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Arbuscular mycorrhizal interactions and nutrient supply mediate floral trait variation and pollinator visitation

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Summary

- Floral traits, including floral display and nutritional rewards from pollen and nectar, drive pollinator visitation. Even within a single plant species, environmental factors can influence the quality and quantity of floral resources. Yet, the ecological interactions driving this variation in floral resources, especially those belowground, remain unknown.
- Here, we investigate how soil microbial community composition and nutrient availability, specifically distinct arbuscular mycorrhizal fungi (AMF) species and phosphorus (P) supply, affect plant growth, AMF traits, floral traits, and how that, in turn, affects bee visitation.
- We found that increased AMF richness of functional diversity enhanced floral display (flower size and number) and rewards (nectar volume and pollen protein) and increased bee visitation. Using structural equation modeling, we found that AMF associations could boost bee visitation by enhancing flower size. However, trade-offs occur; flower size correlates negatively with root colonization but positively with hyphal length, suggesting that AMF traits drive the effects of AMF on flower growth.
- Overall, the effect of AMF on floral traits and bee visitation was not homogenous; instead, AMF trait differences interact with P supply, resulting in varying effects on floral traits and subsequently bee foraging dynamics. These results highlight that focusing on beneficial belowground interactions could provide an opportunity to bolster bee visitation.

Introduction

Floral traits, including floral display and nutritional rewards from pollen and nectar, drive bee visitation (Willmer, 2011; Bauer *et al.*, 2017; Roy *et al.*, 2017; Parachnowitsch *et al.*, 2019) and, in turn, greater bee visitation ensures successful pollination for plants (Willmer, 2011; Bauer *et al.*, 2017; Roy *et al.*, 2017; Parachnowitsch *et al.*, 2019). However, floral resources can vary widely in quality and quantity across environmental contexts (Brunet *et al.*, 2015; Goulnik *et al.*, 2020; Kuppler *et al.*, 2020). Therefore, it is imperative to characterize the ecological mechanisms that can enhance floral resources to increase bee visitation. Recent focus has shifted belowground to examine how microorganisms in the soil can improve plant performance, including floral resource production (Barber & Soper Gorden, 2015; Hyjazie & Sargent, 2024). However, there has been less attention to how functionally distinct microbial communities (and the associated trait variation) can directly or indirectly influence the relationship between floral resources and bee visitation. Here, using an experimental approach, we investigate how trait variation in microorganisms, specifically arbuscular mycorrhizal fungi (AMF) and their traits, affects floral resource production and how that, in turn, affects bee visitation. A secondary goal is to determine how differences in AMF ecological strategies and interactions with phosphorus (P) supply affect these characteristics.

AMF, which grow symbiotically in the roots of most vascular plants (Smith & Read, 2010), are known for often improving plant growth and fitness. In this symbiosis, plants provide carbon to AMF, and in exchange, AMF improve access to nutrients such as P and nitrogen (Smith & Read, 2010). Specifically, AMF acquire and transport nutrients via hyphal networks that extend from outside the root (extraradical hyphae) to inside the root (intraradical hyphae). Nutrients are ultimately transferred to the plant host through structures called arbuscules, attached to intraradical hyphae, that together colonize root cortical cells. In this way, AMF could ultimately influence floral resources by promoting greater uptake of nutrients critical for flower production, including for flower production (size and quantity) and nectar and pollen production (Barber & Soper Gorden, 2015; Hyjazie & Sargent, 2024). In fact, some evidence suggests that AMF can influence flower size and number (Gange & Smith, 2005; Wolfe *et al.*, 2005), flowering duration (Sun *et al.*, 2008), floral volatiles (Barber *et al.*, 2013b), nectar quality and quantity (Kaya *et al.*, 2003), pollen quality and quantity (Poulton *et al.*, 2001; Varga & Kytöviita, 2010; Pereyra *et al.*, 2019), and pollinator behavior (Barber *et al.*, 2013a), including the composition of the

pollinator visitors (Cahill *et al.*, 2008; Bennett & Cahill Jr., 2018).

Improvements to floral resources via AMF (Bennett & Meek, 2020) could increase visitation and pollination services because bees tend to prefer plants with larger and more abundant flowers and flowers with higher nutritional rewards (i.e. greater pollen and nectar quality and quantity; Bauer *et al.*, 2017; Willmer, 2011; Roy *et al.*, 2017; Parachnowitsch *et al.*, 2019). However, previous studies that examined the connection between AMF and floral traits and/or bee visitation have been restricted to experimental systems with only a single AMF taxon or an uncharacterized AMF community (Kaya *et al.*, 2003; Gange & Smith, 2005; Sun *et al.*, 2008; Varga & Kytöviita, 2010; Pereyra *et al.*, 2019); furthermore, few have examined the direct or indirect pathways that can exist between distinct AMF communities, floral traits, and bee visitation (but see Barber *et al.*, 2013a). Because AMF are not functionally homogeneous (Verbruggen & Kiers, 2010; Chagnon *et al.*, 2013; van der Heijden *et al.*, 2016), it is important to assess whether and how compositionally and functionally distinct AMF communities, such as differences in life-history strategies, alter floral resources and affect bee visitation.

In particular, morphological, physiological, and phenological traits can differ among and within AMF species (Kokkoris & Hart, 2019; Chaudhary *et al.*, 2022), which may indicate life-history strategies for AMF (e.g. trade-offs between the extent of root colonization and hyphal biomass production; Hart & Reader, 2002). In different environments, such as nutrient-rich or poor soils, variations in these AMF traits are thought to result in either a net relative cost or benefit to plants (Johnson, 2010; Johnson, 2013). For example, the hypothesized Grime's C-S-R framework for AMF communities (Chagnon *et al.*, 2013) aims to categorize AMF into three life-history strategies: competitor, stress-tolerator, and ruderal. In this framework, *competitor* AMF supersede other AMF at obtaining carbon from plant hosts by optimizing uptake and transfer of nutrients like P to its plant host, which requires greater investment in extradical hyphal production vs root colonization. *Stress-tolerant* AMF prevail in low-resource and stressful conditions (e.g. low carbon supply from the host) by reducing hyphal biomass production, which in turn provides limited nutrient transfer to its plant host in the short-term. *Ruderal* AMF occupy recently disturbed soils through rapid production of spores and reestablishment of hyphal networks and symbiotic interactions (i.e. root colonization), but this high biomass turnover rate may indicate low-resource use efficiency, ultimately resulting in a disadvantage to plants. Thus, variations in these AMF traits could ultimately impact interactions between plants and AMF and thus AMF function.

In this study, we determined how the composition and trait variation of AMF communities affect the relative benefit plants derive from the mycorrhizal associations, including the pathway from plant growth to floral resources to bee visitation, in low vs high P environments. To do this, we conducted a glasshouse experiment comparing how four synthetic AMF communities affected squash (*Cucurbita pepo*) growth and floral resources under two levels of P, and then observed how these changes to experimental plants affected bee behavior in a field setting. The four synthetic communities, which included three pairs of AMF species and a mixture of all six species, were created following the hypothesized Grime's C-S-R framework for AMF communities (Chagnon *et al.*, 2013) to capture trait variation among AMF species. Specifically, we examined three AMF life-history strategies: competitor, stress-tolerator, and ruderal. Importantly, although no conclusive C-S-R designation has been identified for individual AMF species, and it remains debated (Treseder, 2023), this framework offers a starting point to conduct experiments that interrogate how different AMF communities, including the AMF trait variation within these communities, affect floral traits and bee visitation.

