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Density dependence and interspecific interactions between arbuscular mycorrhizal fungi mediated plant growth, glomalin production, and sporulation

Helen A. Violi, Kathleen K. Treseder, John A. Menge, Sara F. Wright, and Carol J. Lovatt

Abstract: Functional differences between the arbuscular mycorrhizal fungi *Glomus intraradices* Schenk and Smith and *Scutellospora heterogama* Nicolson and Gerdemann as they affect *Persea americana* Mill. growth, glomalin, and fungal sporulation were examined by varying the composition and relative density of the two fungi over a gradient of available phosphorus (P). The plant benefit provided by these mycorrhizal fungi together was not a simple sum of the benefits provided by each fungus in monoculture at its respective density. *Glomus intraradices* and *S. heterogama* interacted to reduce plant growth rates and uptake of P, zinc (Zn), and iron (Fe) relative to plants inoculated with *G. intraradices* alone. Thus, for plant growth and nutrition, no evidence for functional complementarity was detected. Instead, interspecific interactions between mycorrhizal fungi resulted in a negative feedback on plants. Under high available P, fungal functional differences were reduced, whereas the overall difference between mycorrhizal and nonmycorrhizal plants was greatest. Overall, *S. heterogama* produced more glomalin than did *G. intraradices.* In a mixture, sporulation of the inferior mutualist, *S. heterogama*, was lower than that of the superior mutualist, *G. intraradices*, but interspecific fungal interactions increased the sporulation of both fungi. Despite the negative impact of interspecific interactions on plants, supporting multiple arbuscular mycorrhizal fungi was of greater benefit than being nonmycorrhizal.

Key words: functional complementarity, mycorrhizae, phosphorus, zinc, iron, glomalin.

Résumé : Les auteurs ont examiné les différences fonctionnelles entre les champignons mycorhiziens arbusculaires *Glomus intraradices* Schenk and Smith et *Scutellospora heterogama* Nicolson and Gerdemann, selon leurs effets sur la croissance du *Persea americana* ainsi que leur production de glomaline et de spores, dans une expérience où ils ont varié la densité relative des deux champignons, en présence d'un gradient en phosphore (P) disponible. Les bénéfices pour la plante provenant de l'ensemble des deux champignons n'est pas une simple sommation des bénéfices découlant de chacun des champignons en monoculture et de leur densité respective. Le *G. intraradices* et le *S. heterogama* interagissent pour réduire les taux de croissance de la plante et l'absorption du P, du zinc (Zn) et du fer (Fe), comparativement aux plantes inoculées avec le seul *G. intraradices*. On observe donc aucune complémentarité fonctionnelle pour la plante et sa nutrition. Au contraire, les interactions interspécifiques entre les champignons mycorhiziens entraînent une rétroaction négative chez les plantes. Sous de fortes disponibilités en P, les différences fongiques fonctionnelles sont réduites, alors que les différences générales entre les plantes mycorhiziennes et non-mycorhiziennes sont à leur maximum. Dans l'ensemble, le *S. heterogama* produit plus de glomaline que le *G. intraradices*. En mélange, la sporulation du mutualiste faible, *S. heterogama*, est moindre que celle du mutualiste supérieur, *G. intraradices,* mais, les interactions fongiques augmentent la sporulation des deux champignons. En dépit de l'impact négatif des interactions spécifiques sur la plante, le fait de supporter plusieurs champignons mycorhiziens arbusculaires est plus utile que de n'en supporter aucun.

Mots-clés : complémentarité fonctionnelle, mycorhizes, phosphore, zinc, fer, glomaline.

