).

Competitive ability of plant species

The partial K values of *M. sativa* were greater than those of *A. gerardii* and *R. pinnata*, showing that the competitive ability of *M. sativa* increased significantly with mycorrhizal and rhizobial co-inoculation (Fig. S2). However, the partial K values of *A. gerardii* decreased significantly with mycorrhizal inoculation, compared to non-mycorrhizal controls.

Root colonization and nodulation

Root colonization by AM fungi was significantly greater for *M. sativa* plants in polyculture than in monoculture, whereas root colonization was significantly greater for *A. gerardii*

and *R. pinnata* in monoculture as compared to polyculture (Fig. 4a, c). In mycorrhizal polycultures, clipping significantly increased AM fungal root colonization of *M. sativa* as compared to not clipped control microcosms (Table 1; Fig. 4b).

The combined effects of AM fungi and rhizobium coinoculation were strongly synergistic for *M. sativa* nodulation in both monoculture and polyculture microcosms (Fig. 5a, b). The effective nodule numbers for *M. sativa* in polycultures were greatest following co-inoculation with AM fungi and rhizobium, in contrast to nodules per plant in monoculture. However, neither clipping nor N-fertilization had a significant influence on *M. sativa* nodulation of any polyculture microcosms (Fig. 5).

Mycorrhizal growth response

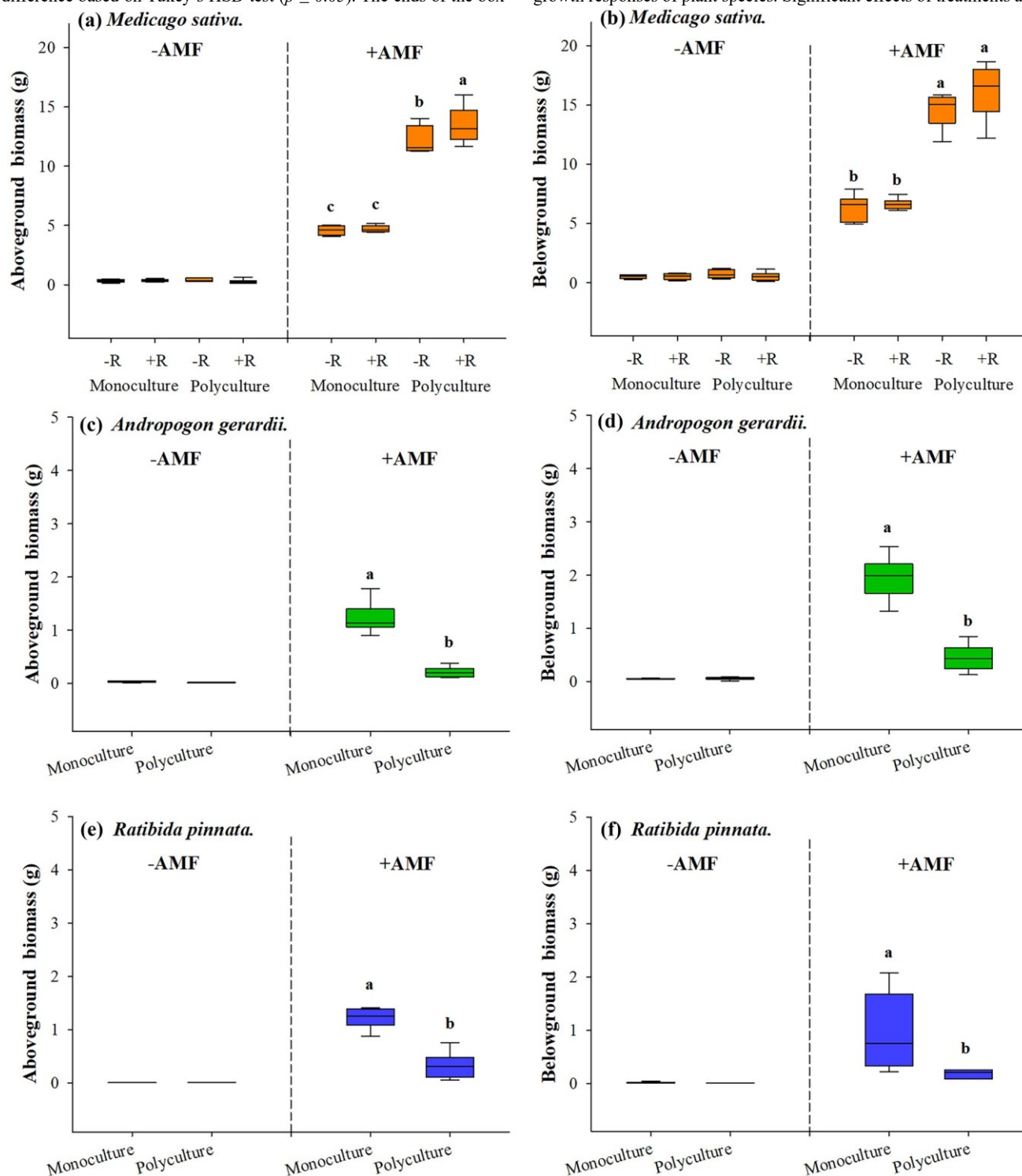
All three species were highly mycotrophic, and all species were characterized by positive mycorrhizal growth responses. The MGR for *M. sativa* showed a stronger positive response in polyculture microcosms following inoculation with rhizobium, compared to *M. sativa* in polyculture microcosms that were not inoculated with rhizobium (Fig. 6a). *M. sativa* was, on average, 2-fold greater in polyculture than in monoculture, whereas the MGR of *A. gerardii* and *R. pinnata* were greater in monoculture than polyculture (Fig. 6c, e).