First, we conducted 'treatment-trait correlations' to examine the effect of the treatment combinations (i.e. distinct AMF communities under different P environments) on the plant (i.e. shoot and root biomass), community-level AMF (i.e. root colonization, hyphal biomass, and spore production), and floral traits (i.e. flower number, flower size, pollen density and protein, and nectar volume and sugar) in addition to bee visitation. We predicted that more resource-competitive AMF would bolster P uptake for plants in both low and high P supply environments and, thus, plants that associate with competitor AMF would have improved plant growth, floral resource quantity, and quality, and ultimately greater bee visitation, compared with either stress-tolerant or ruderal AMF. Additionally, we expected that the effect of competitor AMF species on plant growth, floral resources, and bee visitation would be bolstered when included in a more functionally diverse AMF community. Specifically, a mixture of AMF species with distinct life-history strategies could result in synergistic interactions, positively affecting plant growth and floral resources. By contrast, we expected ruderal AMF would improve floral resources in low P but not in high P environments because high root colonization in high resource environments may result in a net negative effect on plants. When P is not limiting, investing in AMF may be a net carbon cost to plants (Johnson, 2010).

Next, we conducted 'trait–trait correlations' using a path analysis to test the direct and indirect pathways between community-level AMF traits and floral traits and their effect on bee visitation. We predicted that the variation in AMF traits would indirectly influence bee visitation via the effect of AMF on floral resource quantity and quality. Specifically, we expected that greater hyphal biomass relative to AMF root colonization would increase floral resource production. As a result, bee visitation (i.e. number of visits or duration) would respond positively to improvements in the quantity or quality of floral resources (e.g. increased flower size or pollen protein). Therefore, if AMF enhance plant nutrient acquisition and increase floral resource production, then the presence of AMF should ultimately support bee visitation. Overall, by determining how distinct AMF communities alter floral resources and ultimately drive bee visitation, we link belowground interactions to aboveground interactions while taking into account trait differentiation within AMF communities.

Materials and Methods

Study system

In this experiment *Cucurbita pepo* L. var. *cylindrica* (hereafter 'squash') was used to study the relationship between AMF functional groups (following Grime's C-S-R framework in Chagnon *et al.*, 2013) and low-/high P additions on bee visitation and pollination in a glasshouse and experimental field setting at the University of California, Berkeley (Berkeley, CA, USA) between June 29, 2019 and August 31, 2019. We used two nutrient levels (low vs high P supply) and four different synthetic AMF mixtures (competitor, stress-tolerant, and ruderal species plus a mixture of all four species) and a control, in a factorial design for a total of 10 treatment combinations with five replicates each (Fig. 1). Squash is a widely grown, monoecious annual plant, which produces flowers that are only viable for pollination 1 d from sunrise to midday. Squash forms associations with a diversity of AMF species (Smith & Read, 2010). Squash is pollinated by a wide range of bees, including generalist bees (e.g. honey bees, *Apis mellifera* L., and bumble bees, *Bombus* spp., and solitary bees such as Halictadae) and specialist bees (e.g. *Peponapis* sp.).

AMF inoculum

We chose two different AMF species per C-S-R group to create 4 different AMF inoculation mixtures plus a control (Fig. 1): (1) competitor species, *Gigaspora rosea* and *G. albida*; (2) stress-tolerant species, *Acaulospora morrawiae* and *A. spinosa*; (3) ruderal species, *Rhizophagus intraradices* and *Funneliformis mosseae*; (4) all CSR species (competitor, stress-tolerant, and ruderal); and (5) a no AMF species control with an autoclaved (twice 48 h apart at 121°C for 45 min) mixture of all species. AMF richness thus varied across the mixtures: richness of 2 for AMF mixtures 1–3 (competitor, stress-tolerant, ruderal), 6 for mixture 4 (CSR), and effectively 0 for mixture 5 (control), in which inoculum was autoclaved. AMF inoculum was acquired from INVAM (West Virginia University, Morgantown, WV, USA), which prepares the inoculum from roots, spores, hyphae, and the original growth medium. We used 30 g of each of the two species in AMF inoculation mixtures 1–3 (competitor, stress-tolerant, R) and 10 g of each of the six species in mixtures 4–5 (CSR and control) for a total of 60 g of inoculum in each mixture for each pot. At planting, half of the inoculum (30 g) was mixed into the sand–clay mix and the other half (30 g) was put directly into the planting hole, where the seeds were placed, for a total of 60 g of inoculum.

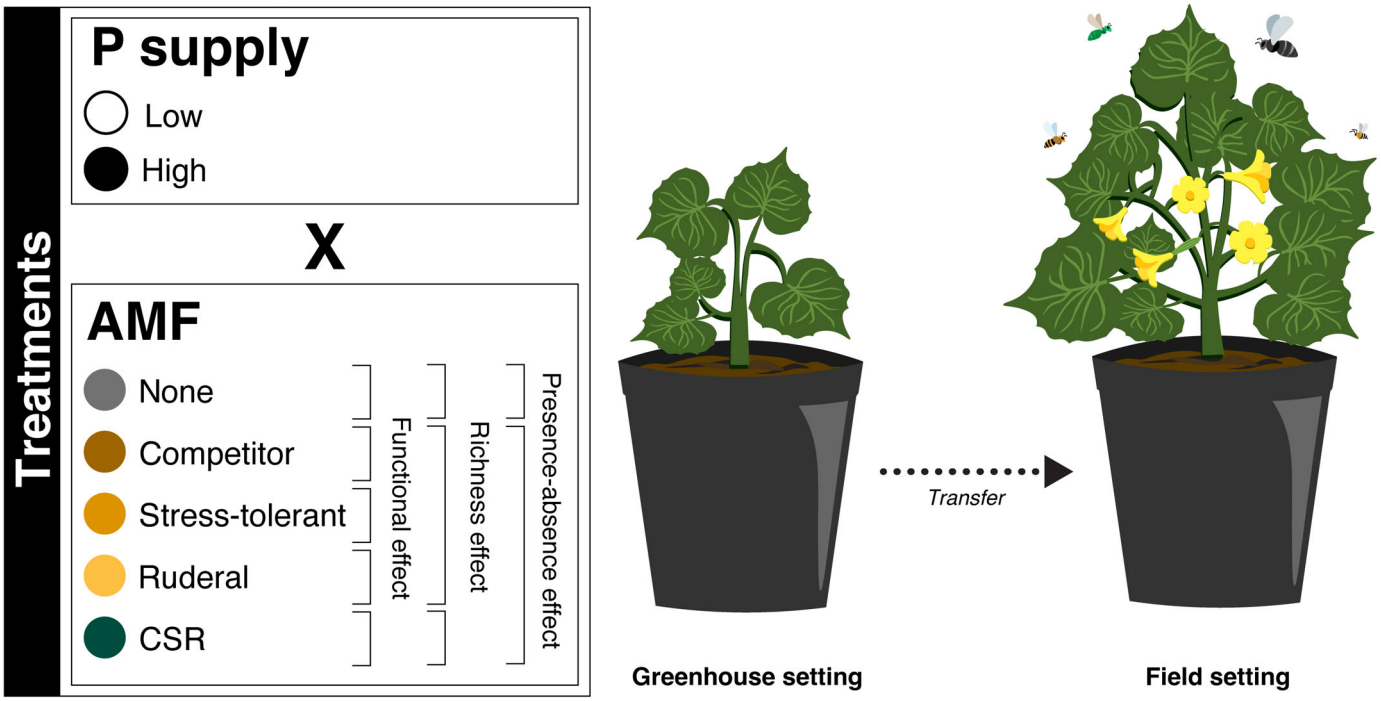

Fig. 1 Cross-factorial experimental design included two treatments: (1) phosphorous (P) supply with low and high levels and (2) arbuscular mycorrhizal fungi (AMF) inoculation with no AMF species control ('none') and four AMF mixtures: (1) competitor species, *Gigaspora rosea* and *G. albida*; (2) stress-tolerant species, *Acaulospora morrawiae* and *A. spinosa*; (3) ruderal species, *Rhizophagus intraradices* and *Funneliformis mosseae*; (4) all CSR (competitor, stress-tolerant, and ruderal) species; and (5) a no AMF species control with an mixture of all species. Each treatment combination had five replicates. Potted plants (squash, *Cucurbita pepo* var. *cylindrica*) were inoculated with the different AMF mixtures and grown in a glasshouse setting. When flowers emerged, the plants were taken to a field setting for floral trait and bee visitation measurements.