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Introduction

Although mycorrhizal plants regulate their interactions with arbuscular mycorrhizal fungi (AMF) (Smith and Smith 1996, 1997; Barker et al. 1998), the symbiosis can range from mutualistic to parasitic (Johnson et al. 1997; Smith and Read 1997). Plant species vary in the nutrients they gain from individual AMF species (Smith et al. 2004) and even AMF isolates (Menge et al. 1980; Munkvold et al. 2004). Arbuscular mycorrhizal fungi can depress growth depending on the availability of soil phosphorus (P) (Peng et al. 1993). Also, improved plant P nutrition may not necessarily be associated with enhanced plant growth (Oliver et al. 1983; Adjoud et al. 1996). Smith et al. (2004) suggested that a lack of correlation between plant P uptake and plant growth in response to AMF might be due to differences in carbohydrate needs of specific AMF species or isolates. Other studies demonstrated that AMF exhibit different P uptake abilities for mycorrhizal plants (Dickson et al. 1999). The interplay between AMF carbohydrate needs and the ability of specific AMF to enhance plant P nutrition is important to predicting the cost of the symbiosis to plants. The relative cost of hosting different AMF taxa to plants can be measured by comparing plant growth relative to plant P nutritional status among the different AMF taxa (Eissenstat et al. 1993). In individual plant root systems, functionally different AMF can, and do, coexist, and the outcome of interspecific interactions between these AMF most certainly impact the overall cost of the symbiosis to plants.

A landmark finding from experiments conducted with grassland species is a positive relationship between AMF community richness and the productivity of plant communities (van der Heijden et al. 1998). Such positive AMF–plant community relationships are, in part, hypothesized to be supported by underlying negative feedbacks generated by individual plant species that shift the soil community towards a community that is less beneficial to their own growth relative to that of competing plant species. Both theoretical and experimental work imply that negative feedbacks are created by plant species that better support sporulation of AMF with which they form inferior mutualistic associations than sporulation of more mutualistic AMF (Bever 2002, 2003; Castelli and Casper 2003). Presumably, the increased density of inferior AMF propagules in the host plant rhizosphere results in a carbon (C) drain for the plant that reduces plant growth. These experiments imply that the increased C cost of the symbiosis is caused by rhizosphere-scale interspecific AMF interactions that result in changes to propagule composition and possibly density of inferior AMF mutualists.

Still others (Koide 2000; Jones and Smith 2004) propose that AMF functional complementarity can benefit plant hosts. For example, in *Persea americana* Mill., two *Glomus* isolates differentially improved leaf zinc (Zn) concentration under low P (Menge et al. 1980). Also, in *Medicago trunculata* Gaertn. different nutrient acquisition strategies were found for *Scutellospora calospora* (Nicol. & Gerd.) Walker & Sanders and *Glomus caledonium* (Nicol, and Gerd.) Trappe and Gerd. (Smith et al. 2000). Based on these results, it is logical to predict that co-inoculation of an AM

plant with functionally different AMF will enhance overall nutrient uptake (as proposed by Koide 2000). If functional complementarity occurred on the community level, then as the number of AMF species in the plant root systems increased, nutrient uptake would increase because of the different but compatible and complementary strategies used by the AMF taxa to acquire nutrients from soil. The study of interspecific AMF interactions remains limited.

One challenge to this work is separating the impact of infectivity from that of AMF interspecific interactions. Evidence that the total density of AMF propagules in soil (inoculum potential or infectivity) leads to interactions among AMF and plant roots that affect the plant benefit gained from the symbiosis has been demonstrated both in the field (Fischer et al. 1994) and in greenhouse experiments (Abbott and Robson 1981). The relative effect of AMF interspecific and intraspecific density-dependent interactions on AMF function requires consideration. In our study, the effects of AMF composition (interspecific density dependence) versus the effects of individual AMF species infectivity over time (intraspecific density dependence) were evaluated independently. Independent evaluation of AMF infectivity and composition enabled tests of functional complementarity for *Glomus intraradices* Schenk and Smith and *Scutellospora heterogama* Nicolson and Gerdemann in *P. americana*. Consistent with the "negative feedback hypothesis", interspecific AMF interactions were predicted to favor sporulation of the inferior AMF mutualist relative to the superior AMF mutualist.