Fig. 2 The effects of arbuscular mycorrhizal fungi (AMF) and rhizobium on above- and belowground biomass of *Medicago sativa* (a, b), *Andropogon gerardii* (c, d), and *Ratibida pinnata* (e, f) in monoculture and polycultures that were neither fertilized nor clipped ($n = 6$). Boxplots topped by the same letter indicate no significant difference based on Tukey's HSD test ($p \leq 0.05$). The ends of the box

the upper and lower quartiles. Boxplots without post hoc test letters indicate no significant differences

Table 1 Effects of clipping

(C), nitrogen amendment (N), rhizobium (R), and their interactions on production, nodulation of alfalfa, root colonization, and mycorrhizal growth responses of plant species. Significant effects of treatments are



represent the upper (75th percentile) and lower (25th percentile) quartiles; the median is shown by a horizontal line inside the box (middle quartile); whiskers extend the interquartile range 1.5-fold from

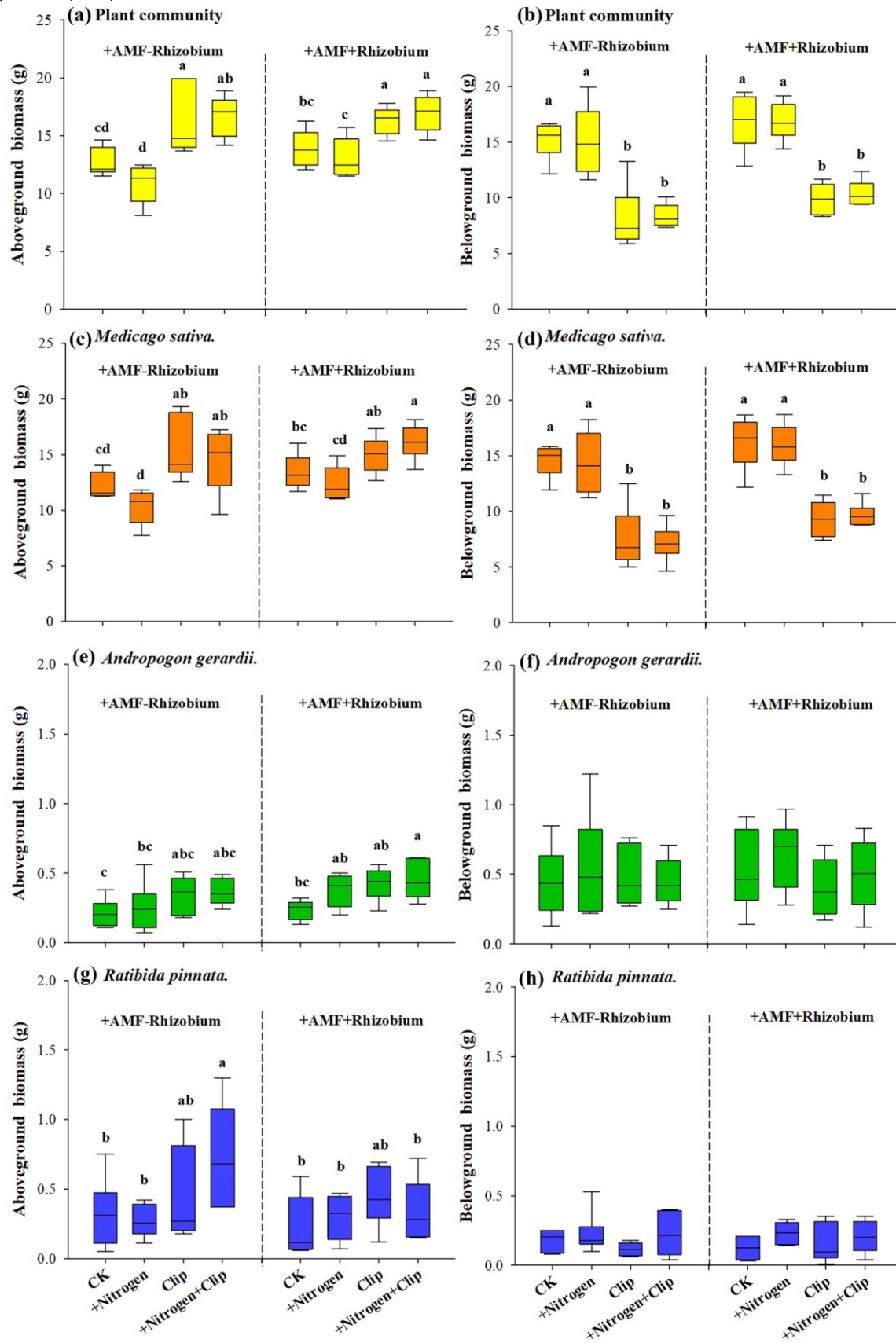
indicated in bold

24	Mycorrhiza (2022) 32:15–32									
	C	N	R	C×N	C×R	N×R	C×N	C×R	N×R	
Df. (factor)	1	1	1	1	1	1	1	1	1	
Df. (error)	47									
Aboveground biomass										
<i>M. sativa</i>	<i>F</i> -value	32.9	1.53	4.22	2					
	0.91	1.61	0.35							
	<i>P</i> -value	<.0001	0.2239	0.0466						
	0.1655	0.3465	0.2114	0.5594						
<i>A. gerardii</i>	<i>F</i> -value	12.9	2.45	4.42	1.23					
	0.76	0.94	0.48							
	<i>P</i> -value	0.0009	0.1258	0.0422						
	0.2748	0.3903	0.339	0.4945						
<i>R. pinnata</i>	<i>F</i> -value	8.81	0.53	2.6	0.32					
	1.26	0.82	3.19							
	<i>P</i> -value	0.005	0.4694	0.1149						
	0.5732	0.2675	0.3713	0.0818						
Plant community	<i>F</i> -value	55.59	3.41	4.7	0.31					
	3	0	1.9							
	<i>P</i> -value	<.0001	0.073	0.0366						
	0.5822	0.0915	1	0.1765						
Belowground biomass										
<i>M. sativa</i>	<i>F</i> -value	139.69	0.04	10.55	0.02					
	0.21	0.12	0.12							
	<i>P</i> -value	<.0001	0.8361	0.0024						
	0.8822	0.6477	0.7329	0.735						
<i>A. gerardii</i>	<i>F</i> -value	1.3	1.04	0.8	0.25					
	0.02	0.91	0.55							
Root colonization										
<i>M. sativa</i>	<i>F</i> -value	267.84	0.14	0.2	9.15					
	7.07	2.64	0							
	<i>P</i> -value	<.0001	0.7099	0.6597						
	0.0043	0.0112	0.1122	0.9576						
<i>A. gerardii</i>	<i>F</i> -value	0.08	0.23	0.05	1.37					
	0.4	1.16	1							
	<i>P</i> -value	0.7849	0.6307	0.8293						
	0.2481	0.5323	0.2876	0.3233						
<i>R. pinnata</i>	<i>F</i> -value	4.35	0.8	0.22	3.87					
	0.26	0.18	0.02							
	<i>P</i> -value	0.0538	0.3758	0.6449						
	0.0566	0.6107	0.6779	0.8842						
Mycorrhizal growth responses										
<i>M. sativa</i>	<i>F</i> -value	7.51	10.42	0.43	0.17					