Experimental conditions

On 29 June 2019, we planted 3 squash seeds (variety 'Black Beauty' zucchini; Baker Creek Heirloom Seed Co., Mansfield, MO, USA) in 5.4-l nursery pots filled with 5 kg of 2 : 1 (v/v) growing medium mix of silica sand and a calcinated, attapulgite clay soil conditioner (Agsorb 5/20 LVM-G, Chicago, IL, USA) modified from (Hodge *et al.*, 2001; Thirkell *et al.*, 2016), hereafter, 'sand–clay mix', and 60 g of AMF inoculum to a final bulk density of 0.923 g cm^{-3}. The sand–clay mix was autoclaved twice 48 h apart at 121°C for 45 min to ensure a sterile growing medium. Drainage holes (9-2 cm^2 circular holes) in pots were covered with 20 µm mesh to prevent roots from growing out while still allowing water to drain. Seeds were surface sterilized using a 10% bleach solution and then rinsed with deionized water.

On 5 July 2019, seedlings were thinned to a single seedling per pot. Pots were routinely rearranged in a random order in rows that were 1 m apart in a glasshouse at *c.* 27°C with a 14 h photoperiod with supplemental lighting (Oxford Tract, UC Berkeley, Berkeley, CA, USA). On 9 August 2019, after at least one flower had emerged for each plant, all plants were transferred to a nearby field setting for bee observations and floral resource measurements (Oxford Tract; UC Berkeley). The field is adjacent to an urban garden which supplies diverse floral resources attracting a diverse group of bees (Wojcik *et al.*, 2008). Pots were placed on the ground and were randomly arranged in rows that were 1 m apart.

Water and nutrient supply

To determine water holding capacity (WHC), a 5.4-l pot was filled with 5 kg of sand–clay mixture, the same amount at the same bulk density used in experimental pots, and then saturated with water and allowed to drain for 48 h; then, the gravimetric water content (GWC) was measured. The GWC of the sand–clay mix at WHC capacity was 17%. Using this information, pots were weighed and watered every other day to maintain WHC with deionized water for the duration of the experiment.

To supply nutrients, we used a modified Long Ashton solution, following Rouphael & Colla (2009), consisting of N (16.0 mM), P (1.5 mM), K (5.5 mM), S (3.5 mM), and Ca (7.0 mM) for the 'high' P supply treatment. For the 'low' P supply treatment, we used one-tenth the concentration of P (0.15 mM) and the same concentrations of the other macronutrients and micronutrients in the 'high' P solution following Valentine *et al.* (2001). The nutrient solution (200 ml) was applied at planting and, thereafter, once every 4 d with watering events.

Floral traits

Between 9 and 19 August 2019, floral trait measurements were taken every day in the field setting. One day before sampling plants, flowers were covered using insect exclusion bags made from a woven polyester fabric to prevent insects from collecting nectar or pollen. Not all plants produce flowers each day. Floral traits per plant were measured as: (1) floral display (flower size and number); (2) nectar resources (volume and sugar concentration); and (3) pollen resources (volume and protein concentration). Flower size refers to the average length of the petals to the base of the flower. Nectar volume was measured using calibrated microcapillary tubes, and sucrose concentration was measured using a refractometer (Eclipse Handheld Refractometer; Bellingham & Stanley Ltd, Tunbridge Wells, UK). For pollen measurements, anthers were collected and frozen at −20°C for later processing. A 1 mg subsample of pollen was used to determine pollen protein concentration using a Bradford Assay following (Vaudo *et al.*, 2016). The remaining sample was suspended in 1 ml 50–50 glycerol water, and a 10 µl aliquot was mounted on a slide to determine the relative density of pollen grains (pollen density) by counting the total number of pollen grains.

Pollinator survey

We surveyed bees for 7 d from August 23 to 30 for a total of 24.5 person-hours of observations. All surveys were performed from 8:30 h to 12:00 h when bees were most active at the site and before flowers closed. We followed individual bees within the experimental plot and used handheld digital voice recorders to flower visitation, measured as the number of flowers visited and time spent per flower in seconds, only if bees probed the stamen, pistil, or nectary following Barber *et al.* (2013a). Since our methods relied on following individual pollinators, our observations only consisted of bees, which were the most actively mobile pollinators at the experimental plot at the time of observations. Bees were identified as honey bees (*Apis mellifera*), squash bees (*Peponapis* spp. and *Xenoglossa* spp.), or within six other flower visitor categories used in observational surveys of flower visitors in this region (Supporting Information Table S1; Kremen *et al.*, 2011); all identified bees are known pollinators of squash. Individual bees were followed as long as possible or until they left the plot. We calculated the number of bee visits as the number of flower visits per day on each plant and bee visitation time as the total time spent by bee per day on each plant.

Plant growth traits

At the end of the pollinator survey, plants were destructively harvested to determine shoot and root biomass. Shoots were cut at the surface of the sand–clay mixture. The root structure was carefully removed from the sand–clay mixture, and any adhered sand and clay particles were rinsed off the roots in dH_2O. All plant material was dried at 60° competitor, and shoot dry weights and root dry weights were determined. The remaining sand–clay mixture was stored at 4°C for extradical hyphal length measurements, and a subsample of the roots was taken before drying for root colonization measurements.

AMF traits

Root colonization We determined root colonization by counting AMF composition in stained roots. Roots were cleared in

10% KOH, acidified in 1% HCl, and stained with trypan blue (Koske & Gemma, 1989). Percent colonization by AMF was determined using the intersections method at 200× magnification (McGonigle *et al.*, 1990). AMF colonization in this study refers to percent root colonization by arbuscules, vesicles, or hyphae over the total intersections counted (*c.* 100 intersections per sample).

Hyphal length As a proxy for AMF hyphal biomass, the total length of extraradical hyphae was measured on extracted hyphae using the membrane filter technique modified after Hanssen *et al.* (1974). Briefly, two 5 g samples of sand–clay mixture from each pot were suspended in 15 ml of dH2O and 20 ml of sodium hexametaphosphate (35%) and stirred overnight. The soil suspension was then sieved through a 32 μm sieve and resuspended with 100 ml dH2O. Next, 10 ml of the suspension was filtered onto a 0.47 μm nitrocellulose filter paper (gridded, 25 mm diameter), which was then stained with trypan blue (Koske & Gemma, 1989). The filters were placed on slides with 50–50 glycerol water. Hyphal length (H) on the slide was calculated using the equation $H = (I\pi A)/(2\ l)$, where I is the average number of intersections per grid, A is the grid area, and L is the total length of the grid lines. Then, the total length of fungal hyphae (F) in each pot (mg^{-1} of sand–clay mixture) was estimated using the equation $F = H \times 10^{-6}(A/B)\ (1/S)$, where A is the area of the filter, B is the grid area, and S is the amount of soil filtered (Bloem *et al.*, 1995).

Ratio of root colonization to hyphal length To account for root colonization vs the production of hyphae, we calculated the ratio of percent root colonization to hyphal length (root colonization : hyphal length) for each pot.

Spore count The number of spores was measured using the sucrose density gradient centrifugation method following Brundrett *et al.* (1996). First, we blended 100 g of the sand–clay mixture with 200 ml of deionized water for 30 s at high speed using a blender. The blended material was poured through a 32 μm and 500 μm sieve. The contents of the 500 μm sieve were transferred to a 50 ml centrifuge tube with a 20–60% sucrose gradient and centrifuged at 960×***g*** for 3 min. The supernatant was decanted into a 32 μm sieve, and the contents were transferred to a gridded Petri dish with 20 ml deionized water. The total number of spores was then counted under the microscope.

Statistical analyses

We first examined the effect of the treatment combinations on the plant, AMF, and floral traits in addition to bee visitation ('treatment-trait models'). Then, using a path analysis, we tested the direct and indirect pathways between AMF traits and floral traits and their effect on bee visitation ('trait–trait models').