We also investigated the effect of available P on the outcome of AMF–plant interactions. Field experiments predict the selection of less mutualistic AMF associations with increasing nutrient availability (Johnson 1993). However, in controlled experiments, comparisons among monocultures of AMF species demonstrated that some AMF were more mutualistic under low P and others were more mutualistic under high P (Graham and Abbott 2000). These results stress the need for comprehensive experiments that simultaneously examine the effects of AMF interspecific and intraspecific density-dependent interactions and P on AMF function. Specifically, we determined the effects of available P, AMF interspecific interactions, and propagule density on plant growth and on P, Zn, and iron (Fe) nutrition.

Enhanced nutrient uptake is not the only function of the AMF symbiosis. Glomalin is an AMF-derived protein that accumulates in soil and may improve soil conditions for plants (Franzluebbers et al. 2000; Rillig et al. 2002; Rillig and Steinberg 2002). This study was designed to determine if two AMF species affect glomalin production differently and independently of their effect on plant size and plant P status. Since AMF root infection can be regulated by host plant P status (Menge et al. 1978), an increase in plant P status may similarly limit glomalin production. Thus, there may be a tradeoff between AMF that function better to improve plant P status and those that are more efficacious contributors to glomalin production.

Defining AMF function in terms of AMF propagule density and P serves several purposes. Both AMF density and available P vary in natural systems. Estimating AMF function per number of propagules and available P extends the ability to predict AMF function to a broader range of sce-

narios. Similarly, controlling for plant P status (1) provides a means to compare the relative cost of the symbiosis among AMF species of differing mutualistic capabilities and (2) leads to a better understanding of AMF functional differences by accounting for the potential confounding effects of improved plant P status on other potentially independent AMF functions.

Plant P status can influence concentrations of other nutrients in the plant (Rubio et al. 2003) and, as already discussed, may impact glomalin production. Our approach was to use a gradient of available P to generate mycorrhizal and nonmycorrhizal plants that varied in P status (concentration of P in plant leaf tissue) and then use plant P status as a predictor variable in our models. Using plant P status as a predictor variable enabled tests of AMF intraspecific and interspecific function both with respect to, and independently of, P.

If functional complementarity is occurring for the AMF–host complexes tested, then AMF interspecific interactions may function such that the contribution of a given AMF species in a mixture of two AMF species to plant growth, glomalin production, and plant nutrient status will be the sum of the contributions of the AMF at the same proportions in monoculture (when comparing plants of similar P status). Alternatively, an increase in the propagule density of the inferior mutualist coupled with a negative impact of AMF interspecific interactions on plants would provide more support for the "negative feedbacks" hypothesis.

In this study, we addressed the following questions. When controlling for plant P status (% leaf P), how do inocula of different AMF species composition and density (infectivity) affect (*i*) plant growth, (*ii*) glomalin production, and (*iii*) AMF sporulation? Further, for a given value of available P in the soil, does the difference in inoculum composition affect the ability of an AMF species to improve plant P, Fe, and Zn nutrient status and growth.

Materials and methods

Establishment of experimental conditions

The experimental system was a solid-phase buffered silica sand culture system containing 128, 12 L pots (based on Lynch et al. (1990)). An automated irrigation system supplied the nutrient solution to seedlings from 32 separate 100 L tanks (each tank provided solution to four pots). Following irrigation, nutrient solution drained from the pots back into their respective tanks.

The alumina (Al_2O_3) solid-phase buffer was used to attain more realistic P concentrations in the soil solution and spatial distributions in the sand culture system. This buffer was used to provide a constant supply of available P at low and moderate rates. Sand was ~99% pure silica sand (SiO_2 75:25 grade no.12:no.16, $m \cdot m^{-1}$, for an average grain size of 1 mm). Alumina was mixed with silica sand with that had been previously autoclaved twice for 45 min within a 24 h period, to a final 2% mass to volume ratio. Methods for acid washing sand and establishing P regimes followed Lynch et al. (1990). To the buffer was added 0.4, 100, and 200 μmol of P. The pH of the nutrient solution reached 6.0 after P was adsorbed onto the buffer and was thereafter maintained at 6.0 by the buffer. Phosphorus concentration

of the buffer–sand mixture in each pot was estimated according to Olsen and Sommers (1982). The mean (±1 SE) concentration of available P for pots treated with the 0.4, 100, and 200 μmol was 3.0 ± 0.0, 9.5 ± 0.23, and 29.1± 1.42 μg·g^{-1} sand, respectively. Thus, this system made it possible to establish and maintain a low concentration gradient of P that is comparable to the field condition under which *P. americana* naturally occurs.