	5.53	0.2	3.67							
	<i>P</i> -value	0.0092	0.0025	0.5152						
	0.6866	0.0239	0.6607	0.0628						
Soil microbial communities										
<i>A. gerardii</i>	<i>F</i> -value	0.03	0.14	0.37	7.43					
	0.47	0.01	0.03							
	<i>P</i> -value	0.8575	0.7111	0.5477						
	0.0096	0.4952	0.9198	0.8568						
<i>R. pinnata</i>	<i>F</i> -value	9.57	0.35	1.44	0.35					
	0.3	0.61	2.48							
	<i>P</i> -value	0.0036	0.5575	0.2376						
	0.5589	0.5871	0.4395	0.1231						

In the polyculture microcosms, in the absence of rhizobium, the MGR of *M. sativa* significantly increased with clipping, and N-addition decreased production regardless of rhizobial inoculation (Fig. 6b). Generally, the MGR of *A. gerardii* or *R. pinnata* was not influenced by any treatment. However, *R. pinnata* MGR increased following combined N-fertilization and clipping in the absence of rhizobium (Fig. 6).

Soil microbial communities

In polyculture microcosms, regardless of rhizobial inoculation, clipping, or N-fertilization, AM fungal inoculation had a significant effect on microbial biomass, with increased abundance of gram-positive bacteria, gramnegative bacteria, AM fungi, and saprophytic fungi (Tables S2 and S3).



3 The effect of clipping and nitrogen amendments on above- and belowground biomass of total model plant communities (**a, b**), as well as by species: *Medicago sativa* (**c, d**), *Andropogon gerardii* (**e, f**), and *Ratibida pinnata* (**g, h**) in polyculture microcosms with arbuscular mycorrhizal fungi (AMF) with or without rhizobial inoculation ($n = 6$). CK indicates neither clipping nor fertilization. Boxplots topped by the same letter indicate no significant difference based on Tukey's HSD test ($p \leq 0.05$). The ends of the box represent the upper (75th percentile) and lower (25th percentile) quartiles; the median is shown by a horizontal line inside the box (middle quartile); whiskers extend the interquartile range 1.5-fold from the upper and lower quartiles. Boxplots without post hoc test letters indicate no significant differences

RDA was used to examine the variation in soil PLFA biomarkers relative to AM fungal and/or rhizobial inoculation (Fig. S3). The first axis explained 69.0% of total variance. Soils inoculated with AM fungi had greater gram-positive bacteria, gram-negative bacteria, AM fungi, saprophytic fungi, and total microbial biomass (RDA axis 1, $R^2 = 0.69$, $F = 204.96$, $p = 0.002$), compared to non-mycorrhizal soils. Nitrogen fertilization and/or clipping of polycultures, however, only explained 4% (RDA axis 1, $R^2 = 0.04$, $F = 3.812$, $p = 0.014$) of the total variation in microbial communities (Fig. S3b).