Treatment-trait models We tested the effect of AMF inoculation, P addition (low and high P supply), and their interaction on the multiple plants, floral, AMF traits measured, and bee visitation. Bee visitation was modeled for all bee groups (e.g. honey bees and other wild bees) combined because there was insufficient data for each bee group to model them separately (Table S1). All models had the same model structure: AMF inoculation treatment, P supply treatment, and their interaction as the fixed effects. We used generalized linear models (GLM) for all treatment-trait tests except for floral traits and bee visitation; these variables were measured on individual plants over multiple days and, thus, we used generalized linear mixed models (GLMM), with individual plant identity and date as random effects to account for the variation between sampling dates (Bates *et al.*, 2014; Kuznetsova *et al.*, 2017). Models were constructed using LME4 and LMERTEST packages in R. Root colonization models assumed a binomial error distribution, and models with count data (i.e. spore count, number of flowers, pollen density, and number of bee visits) assumed a Poisson error distribution. All other models assumed a Gaussian error distribution. To determine the significance of the fixed effects, we used an F-test for models with continuous variables and a likelihood ratio test for models with count data. Type II sums of squares were used for each test (Langsrud, 2003). Degrees of freedom were calculated using the Kenward & Roger (1997) method.

While our experiment focuses on the 'functional' effect of the AMF inoculation (competitor, stress-tolerant, ruderal, and the combined CSR species, plus the control; model AMF_{CSR}), we also tested whether there was a 'richness' effect (model $AMF_{richness}$) or 'presence–absence' effect of AMF inoculation (model AMF_{pa}) on AMF traits, floral traits, and bee visitation. For these models, we ran the same GLM or GLMM (with the same fixed/random effects structure) for each variable with the levels of AMF inoculation treatment effect regrouped as follows (Table 1): (a) AMF richness of 0 sp. (none) vs 2 sp. (competitor + stress-tolerant + ruderal) vs 6 sp. (CSR) for $AMF_{richness}$ model; (b) and presence (none) vs absence (competitor + stress-tolerant + ruderal + CSR) of AMF inoculum for the AMF_{pa} model.

Trait–trait models Next, we determined the trait–trait relationship between AMF traits (hyphal length, root colonization, and root colonization : hyphal length) and floral traits (flower number, flower size, pollen density and protein, and nectar volume and sugar) on bee visitation (number of bee visits and bee visitation time) using a piecewise structural equation model (PSEM, or path analysis). In contrast to the traditional structural equation modeling (SEM) method, piecewise SEM provides an important advantage as it permits the analysis of data with non-normal error distributions, such as bee visitation count data (Lefcheck, 2016). For both bee visitation response variables, we constructed the same *a priori* model, considering all possible mechanisms whereby AMF traits and floral traits influence bee visitation. We simplified the initial models by eliminating nonsignificant pathways before developing the final models. Model adequacy was determined using the chi-squared test and AIC. Because the

Table 1 Statistics for treatment-trait models that examined the effect of arbuscular mycorrhizal fungi (AMF) at different levels: AMF_{CSR} tested the functional effect of the different AMF functional group mixtures (none, competitor, stress-tolerant, ruderal, and CSR (competitor, stress-tolerant, and ruderal) mixture), $AMF_{richness}$ tested the richness effect of AMF inoculation (richness of 0, 2, or 6 AMF species), and AMF_{pa} tested the presence–absence effect of AMF inoculation.

				AMF functional groups				AMF richness				AMF presence–absence			
		P supply		AMF_{CSR}		AMF_{CSR} × P supply		$AMF_{richness}$		$AMF_{richness}$ × P supply		AMF_{pa}		AMF_{pa} × P supply	
		Statistic	*P*	Statistic	*P*	Statistic	*P*	Statistic	*P*	Statistic	*P*	Statistic	*P*	Statistic	*P*
Plant traits	Shoot (g)	**61.322**	**<0.001*** **	**3.030**	**0.028** *	0.523	0.719	**6.269**	**0.004** **	0.971	0.387	**12.767**	**<0.001*** **	0.877	0.354
	Root (g)	**11.341**	**0.002****	1.099	0.370	0.511	0.728	1.395	0.259	0.546	0.583	0.000	0.987	0.639	0.428
	Root : shoot	1.354	0.251	1.148	0.348	0.708	0.591	1.997	0.148	1.096	0.343	3.894	0.054	0.252	0.618
AMF traits	Hyphal length (m g^{-1})	**39.383**	**<0.001*** **	**28.215**	**<0.001*** **	**14.518**	**<0.001*** **	**3.601**	**0.036***	1.221	0.305	**7.164**	**0.010***	2.494	0.121
	% root colonization	**349.258**	**<0.001*** **	**256.065**	**<0.001*** **	**13.183**	**0.010***	**201.545**	**<0.001*** **	**9.994**	**0.007****	**6.732**	**0.009****	0.001	0.971
	Root colonization : hyphae	**34.668**	**<0.001*** **	**25.482**	**<0.001*** **	**11.060**	**<0.001*** **	**24.875**	**<0.001*** **	**11.827**	**<0.001*** **	**6.855**	**0.012***	3.210	0.080
	Spore count (grains ml^{-1})	3.201	0.081	**2.946**	**0.032***	**4.476**	**0.004****	1.337	0.273	**3.407**	**0.042***	1.830	0.183	**6.343**	**0.015***
Floral traits	Flower size (cm)	3.397	0.073	**2.638**	**0.048***	1.955	0.121	**4.395**	**0.018***	3.009	0.060	**7.652**	**0.008****	3.015	0.089
	Number of flowers	**9.547**	**0.002****	**16.967**	**0.002****	2.666	0.615	**8.093**	**0.017***	1.224	0.542	**5.197**	**0.023***	1.167	0.280
	Nectar volume (μl)	**9.215**	**0.004****	**3.635**	**0.014***	0.626	0.647	0.855	0.432	0.353	0.704	0.962	0.333	0.706	0.406
	Nectar sugar (% brix)	0.883	0.354	2.633	0.051	0.977	0.432	2.412	0.102	0.171	0.844	2.086	0.156	0.359	0.552
	Pollen density (grains μl^{-1})	0.493	0.483	8.701	0.069	**11.100**	**0.025***	1.687	0.430	1.031	0.597	1.541	0.215	0.771	0.380
	Pollen protein (mg l^{-1})	1.078	0.306	**4.853**	**0.003****	1.176	0.336	2.700	0.078	0.889	0.419	2.507	0.120	0.327	0.570
Pollinator visitation	Number of pollinator visits	2.318	0.128	7.981	0.092	1.750	0.782	**7.288**	**0.026***	0.005	0.997	**4.163**	**0.041***	0.000	0.995
	Pollinator visitation time (s)	0.839	0.366	2.114	0.100	0.221	0.925	**3.695**	**0.033***	0.463	0.632	3.550	0.067	0.894	0.350

The test statistic corresponds with the *F*-value (for continuous variables) and chi-squared (for count data). Bolded text represents significant values. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$.
The test statistic corresponds with the *F*-value (for continuous variables) and chi-squared (for count data) for *F*-test or likelihood ratio test, respecitively, of generalized linear model or generalized linear mixed model.