Based on the P concentration of the buffer–sand mixture in each pot, pots were moved to new locations within the system using a stratified random design, such that all four pots associated with a single tank had received the same P treatment. Grouping pots with strongly contrasting available P concentrations on the same tank was avoided, because P desorbed from pots with the highest P concentration increase the P concentration of the tank and subsequently increase the available P in pots with a lower P concentration.

After the pots were randomized, a modified (–P) half-strength Hoagland's solution (Hoagland and Arnon 1938) was added to the tanks. The solution was replaced halfway through the experiment. Temperatures were held between 24 and 27 °C. Light photon flux density was between 750 and 1100 μmol·m^{-2}·s^{-1} midday, depending on cloud cover. Twelve-hour days were maintained during the course of the experiments by supplementing natural light with high-pressure sodium lights.

The experiment was repeated during the same months of consecutive years. The first experiment included the 0.4 μmol and 100 μmol P loading rates and all inoculum treatments excluding the 0:50 and 50:0 *G. intraradices* – *S. heterogama* inoculum treatments (*n* = 96). In the second experiment, P was loaded onto the buffer at 100 and 200 μmol P, and all inoculum treatments were included (*n* = 128). There were 16 replicates per inoculum treatment for each experiment. No significant differences in plant growth were detected between experiments for plants of similar P status that received the same inoculum treatments. Thus, data from both experiments were combined and analyzed together.

Mycorrhizal inocula production and estimation of infectivity

Whole soil inocula of *G. intraradices* (INVAM DN989) and *S. heterogama* (INVAM BR154) were obtained from the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (West Virginia University, Morgantown, West Virginia). To produce adequate quantities of inocula for experiments, approximately 20 mL of either inoculum was used to inoculate 10-day-old *Sorghum vulgare* L. roots, which were potted in silica sand in 0.4 L pots. At 2 months, plants and soil were transferred with minimum disturbance to 1 L pots containing silica sand, and additional seeds were sown around the plants. Plants were grown in chambers under 14 h days and fertilized once per week with a modified (phosphorus omitted, –P) half-strength Hoagland's solution and watered as needed. After 4.5–5.5 months, watering was stopped and plants were allowed to senesce for 2 weeks before being harvested. Resulting soil and *S. vulgare* roots constituted inocula for *G. intraradices* or *S. heterogama*. Inocula were stored in pa-

per bags in a cold room (20 °C) for under 1 month before being used in the experiment.

A most probable number (MPN) test (Alexander 1982) was performed to quantify the infectivity (expressed as infective propagules·g⁻¹ soil) of *G. intraradices* and *S. heterogama* whole soil inocula, respectively. The MPN test included three 10-fold dilutions of inoculum and five replicates (plants) per dilution. Individual *Persea americana* Mill. var. *drymifolia* (Schltdl, and Cham.) S.F. Blake seedlings were inoculated with either *G. intraradices* or *S. heterogama* and grown in separate pots of buffer–sand mixture and watered with modified (phosphorus omitted, –P) half-strength Hoagland's solution. To detect infection, 50 roots per plant were stained with trypan blue and evaluated for AMF infection under a compound microscope (methods modified after Phillips and Hayman (1970)). Any detectable infection resulted in a positive score for that plant. A MPN test was applied to these presence or absence data. The resulting infectivity values (infective propagules·g⁻¹ soil) were used to calculate the amount of whole soil inoculum required for each AMF species to achieve infectivity ratios tested below.