Correlations between partner responses

Root colonization by AM fungi positively correlated with aboveground biomass of all three species. Positive relationships were detected between root colonization and belowground biomass of *A. gerardii* and *R. pinnata*, whereas a negative relationship was observed between root colonization and belowground biomass of *M. sativa* (Fig. S4). Effective nodule number of *M. sativa* was positively correlated with above- and belowground biomass of *M. sativa* in microcosms that were co-inoculated with AM fungi and rhizobium (Fig. S5), and a positive relationship between AM fungal root colonization and effective nodule number of *M. sativa* was observed in microcosms that were co-inoculated with AM fungi and rhizobium (Fig. S6). However, there was no significant relationship between nodulation and above- or belowground biomass of *M. sativa* in microcosms with single symbiont inoculation. Colonization of *M. sativa* roots by AM fungi was positively correlated with shoot tissue P, K, Zn, and Cu (Fig. S7).

Discussion

Our findings suggest that microbial drivers are critical for the success of *M. sativa* in polyculture systems by demonstrating how AM fungi, rhizobium, and their

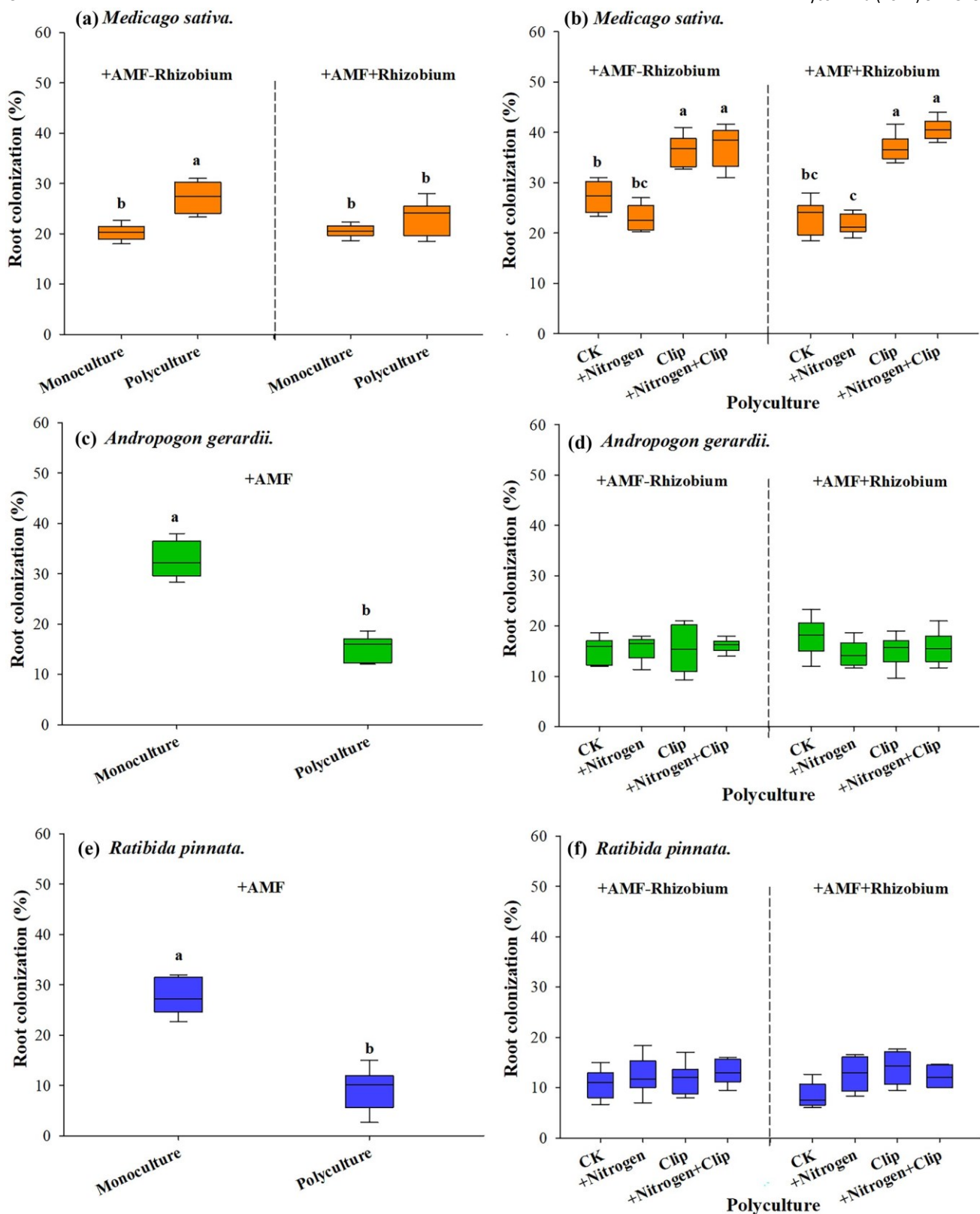
interactions influence competitive relationships among grassland plants. A reduction in intraspecific competition of *M. sativa* in polycultures (two individuals versus six in monocultures) greatly facilitated its growth and increased interspecific effects on each of the other two plant species. Clipping and N-fertilization had fewer effects than microbial symbionts on interspecific relationships in our model plant community. Mycorrhizal and rhizobial coinoculation was synergistic for *M. sativa* nodulation and productivity, and plant community competitiveness (proportional biomass of *M. sativa*) was enhanced in microcosms where both symbionts were present compared with single symbiont inoculation. Conversely, clipping shifted plant community competition in the opposite direction, reducing proportional biomass of *M. sativa* and increasing the proportional biomass of *A. gerardii* and *R. pinnata* in polycultures. Although N-fertilization did not significantly influence plant species dominance, we observed a negative effect on mycorrhizal growth responses of *M. sativa* following fertilization in the absence of clipping. N-fertilization may result in investment of fewer resources to support rhizobia, reducing the likelihood of microbial synergism (Shantz et al. 2016). Previous research demonstrated independent contributions of AM fungi and rhizobium to plant productivity and community composition (Bahadur et al. 2019; Beyhaut et al. 2014) as well as interactive effects of AM fungi and rhizobium on plant (mainly legumes) growth (Larimer et al. 2010; 2014). Nevertheless, our work contributes to deepen the understanding of interactive and synergistic effects of both symbionts, which drive plant community dynamics more than independent abiotic (N-fertilization) or biotic (clipping) influences.