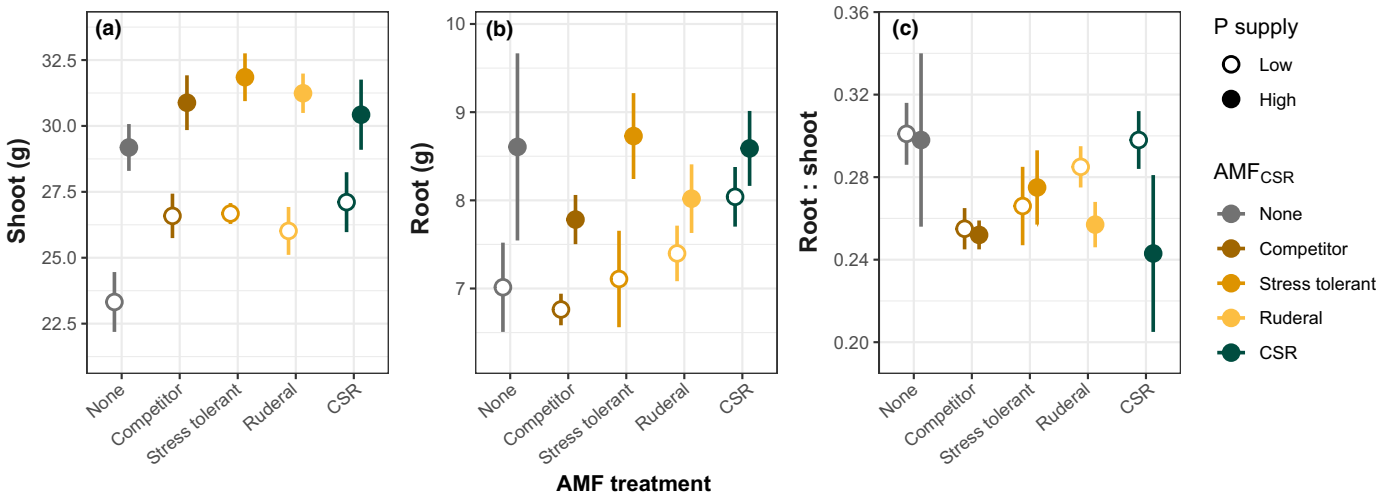

Fig. 2 Mean ± SE of plant traits for AMF_{CSR} functional groups (none, competitor, stress-tolerant, ruderal, and CSR (competitor, stress-tolerant, and ruderal) mixture) between low (open) and high (filled) P supply: (a) dry shoot biomass (g), (b) dry root biomass (g), and (c) ratio of dry biomass of shoot (g) to root (g).

AMF traits were measured once per individual plant, but floral traits and bee visitation were measured across multiple days (but not overlapping days), we averaged all floral traits for each individual plant and summed bee visits across days. We accounted for the number of observation days (log-transformed) for bee visitation in the model using the offset function. Structural equation modeling was conducted with the R package PSEM (Lefcheck, 2016).

In all GLM, GLMM, and PSEM models, we used Gaussian and Poisson error distributions, respectively, for continuous and count variables. We performed all statistical analyses in R v.4.4.1 (R Core Team, 2024).

Results

Plant growth traits

Shoot biomass varied significantly between AMF_{CSR} functional groups ($F = 3.03$, $P = 0.03$; Fig. 2; Table 1). Plants inoculated with stress-tolerant AMF had 11% greater shoot biomass on average than the control (Table S2). The AMF_{CSR} inoculation treatment had no effect on root biomass and root-to-shoot biomass (Fig. 2; Table 1).

The richness and presence–absence of AMF inoculation also had a significant effect on shoot biomass (Table 1), with the largest shoot biomass when inoculated with the richest AMF inoculum (Table S2). Root biomass and root-to-shoot biomass did not significantly vary between the richness and presence–absence levels (Table 1).

P supply had a strong effect on shoot ($F = 61.32$, $P < 0.001$) and root biomass ($F = 11.34$, $P < 0.05$) but not root-to-shoot biomass (Fig. 2; Table 1). Specifically, in the high P supply treatment, shoot biomass was 18% greater on average, and root biomass was 15% greater on average (Table S2). Across all AMF_{CSR}, $AMF_{richness}$, and $AMF_{p\text{-}a}$ models, there was no interactive effect of P supply and AMF treatments on the plant traits measured.

AMF traits

There was a strong effect of the AMF_{CSR} inoculation treatment on all AMF traits (hyphal length (m g^{-1}): $F = 28.22$, $P < 0.001$; root colonization (%): $F = 256.07$, $P < 0.001$; root colonization : hyphae: $F = 25.48$, $P < 0.001$; spore count (grains ml^{-1}): $F = 2.95$. $P = 0.03$; Table 1; Fig. 3). For example, hyphal length was 300% higher than the control in pots with ruderal type AMF inoculum, followed by CSR, stress-tolerant, and ruderal (Fig. 3a; Table S3). We observed a similar trend for root colonization and the ratio of root colonization to hyphae, where plants/pots inoculated with CSR and ruderal type AMF inoculum had the highest values, followed by stress-tolerant and competitor type AMF inoculum. By contrast, spore production was highest for plants inoculated with CSR-type AMF inoculum, followed by ruderal stress-tolerant, and competitor type AMF inoculum, but root colonization for plants inoculated with ruderal type inoculum. There was also a richness and presence–absence effect of AMF inoculation on all AMF traits.

AMF_{CSR} functional groups and P supply also had an interactive effect on all AMF traits (hyphal length (m g^{-1}): $F = 14.52$, $P < 0.001$; root colonization (%): $F = 13.18$, $P = 0.01$; root colonization : hyphae: $F = 11.10$, $P < 0.001$; spore count (grains ml^{-1}): $F = 4.48$, $P < 0.01$; Table 1; Fig. 3). While hyphal length was larger in AMF groups stress-tolerant, ruderal and CSR with low P supply, in competitor type AMF groups, hyphal length was larger with high P supply. Root colonization was substantially greater on average (*c.* 99% more; Table S4) in pots with low P supply with the highest levels observed in ruderal and CSR-type AMF mixtures, but root colonization was generally low for plants that received high P supply regardless of AMF_{CSR} inoculation treatment (Fig. 3b). Similarly, the ratio of root colonization to hyphal production was greatest in pots with low P supply and was the highest in pots inoculated with the CSR-type AMF (Fig. 3c). Spore production was higher in AMF inoculated pots, with stress-tolerant

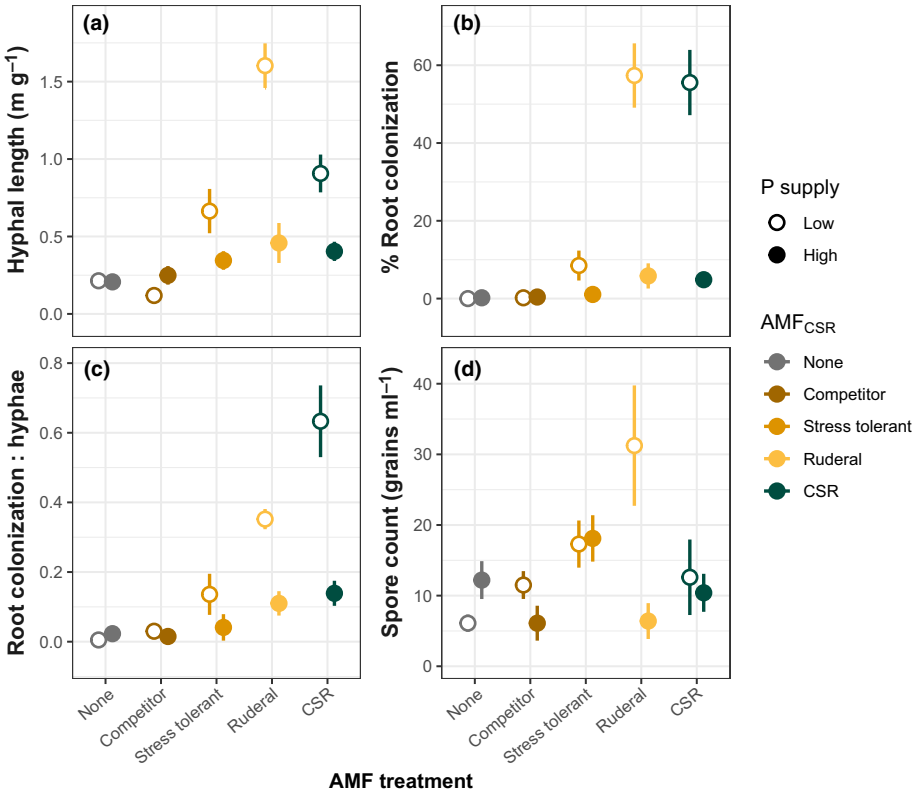

Fig. 3 Mean ± SE of arbuscular mycorrhizal fungi (AMF) traits for AMF_{CSR} functional groups (none, competitor, stress-tolerant, ruderal, and CSR (competitor, stress-tolerant, and ruderal) mixture) between low and high phosphorus (P) supply: (a) hyphal length (m g^{-1}) (b) % root colonization, (c) root colonization : hyphal length, and (d) spore count (grains ml^{-1}).

and ruderal type AMF producing the most spores (Fig. 3d). We observed low background levels of spores and hyphae in control pots, likely due to the autoclaved AMF inoculum containing residual spores and hyphae, yet there was virtually no AMF colonization in control pots (i.e. only one plant with 1% root colonization; Fig. 3).