Plant cultivation and inoculation

Persea americana seeds were washed and then surface sanitized in 1% household bleach solution. Approximately 600 seeds were planted in sterile vermiculite in 0.5 L pots. To induce germination, the top quarter of each seed was cut off. After 2 months, seedlings of similar size were selected for the experiment, and a subsample was harvested to determine the degree of variation among seedlings (plant fresh mass (±1 SE, n = 128) mean = 114.0 ± 3.22 g). Vermiculite was removed from seedling roots by gentle washing in distilled water directly before inoculation.

Ratios of *G. intraradices* – *S. heterogama* propagules used to inoculate plants were 100:0, 50:0, 50:50, 75:25, 25:75, 0:50, 0:100, and a 0:0 control. Propagules were obtained from whole soil inoculum that was homogenized by mixing and then divided into volumes that corresponded with the infectivity values needed for each treatment. Dispersing propagules in the large pots used in the experiments might differentially affect the infectivity of the fungi studied. Thus, to maintain AMF infectivity ratios, propagules (spores, hyphae, and *S. vulgare* roots) in the buffer–sand mixture (inoculum) were applied directly to plant roots via filter paper. Controls were inoculated with soil filtrate produced by soaking inocula in distilled water for 45 min and then twice passing the solution through a 40 µm filter (modified after methods in Graham et al. (1981)).

Seedlings were then randomly assigned to 12 L pots within the sand culture system. Thus, each inoculum treatment was subjected to each P concentration in the gradient of available P. To verify the mycorrhizal status of seedlings, root samples were taken from each pot approximately 3 months postinoculation. Roots were stained as previously described and observed under a compound microscope (40×). Infection was verified for plants receiving AMF inocula, and no mycorrhizal structures were observed in the roots of control plants.

Plant measurements

All trees were destructively harvested after 8 months. Leaf area was measured photometrically with a leaf area meter (model Li-3100, LI-COR, Lincoln, Nebraska). Dry masses for plant parts were determined after material was oven-dried for 2 d at 65 °C. At the end of each experiment, dried leaf tissue from all trees was analyzed at the Agricultural and Natural Resources Analytical Laboratory of the University of California (Davis, California). Following nitric acid – hydrogen peroxide microwave digestion (Sah and Miller 1992), Zn and Fe were quantified by atomic absorption spectrometry, and P was quantified by inductively coupled plasma atomic emission spectrometry (Meyer and Keliher 1992). The method is generally reproducible within 8% for all analyses. Leaf nutrient concentrations and measures of dry leaf mass were used to calculate total nutrient uptake for leaves for each study plant.

Fungal sporulation

At the end of each experiment, and following the removal of plant roots from sand, sand was thoroughly mixed and a subsample of 200 mL was retained to estimate spores produced per millilitre of sand for each AMF. Spores of both AMF were extracted from sand using a sucrose flotation method (Furlan et al. 1980), identified, and counted. For *G. intraradices*, sporulation was simply calculated as: ln(spores·mL⁻¹)$_{t1}$– ln(spores·mL⁻¹)$_{t0}$. For the purposes of the analyses herein, *S. heterogama* spores were coded as present or absent (see Statistical analyses below for further explanation).

Glomalin production

To measure glomalin production, sand-filled plastic cores with mesh ends were inserted 10 cm from the sand surface at a point equidistant from the seedling stem and pot edge. Cores were constructed from plastic cylinders covered with an inner plastic horticultural mesh and an outer 40 µm nylon mesh that allowed entry of fungal hyphae but excluded plant roots (Lovelock et al. 2004). Cores were filled with silica sand that had been autoclaved at 121 °C for 60 min in 50 mmol·L⁻¹ sodium citrate (pH 8.0) and rinsed with distilled water. At the end of the experiment, cores were collected and immediately frozen at –20 °C until processed. Sand was removed from cores, and glomalin was extracted from the sand by autoclaving in 8 mL of 50 mmol·L⁻¹ sodium citrate buffer (pH 8.0) for 60 min. The cooled extract was centrifuged at 6000 r/min (10 000g) for 15 min (Wright et al. 1996). Glomalin content (µg·g⁻¹ dry sand) was quantified using the monoclonal antibody MAb32B11 in an enzyme-linked immunosorbent assay (ELISA) (Wright and Upadhyaya 1996).