We observed interactive benefits of AM fungi and rhizobium in both monoculture and polyculture microcosms, indicating nutrient uptake gains outweighed carbon costs of maintaining microbial symbioses (Bahadur et al. 2019). Synergism may occur in legume-rhizobia-AM fungal interactions as plants are able to acquire complementary resources (nitrogen and phosphorus) from associations with both AM fungi and rhizobia (Primieri et al. 2021). Associating with both AM fungi and rhizobia simultaneously provides both of these potentially limiting, complementary nutrients (Afkhami et al. 2020). In our current study, synergistic benefits occurred for *M. sativa* under dual inoculation in polyculture, as growth and nodulation exceeded the additive effects of either symbiont independently. Generally, AM symbioses contribute P and other limiting nutrients to legumes (Liu et al. 2020) and facilitate the establishment of rhizobial symbioses that contribute to nodule formation (Antunes and Goss 2005;

Fig.

Larimer et al. 2014). AM fungi and rhizobium are both essential for effective N-fixation in many contexts, and previous research has shown that in the absence of AM fungi

or rhizobium, only inefficient and amorphous nodules are formed (Bournaud et al. 2018). Our



results concur and expand these ideas, as effective nodule In the presence of AM fungi, *M. sativa* produced more number and shoot tissue P, K, Zn, and Cu of *M. sativa* were biomass in polyculture plant communities than in monocultightly linked with AM fungal root colonization. ture plant communities, whereas *A. gerardii* and *R. pinnata*

◀ Fig.

4 The influence of rhizobium on AM fungal root colonization of *Medicago sativa* (a, b), *Andropogon gerardii* (c, d), and *Ratibida pinnata* (e, f) in monoculture and polyculture (a, c, e) and within polyculture across clipping and nitrogen amendments (b, d, f). All are inoculated with arbuscular mycorrhizal fungi (AMF) ($n = 6$). CK indicates neither clipping nor fertilization. Boxplots topped by the same letter indicate no significant difference based on Tukey's HSD test ($p \leq 0.05$). The ends of the box represent the upper (75th percentile) and lower (25th percentile) quartiles; the median is shown by a horizontal line inside the box (middle quartile); whiskers extend the interquartile range 1.5-fold from the upper and lower quartiles. Boxplots without post hoc test letters indicate no significant differences

monocultures were more productive than polycultures. The competitive ability of *M. sativa* was greater than *A. gerardii* and *R. pinnata* in polyculture, as the partial K values of *M. sativa* were much greater than those of the other two plant

species, suggesting that *M. sativa* is a highly successful competitor for soil resources in polyculture. As we observed a negligible number of plants die or respond negatively to transplant, and all plants produced sufficient growth in monocultures, these differences do not appear to be a result of differential abilities in recovering from transplant. Belowground biomass of *M. sativa* was also greater than that of *A. gerardii* or *R. pinnata*. There clearly are differences of carbon investment strategies regarding AM fungal-mediated versus root-mediated nutrient acquisition in mycorrhiza-dependent plant species (Bahadur et al. 2019). In polyculture, we observed increased AM fungal root colonization of *M. sativa* roots, while colonization of *A. gerardii* and *R. pinnata* decreased,