In the $AMF_{richness}$ models, a significant interactive effect of AMF inoculation and P supply was present for all AMF traits (root colonization (%): $F = 3.60$, $P = 0.04$; root colonization : hyphae: $F = 201.55$, $P < 0.001$; spore count (grains ml^{-1}): $F = 3.41$, $P = 0.04$) except hyphal length production. By contrast, for $AMF_{p\text{-}a}$ models, a significant interactive effect of AMF inoculation and P supply was only present for spore production ($F = 6.34$, $P = 0.02$).

P supply alone had a significant effect on hyphal production ($F = 39.38$, $P < 0.001$), root colonization ($F = 349.26$, $P < 0.001$), and the ratio of root colonization to hyphal production ($F = 34.67$, $P < 0.001$). Plants grown with a low P supply produced 145% more hyphae on average and had a higher ratio of root colonization to hyphal production than those that received a high P supply.

Floral traits

AMF_{CSR} inoculation treatments significantly affected the flower size ($F = 2.64$, $P = 0.05$), total number of flowers ($F = 16.97$, $P < 0.01$), nectar sugar nectar volume ($F = 3.64$, $P = 0.01$), and pollen protein ($F = 4.85$, $P < 0.01$). Plants inoculated with stress-tolerant AMF had 13% higher nectar sugar concentration than the control (Fig. 4; Table S4). By contrast, plants inoculated with ruderal and CSR types had greater nectar volume (up to 318% more nectar than the control on average; Table S2). For pollen protein, plants inoculated with competitor and CSR types had up to 21% greater pollen protein than the control on average (Table S2). The interaction between AMF_{CSR} and P supply – not AMF_{CSR} alone – significantly affected spore density ($F = 11.10$, $P = 0.03$; Table 1).

Among the traits that significantly varied among AMF_{CSR} functional groups, only variation in flower size ($AMF_{richness}$: $F = 4.40$, $P = 0.02$; AMF_{pa}: $F = 7.65$, $P < 0.01$) and the number of total flowers ($AMF_{richness}$: $F = 8.09$, $P = 0.02$; AMF_{pa}: $F = 5.20$, $P = 0.02$) could also be explained by the richness and presence–absence of AMF inoculation (Table 1). In general, plants grown with AMF (Fig. 4b; Table S4) had *c.* 29% more flowers on average (Table S4), while plants inoculated with the richest assemblage of AMF (i.e. CSR type) had the largest number of flowers (Fig. 3b).

The P supply treatment also had a strong effect on nectar volume ($F = 9.22$, $P < 0.01$), with plants that received a higher supply of P producing a greater amount of nectar (Table 1; Fig. 4). We observed a similar effect of P supply on the number of flowers ($F = 9.55$, $P < 0.01$), with an average of *c.* 27% more flowers on plants grown with high P supply. Across all AMF_{CSR}, $AMF_{richness}$, and $AMF_{p\text{-}a}$ models, there was no interactive effect of AMF inoculation and P supply on floral traits.

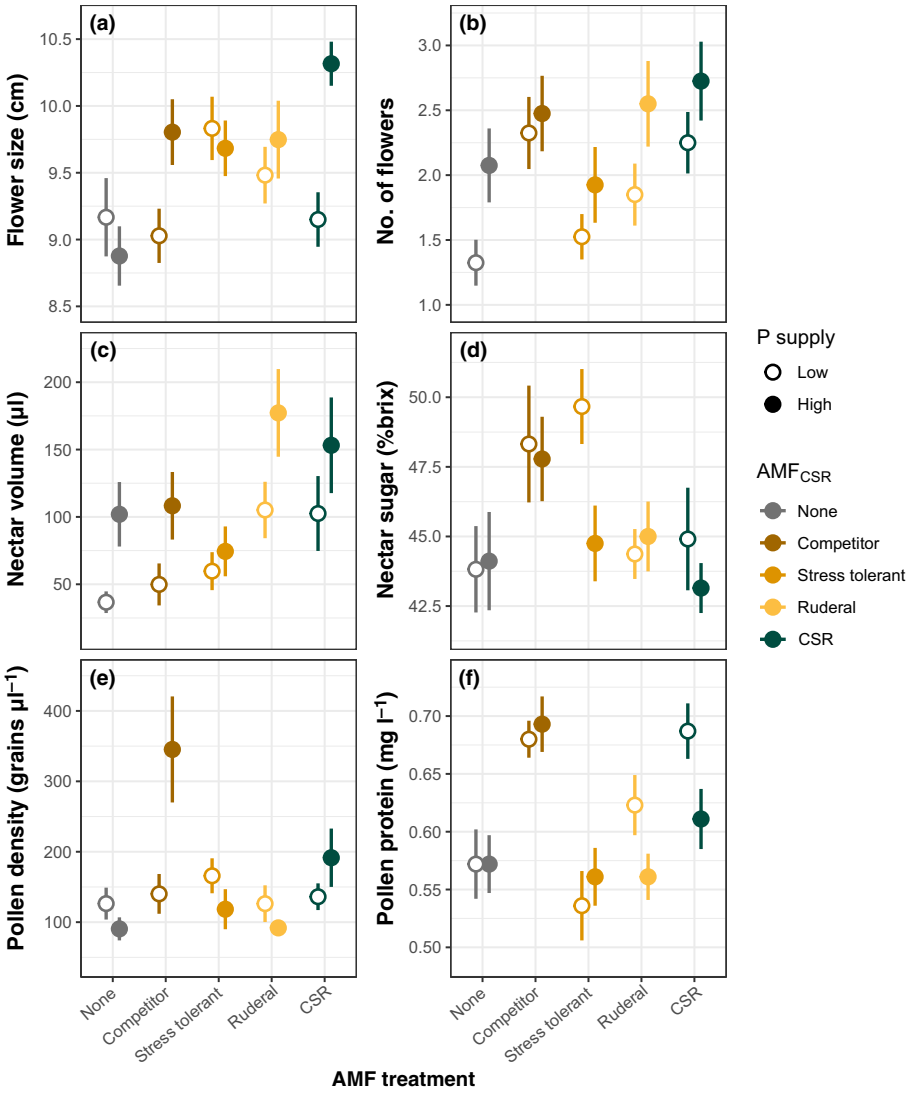

Fig. 4 Mean ± SE of floral traits for AMF$_{CSR}$ functional groups (none, competitor, stress-tolerant, ruderal, and CSR (competitor, stress-tolerant, and ruderal) mixture) between low and high phosphorus (P) supply: (a) flower size (cm), (b) number of flowers, (c) nectar volume (µl), (d) nectar sugar concentration (%brix), (e) pollen density (grains µl^{-1}), and (f) pollen protein concentration (mg L^{-1}).