Statistical analysis

All analyses were performed in JMP version 5.1.1 (SAS Institute Inc. 1989–2002). A response surface model was used to test the strength of AMF interspecific density-dependent factors relative to intraspecific density-dependent factors in predicting plant response variables and production of glomalin. The model included linear and quadratic effects

Table 1. Results of standard least squares fit for the effects of arbuscular mycorrhizal fungal infectivity and percent leaf P on ln plant growth rate.

Source of variation*	df	t ratio	P
Model (F ratio)	6	7.15	<0.0001
Glomus intraradices (G) infectivity	1	–1.18	0.2375
Scutellospora heterogama (S) infectivity	1	–2.00	0.0466
G × G	1	–2.51	0.0127
G × S	1	–3.21	0.0015
S × S	1	–1.69	0.0918
% Leaf P	1	4.27	<0.0001
Error (MS)	216	0.32	

Note: df, degrees of freedom. $r^2 = 0.17$.

*Terms in the model were reduced from a full response surface analysis with a forward stepwise regression.

of *G. intraradices* and *S. heterogama* and combinations of the fungi at all starting inocula infectivities.

Prior to response surface analyses, a forward combine stepwise regression method was used to determine which predictor variables best explained the variation in the response variables. Variables included in the regression (which could include linear, quadratic, and two-way interaction terms) were then used in a least squares fit regression. Plant response variables were transformed where test assumptions could not be met with raw data. Data were transformed by ln transforming data. Where this transformation still failed to meet test assumptions, Box-Cox transformations were performed.

For determining the effects of inoculum composition and infectivity on plant growth rate, % leaf P was used as a term in the standard least squares fit model. In testing for differences in leaf Zn and Fe concentrations across AMF treatments, % leaf P was also included as a predictor variable in the model. For examining AMF effects on % leaf P, the amount of available P was used as a predictor variable. To determine if model fit was adequate, a "lack of fit test" was performed. Specifically, this involved a χ^2 test that determined whether the pure error log-likelihood was significantly better than the fitted model (SAS Institute Inc. 1989–2002).

Where the effects of % leaf P were significant in the least squares fit model, scores were adjusted for the effect of % leaf P and then used in post hoc tests. Specific differences among the AMF inoculum treatments tested could then be identified independently of plant P status by comparing adjusted means with Tukey's HSD test (SAS Institute Inc. 1989–2002). Adjusting scores was accomplished by saving the residuals from a regression that included the response variable and % leaf P. When presenting raw data with adjusted data, the unadjusted score was added to the residual score. The procedure can be represented by the following equation: Adjusted score = $Y_i – \beta(X_i – X...)$ where Y_i is the unadjusted score, β is the regression coefficient, and $(X_i – X...)$ represents the deviation due to % leaf P. Normality was determined by inspecting histograms of residuals for each test where these data characteristics were necessary for meeting test assumptions.

Test assumptions could not be met for the regression analysis on *S. heterogama* spore data because of a high variability among scores. For this reason, scores were coded as

either "1" (spores detected) or "0" (no spores detected). A nominal logistic regression was performed with these recoded data.

Results

Plant growth

When plant growth rates are adjusted for % leaf P (plants of similar P status), only plants inoculated with *G. intraradices* monocultures were significantly faster growing than control plants (Fig. 1a). Plants treated with inoculum containing *S. heterogama* had reduced growth rates that were not significantly different from the control plants (Table 1; Fig. 1a). This is supported by the significant and negative t ratios associated with *S. heterogama* infectivity and the *G. intraradices* × *S. heterogama* interaction (G × S) on plant growth rates (Table 1).

The negative G × S interaction can be specifically attributed to the lower plant growth rates associated with mixtures (*G. intraradices* and *S. heterogama*) relative to what was predicted for plant growth rates associated with *G. intraradices* monocultures of the same infectivity (Table 1; Fig. 1a). For example, the adjusted plant growth rate for plants inoculated with monocultures of *G. intraradices* at intermediate levels of infectivity (50:0 *G. intraradices* – *S. heterogama*) differed from growth rates for control plants, whereas mixtures of the same *G. intraradices* infectivity (50:50 *G. intraradices* – *S. heterogama*) did not (Fig. 1a).