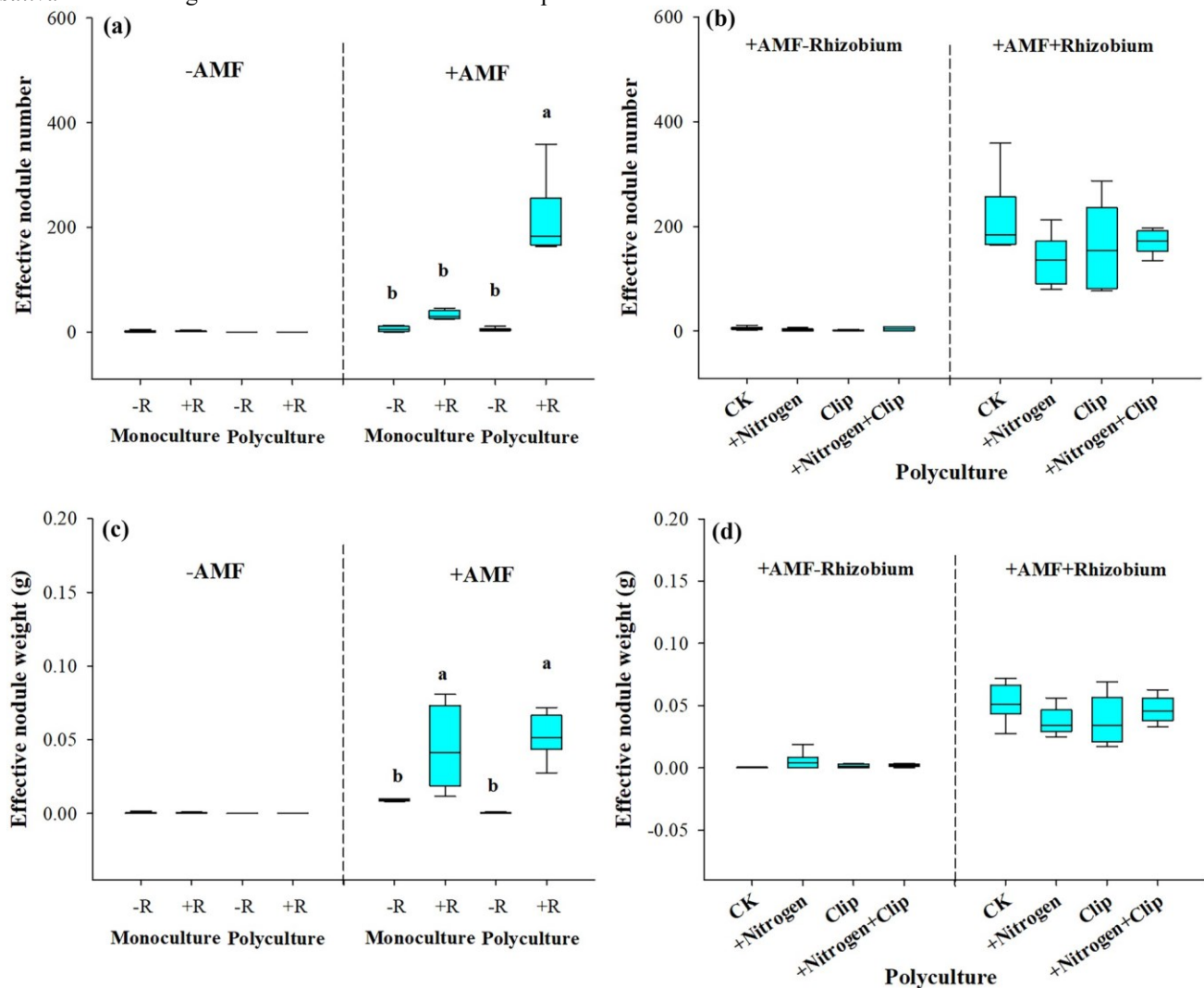
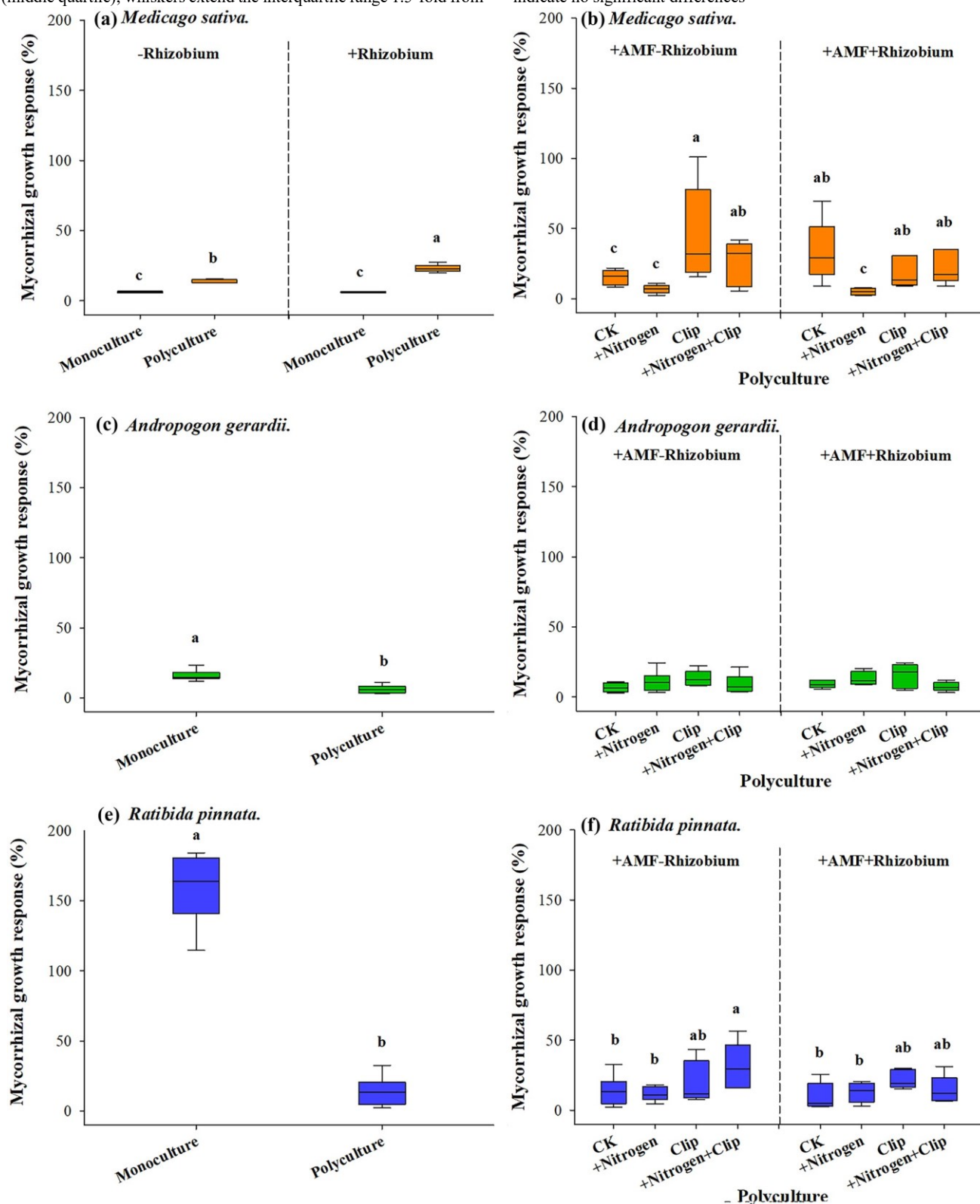