Bee visitation

AMF functional types (i.e. AMF$_{CSR}$) did not affect bee visitation and the number of bee visits. However, both AMF$_{richness}$ had an effect on the number of bee visits (number of bee visits: $F = 7.29$, $P = 0.03$; bee visitation time: $F = 3.70$, $P = 0.03$), but only AMF$_{p\text{-}a}$ had an effect on the number of bee visits ($F = 4.16$, $P = 0.04$). Plants grown with AMF inoculum received 28% more bee visits and 47% more bee visitation time (Table S5). Plants inoculated with six AMF species (i.e. representing all functional groups) received the highest bee visitation time was highest (Fig. 5). P supply did not affect bee visitation or number of bee visits.

Effect of belowground and aboveground traits on bee visitation

PSEM revealed a direct link between the number of bee visits and flower size and further revealed that flower size was associated negatively with AMF root colonization and positively with hyphal length (Fig. 6). No significant pathways to bee visitation time emerged in the PSEM.

Discussion

In this study, we demonstrate that belowground interactions between a plant and AMF impact floral traits, which in turn affect bee foraging dynamics on that plant. In general, we observed positive effects of AMF not only on plant growth but also on floral traits, such as display size and floral resource quantity and quality, and in turn, on bee visitation. Importantly, however, the effect of AMF on some floral traits varied between compositionally distinct AMF inoculation mixtures. Bee visitation was also highest for plants inoculated with the richest assemblage of AMF species, which included AMF representing different life-history strategies. Because our experimental design included a range of distinct AMF communities, we were able to examine the wide expression of AMF traits. Yet, our design did not distinguish the effects of AMF functional diversity from species richness (as the CSR mixture had 6 AMF species whereas the

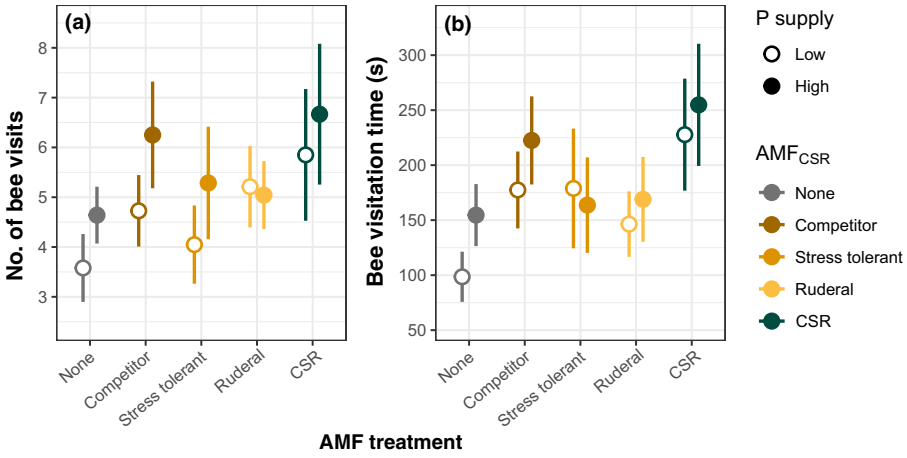

Fig. 5 Mean ± SE of pollinator visitation for AMF_{CSR} functional groups (none, competitor, stress-tolerant, ruderal, and CSR (competitor, stress-tolerant, and ruderal) mixture) between low and high phosphorous (P) supply: (a) pollinator visitation time (s) and (b) number of pollinator visits.

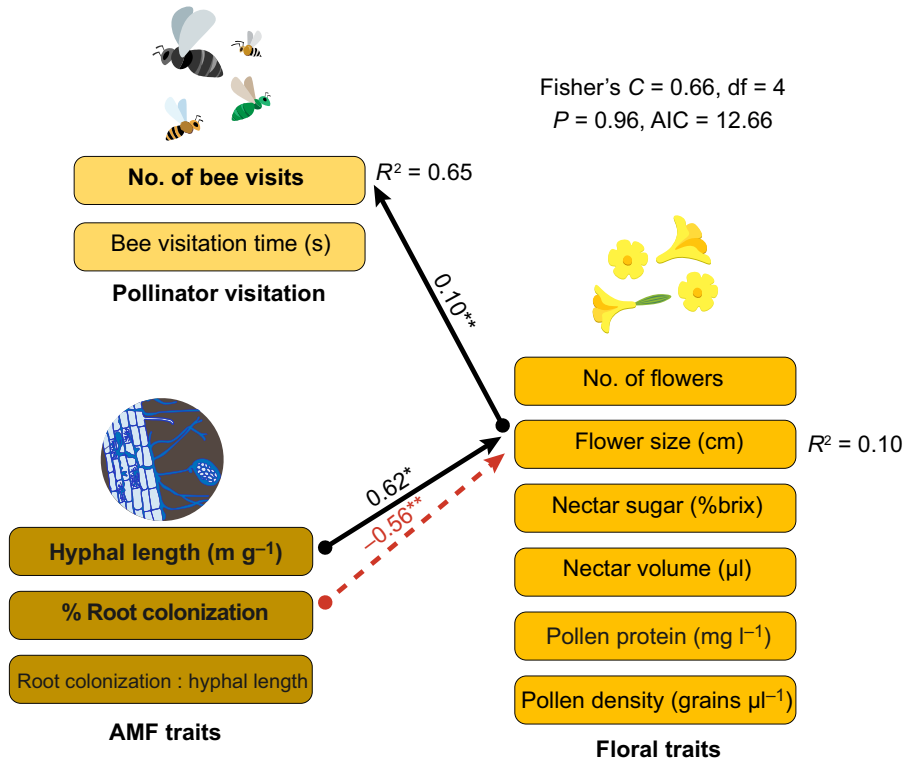

Fig. 6 Structural equation models consider the direct and indirect pathways via which arbuscular mycorrhizal fungi (AMF) and floral traits impact bee visitation. Black (solid) and red (dashed) arrows indicate significant positive and negative pathways, respectively (*, $P < 0.05$; **, $P < 0.01$). Numbers along the arrows indicate standardized path coefficients. R^2 represents the proportion of variance explained for each dependent variable. The global goodness-of-fit is represented by Fisher's $C = 0.66$, and $P > 0.5$ also indicates the model is well-fit.

competitor, stress-tolerant, or ruderal mixtures each had only 2 AMF species), and in some cases, the effects of AMF inoculation varied across both richness and functional diversity of AMF. Nevertheless, our results demonstrated that AMF traits varied among the different AMF communities and how this variation was ultimately linked to floral traits and bee visitation.

AMF traits (i.e. spore production, hyphal length, and root colonization) strongly varied between the AMF inoculation mixtures and interactively with the P supply treatment, suggesting different ecological strategies among the AMF mixtures. For example, the ruderal AMF group had the highest root colonization and production of spores and hyphae, especially in low P supply conditions. This follows the CSR framework, which indicates ruderal species will flourish in high-disturbance environments by growing quickly (i.e. production of spores and hyphae) and establishing symbiotic associations via root colonization. Surprisingly, the competitor AMF species group had the lowest values across all the AMF traits measured – even lower than the stress-tolerant AMF species group, which is expected to grow slower than the competitor or ruderal species. While individual AMF species do not have conclusive CSR designations, the strong differences in AMF traits between the AMF inoculation mixtures and P supply treatment signals that the AMF inoculation mixtures in our study represent functionally distinct ecological strategies with important implications for the plant host.

While plant growth responded positively to AMF inoculation, plant growth varied minimally between the AMF functional groups – regardless of the differences in AMF traits (i.e. root colonization, hyphal biomass, and spore production) between the

AMF functional groups. Instead, P supply had a stronger impact on plant growth. Plant growth was greater (i.e. greater shoot biomass) when P supply was high (Fig. 2). In this case, our application of the CSR framework for AMF was minimally predictive of the variations among plant traits, contrasting previous studies examining the effect of AMF functional differences on plant growth (Smith *et al.*, 2004); instead, we found that the CSR framework was more predictive for floral traits (Table 1).