The effect of adjusting for % leaf P is evident when comparing Figs. 1a and 1b. When growth rate is evaluated independently of plant P status, plants inoculated with the *S. heterogama* monoculture 0:50 and with mixtures of 25:75 *G. intraradices* – *S. heterogama* infectivity had higher growth rates than control plants (Fig. 1b). Differences between controls and these inoculum treatments (0:50 and 25:75 *G. intraradices* – *S. heterogama*) were no longer significant when plant growth rates were adjusted for % leaf P (Fig. 1a).

Both *G. intraradices* and *S. heterogama* interacted with available P to affect plant growth rates (G × P: t ratio = 3.47, $P = 0.001$; S × P: t ratio = 2.62, $P = 0.009$) and % leaf P (G × P: t ratio = –2.82, $P = 0.005$; S × P: t ratio = –2.38, $P = 0.018$). However, fungi were only important in improving plant P status at low available P, as indicated by the negative

Fig. 1. The effect of inoculum type (composed of *Glomus intraradices* and (or) *Scutellospora heterogama* propagules) on plant response variables and glomalin. Post hoc tests used include ANOVA *F*, *P* values followed by pairwise Tukey's HSD comparisons performed on either raw data or means of residuals from regressions of plant response variables on % leaf P. Means of residuals were used where % leaf P was determined to significantly impact a plant response variable in least squares fit models. Response variables are as follows: (*a*) adjusted plant growth rate with scores adjusted to reflect plants of similar P status using the regression equation (Adj. mean = $Y_i - \beta(X_i - X...)