Fig. 5 The influence of arbuscular mycorrhizal fungi (AMF) and rhizobium on effective root nodule number (a, b) and effective nodule weight (c, d) of *Medicago sativa* in monoculture and polyculture (a, c), and in AM-inoculated polyculture, across clipping and nitrogen

amendments (b, d) ($n = 6$). CK indicates neither clipping nor fertilization. Boxplots topped by the same letter indicate no significant difference based on Tukey's HSD test ($p \leq 0.05$). The ends of the box represent the upper (75th percentile) and lower (25th percentile)

quartiles; the median is shown by a horizontal line inside the box (middle quartile); whiskers extend the interquartile range 1.5-fold from

the upper and lower quartiles. Boxplots without post hoc test letters indicate no significant differences



◀ Fig.

compared to corresponding monocultures. This is likely Although all three species exhibited positive response driven by host-plant photosynthetic capacity, as large plants siveness to AM inoculation when grown in monoculture, can energetically support greater AM fungal root colonization stronger mycorrhizal growth responses were measured for than small plants (Moora and Zobel 1996). *M. sativa* than *A. gerardii* or *R. pinnata* in polycultures.

6 The influence of rhizobium on mycorrhizal growth responses (MGR) of *Medicago sativa* (a, b), *Andropogon gerardii* (c, d), and *Ratibida pinnata* (e, f) in monoculture and polyculture (a, c, e), and in polyculture across clipping and nitrogen amendments (b, d, f). CK indicates neither clipping nor fertilization. $MGR (\%) = \log(e)(Myc/Nonmyc)$, where Myc is the aboveground biomass in the mycorrhizal treatment, and Nonmyc is the mean value of aboveground biomass in the non-mycorrhizal treatment. Boxplots topped by the same letter indicate no significant difference based on Tukey's HSD test ($p \leq 0.05$). The ends of the box represent the upper (75th percentile) and lower (25th percentile) quartiles; the median is shown by a horizontal line inside the box (middle quartile); whiskers extend the interquartile range 1.5-fold from the upper and lower quartiles. Boxplots without post hoc test letters indicate no significant differences

Several previous studies found AM fungi favored legumes when in competition with grass or non-N₂-fixing forb species (Bahadur et al. 2019; van der Heijden et al. 1998; 2016). Plants with relatively greater mycorrhizal responsiveness typically show enhanced nutrient uptake, compared to less responsive species, often with a concomitant growth depression in neighbors (Zabinski et al. 2002). These belowground drivers can be enhanced further by aboveground competition, as *M. sativa* is characterized by substantial biomass production and an extensive canopy that might reduce light resources for neighboring species (Klabi et al. 2014).