Specifically, our study shows that the effect of AMF on the quantity and quality of individual floral resources depends on the identity or composition of AMF. A key pattern observed was that no singular AMF inoculum mixture held the highest value for all floral traits (Fig. 4; Table S2), which conflicted with our expectations that all floral resources would be most enhanced by the CSR mixture. Instead, our prediction that floral resources could be bolstered by more functionally diverse AMF communities (i.e. an additive effect of the richer CSR mixture) was only observed for some floral traits in our study (e.g. plants inoculated with the CSR mixture had the largest flower size on average; Fig. 4a). On one occasion, we observed that antagonistic effects may result from a functionally diverse AMF community (e.g. plants inoculated with the CSR mixture with the lowest nectar sugar). Overall, these results indicate that the effect of AMF inoculation on individual floral resources is not equal across distinct AMF communities and, thus, emphasize the important role of AMF identity in mediating aboveground processes. Importantly, these results show that at the whole plant level, responses to AMF functional differences may be obscured, whereas, upon closer inspection of plant structures, such as floral traits, they may come to light.

In our study, we suspect that the overall benefit of AMF to floral traits is also due to the increased transfer efficiency of P by competitor AMF. P is a necessary nutrient for plant growth and an important building block for pollen (Lau & Stephenson, 1994). For example, pollen protein concentration was highest on average for plants inoculated with competitor AMF whereas plants inoculated with stress-tolerant AMF had the lowest pollen protein concentration on average, even compared with the control (Fig. 3f). This result follows our expectation that competitor AMF would be most beneficial to plants. Surprisingly, however, we measured the lowest hyphal production for competitor AMF (Fig. 3b). While this may suggest that hyphal production may not necessarily track with the rate of P transfer as previously suggested (Jansa *et al.*, 2005; Avio *et al.*, 2006). One possibility is that competitor AMF, may be more efficient in P translocation and transfer to plant roots despite low hyphal production.

Previous studies have also shown that P availability is a determining factor for mycorrhizal responses (Smith & Read, 2010). In some cases, P supply did influence the role of AMF on floral traits in this study (Fig. 4). For example, while plants inoculated with the CSR AMF mixture had the largest flowers on average, these plants had smaller flowers in low vs high P supply conditions (Fig. 4b). Despite these differences, we found that P supply alone had a minimal impact on floral resources (Table S5), suggesting that plants that form associations with AMF were able to counteract the potentially detrimental impact of low P supply on the production of floral resources (e.g. low P supply in control plants results in lower nectar volume and the number of flowers).

We speculate that AMF-mediated variations in floral traits may also influence pollinator health. Because bees depend on pollen and nectar to meet critical nutritional requirements (Willmer, 2011; Bauer *et al.*, 2017; Roy *et al.*, 2017; Dolezal & Toth, 2018; Parachnowitsch *et al.*, 2019), our results suggest that plants that form associations with AMF may be more nutritionally beneficial to foraging bees via improvements to pollen and nectar (Fig. 4b–f) and, thus, could potentially improve bee health. If nectar volume is relatively higher for plants forming AMF associations, then bees may be able to meet their caloric needs in fewer flower visits (i.e. with less energetic expenditure and risk of predation during foraging) by visiting those plants (Jha & Kremen, 2013). Pollen protein, in particular, is necessary for brood rearing and reproduction (Roulston *et al.*, 2000; Human *et al.*, 2007; Brodschneider & Crailsheim, 2010; Li *et al.*, 2012, 2014) and thus connects directly to bee fitness. Importantly, few studies have addressed how pollen quality, much less pollen protein, is impacted by AMF (Bennett & Meek, 2020), and to the best of our knowledge, our study provides the first evidence that AMF can improve pollen protein concentration in flowers. Therefore, the 9–21% increase in pollen protein by AMF associations (Table S2) provides an opportunity to support bee health by focusing on beneficial belowground interactions.

Beyond floral traits, our results provide evidence that AMF inoculation, in general, could have some positive effects on bee foraging dynamics. Plants inoculated with AMF received the highest number of bee visits and bee visitation time (Fig. 5b). Even though our CSR framework was not predictive for bee visitation (Table 1), the number of bee visits and bee visitation time responded positively to the richest assemblage of AMF species (i.e. the CSR mixture). Since our experimental design did not differentiate the effects of AMF functional diversity from species richness, it is possible that the effect of AMF on bee visitation varied across both the richness and functional diversity of AMF.

The study also suggests that one of the principal ways AMF could influence bee foraging dynamics is via floral display size. In our pathway analysis (structural equation model), flower size increased with AMF hyphal biomass, and, in turn, plants received a greater number of bee visits when flowers were larger (Fig. 6). Floral display size is well-known to influence bee foraging dynamics (Herrera, 2020) and is considered an important visual cue for the quality and quantity of floral resources (Ortiz *et al.*, 2021). However, trade-offs did emerge for plant host and AMF associations because plants with greater root colonization had reduced flower size (Fig. 6). These opposite trends signal potential carbon expenditure trade-offs for an individual plant: between producing flowers vs forming an association with AMF. Floral resource production costs plants a lot of carbon. For example, in some cases, plants allocate up to 30% of net primary productivity to floral nectar (Obeso, 2002). Similarly, plants can transfer up to 30% of net primary productivity to AMF (Frey, 2019). Our pathway analysis suggests that in more highly colonized roots, the relative carbon cost per unit of nutrients

delivered by AMF may be higher. This leads to smaller flowers as plants shuttle more carbon belowground to obtain needed nutrients. Conversely, more extraradical hyphae may indicate relatively more nutrient transport via AMF to plants (Smith & Read, 2010) and possibly a lower marginal carbon cost. Extraradical hyphal production may be a better predictor of nutrient acquisition and uptake benefits to the host plant (Jakobsen *et al.*, 1992; Sawers *et al.*, 2017; Charters *et al.*, 2020) and, in this case, floral resources. These results suggest that AMF traits (i.e. root colonization vs hyphal length; Kiers *et al.*, 2011; Hart *et al.*, 2013; Treseder, 2013; Treseder *et al.*, 2018) affect floral traits and, in turn, bee foraging dynamics.

Overall, our study suggests that functional diversity underscores below- to aboveground interactions. The different AMF inoculation treatments did not have an equal effect on floral traits and bee foraging dynamics. Applying trait-based frameworks may reveal ecological patterns that could otherwise be obscured, especially when multiple mutualistic interactions are involved (Afkhami *et al.*, 2014). Furthermore, variations in the plant–mycorrhizal and plant–pollinator relationships that we observed can have important implications for conservation management of natural and managed systems. Consideration of below- to aboveground linkages could inform and guide restoration efforts of natural habitats aiming to improve plant growth and bee visitation. In agricultural systems, targeting practices to enhance plant–mycorrhizal relationships, such as cover crops (Higo *et al.*, 2019) and crop diversification (Guzman *et al.*, 2021), may lead to several beneficial impacts on plant growth and floral traits, influencing the frequency and duration of bee visitations important for plant reproduction. In general, incorporating belowground interactions into predictive models of floral trait variations may assist in predicting changes in plant–pollinator interactions.

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Competing interests

None declared.

Author contributions

AG, MF, CK and TB designed the study. AG collected the data with substantial assistance from MM, NL, MB, GD and IS-G. AG conducted the analyses and wrote the first manuscript draft. All co-authors provided feedback and approved the final manuscript.

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Data availability

Data are available at https://github.com/aideeguzman/amf_bees.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1 Total number of occurrences of different bee groups per AMF functional group (none, competitor, stress-tolerant, ruderal, and CSR mixtures) and P supply (low vs high) combination.

Table S2 Mean ± SE of all plant traits. Same letters indicate nonsignificant difference between means based on *post hoc* Tukey HSD tests.

Table S3 Mean ± SE of AMF traits. Same letters indicate nonsignificant difference between means based on *post hoc* Tukey HSD tests.

Table S4 Mean ± SE of floral traits. Same letters indicate nonsignificant difference between means based on *post hoc* Tukey HSD tests.

Table S5 Mean ± SE of bee visitation measurements. Same letters indicate nonsignificant difference between means based on *post hoc* Tukey HSD tests.

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