In polycultures, rhizobium in combination with AM fungi enhanced growth of *M. sativa*, but also enhanced growth of *A. gerardii*. This positive effect of rhizobium on neighboring grasses and overall plant community productivity is likely due to increased nitrogen obtained with rhizobial symbioses. An increase of available N may satisfy the N-requirements of *M. sativa* and provide additional plant-available nitrogen to *A. gerardii*. In addition to symbiotic N₂-fixation in legumes, rhizobium is also capable of plant growth promotion in neighboring non-N₂-fixing grasses (Arora et al. 2001; Hayat et al. 2012). However, rhizobium had no effect in the growth of neighboring *R. pinnata* in our study. NonN₂-fixing herbaceous species have been reported to generally benefit from the presence of legumes to a lesser extent than grasses, presumably because of different capacities to utilize legume-derived N (Temperton et al. 2007). Differential capacities between plant species to utilize legumederived N may be due to different root system architecture (Bartelheimer et al. 2008). Both *M. sativa* and *A. gerardii*

have relatively deep rooting systems that may be favorable to root nutrient exchange, whereas the shallower rooting of *R. pinnata* may result in less rhizosphere contact and resource sharing. These physical differences may suggest potential N transfer from *M. sativa* to *A. gerardii* to be effective in our study. Furthermore, *R. pinnata* is the only species in our model communities that benefited from N-fertilization, with greater belowground biomass production compared to non-fertilized plants. Building on previous research, we suggest *R. pinnata* is relatively more competitive than our model grass species (*A. gerardii*) for plant-available, nonlegume derived soil N, likely due to its rapid early growth and relatively high N demand (Rothrock and Squiers 2003).

We observed pronounced increases in plant biomass following inoculation with AM fungi and rhizobium, especially following clipping and N-fertilization. As the dominant species in our model communities, *M. sativa* biomass increased further due to its high growth responses to AM fungi and reliance on rhizobium, supporting the idea that microbial symbioses can suppress plant diversity when dominant species are more dependent on mycorrhizas than subordinate species (Hartnett and Wilson 1999). In contrast, van der Heijden et al. (2016) demonstrated that AM fungi and rhizobium can promote plant diversity and seedling recruitment of legumes in microcosms that simulated European grasslands. Key plant community differences may explain these different experimental outcomes, as C₃ grasses that characteristically dominate in European systems typically show relatively weaker responses to AM fungi and rhizobium than C₄ grasses such as *A. gerardii*.

While clipping and inoculation with both AM fungi and rhizobium significantly increased aboveground biomass of all plant species in our polyculture microcosms, the proportional biomass of *A. gerardii* and *R. pinnata* increased to a greater extent following clipping than that of *M. sativa*. This does not support our hypothesis that legume competitiveness would increase in clipped communities. Clipping increased light availability for *A. gerardii* and *R. pinnata*, potentially reducing competitive exclusion by *M. sativa*, resulting in enhanced complementarity and coexistence in the polycultures (Borer et al. 2014; Zhou et al. 2019a). In addition, negative effects of clipping on belowground biomass of *M. sativa* indicate potential alterations in microbial carbon resources. We also observed

AM fungal root colonization of *A. gerardii* and *R. pinnata* were positively related to belowground biomass, in contrast to *M. sativa* in which root colonization was negatively related to belowground biomass.

We also hypothesized N-fertilization would result in significant alterations to plant community dynamics and productivity. However, we found little effects of N-fertilization on growth or community dynamics. We found evidence that N-fertilization reduced mycorrhizal growth response of *M. sativa*, potentially attenuating legume competitiveness in fertilized communities. Legume nodulation often decreases in response to N-fertilization. However, in our study, nodule development was not reduced when AM fungi also were present. Liu et al. (2021) reported that AM fungi benefited legumes following N-fertilization, resulting from AM fungi and other soil micro-organisms improved nutrient availability for the legume by stimulating activity of the enzyme responsible for soil organic matter mineralization. Generally, plants with high mycorrhizal growth responses can be effectively adapted for production in nutrient-deficient environments, given sufficient light-availability (sufficient carbon supply). We detected few negative effects of N-fertilization on aboveground *M. sativa* biomass and total plant community productively, and only when grown in polyculture and inoculated with only AM fungi, possibly because co-inoculated with AM fungi and rhizobium increased legume biomass and limited potential detrimental effects of N-fertilization on *M. sativa*. The lack of effects of N-fertilization on nodule production of *M. sativa* could be an artifact of our selection of relatively low levels of N fertilization applied as a single application at week 12 following aboveground biomass removal. Our results are consistent with those of Larimer et al. (2014); however, in that biotic interactions were more influential than abiotic nutrient additions for promoting legume biomass.

Degraded grasslands commonly are characterized by loss of perennial legume species, and perennial legumes, such as *M. sativa*, are typically difficult to re-establish in grassland restorations (Zhou et al. 2019a). Our results indicate the potential for managing microbial partnerships to facilitate legume productivity and competitiveness. This provides a foundation for further in situ research into grassland restoration via inoculation of legumes with beneficial microbial symbionts. Our results demonstrate greater interactive effects of AM fungi and rhizobium on the dynamics of plant coexistence and grassland productivity due to complementary roles in supplying likely limiting nutrients. Indeed, AM fungi and rhizobium altered plant community structure by favoring the legume over a neighboring C_4 grass and C_3 forb. Therefore, our work

demonstrates how microbes can drive competitive relationships between legumes and neighboring plant species, ultimately shaping grassland plant community structure.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1007/s00572-021-01061-2>.

Author contribution JZ, GW, AC, and YZ conceived the ideas and designed the methodology; JZ, GW, and AC collected the data; JZ and LL analyzed the data; JZ led the writing of the manuscript; GW, AC, XZ, and FS revised the manuscript for intellectual content; all the authors contributed to drafts and gave final approval for publication.

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◀ Fig.

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