$) where X = % leaf P, Y_i is the unadjusted score, $F = 2.52$ and $P = 0.017$; (*b*) absolute plant growth rate in g dry mass·month^{-1}, $F = 3.79$ and $P = 0.0007$; (*c*) glomalin production (immunoreactive protein to the antibody MAb32B11 in µg·g^{-1} sand·month^{-1}), $F = 3.98$ and $P = 0.0004$; (*d*) total P uptake in g·month^{-1} (in total dry leaf mass), $F = 3.18$ and $P = 0.003$; (*e*) total Zn uptake in µg·month^{-1} (in total dry leaf mass), $F = 3.13$ and $P = 0.002$; (*f*) total Fe uptake in µg·month^{-1} (in total dry leaf mass), $F = 3.14$ $P = 0.004$; (*g*) leaf Zn concentration residuals $F = 2.41$ and $P = 0.02$; and (*h*) leaf Fe concentration residuals, $F = 2.06$ $P = 0.05$. Bars not sharing identical letters are significantly different at Tukey's $P < 0.05$. Error bars are ±1 SE.
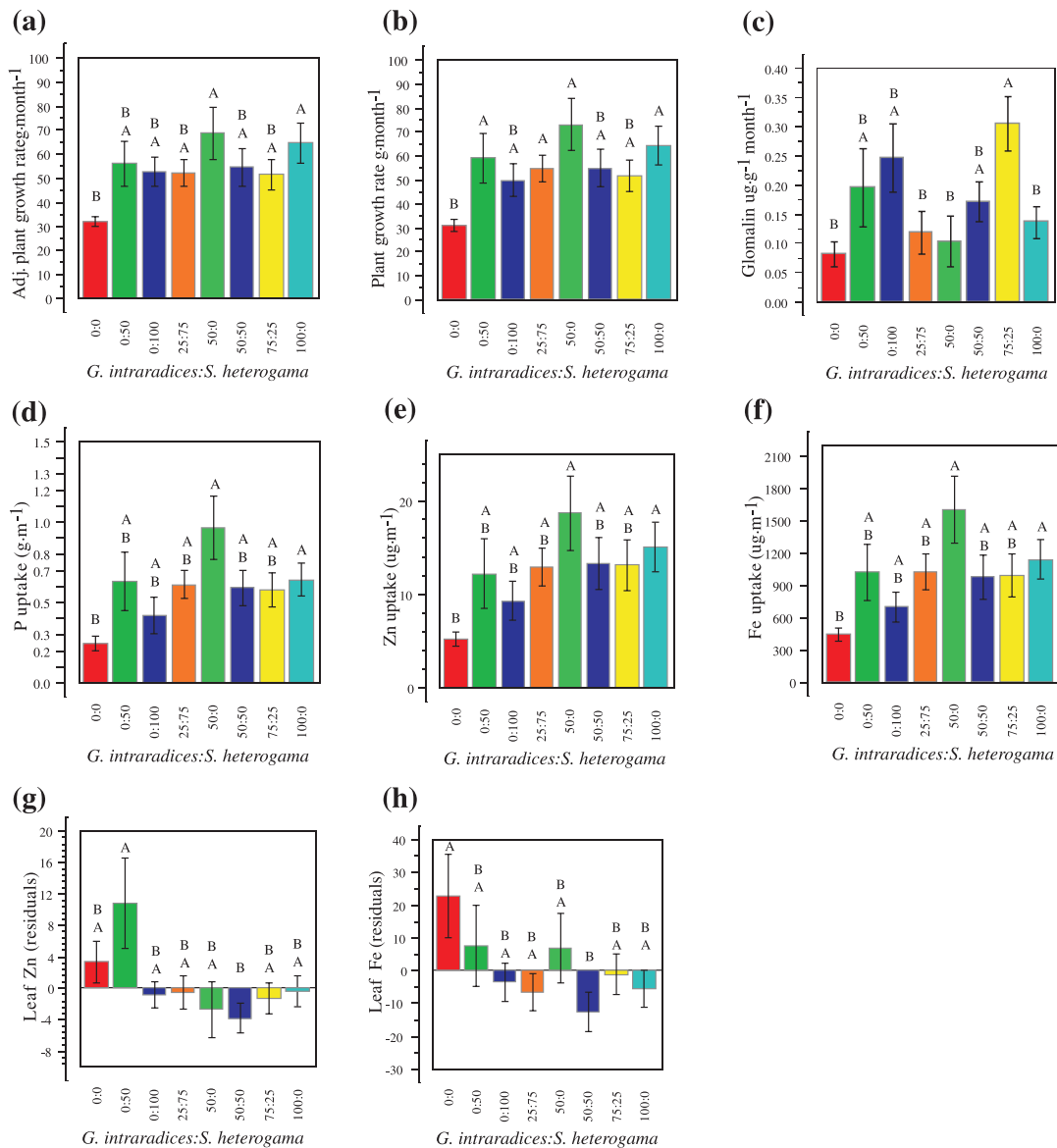
interactions (*t* ratios) between both *G. intraradices* and *S. heterogama* with available P (see above and Figs. 2*a*, 2*c*, 2*e*). Strong growth responses and increases in % leaf P to available P were detected (plant growth rate *t* ratio = 9.48, *P* < 0.0001; % leaf P *t* ratio = 9.18, *P* < 0.0001), but these relationships were asymptotic (P × P: plant growth rate *t* ratio = –3.11, *P* = 0.002; % leaf P *t* ratio = –5.33, *P* < 0.0001) indicating that P was not limiting throughout the entire range of available P tested (Fig. 2). Another in-

dication that P was not limiting at the highest P availability tested was that *G. intraradices* and *S. heterogama* infectivity interacted with available P such that differences in % leaf P among inoculum treatments decreased with increasing available P (Figs. 2*a*, 2*c*, 2*e*). Despite an available P gradient that appears to range from limiting to nonlimiting, differences in plant growth rates among AM and nonmycorrhizal plants strongly increased with increasing available P (Figs. 2*a*–2*f*). The two AMF differed in that

Table 2. Results of standard least squares fit showing t ratios and significance levels (in parentheses, $P \leq 0.05$) and total percentage of variance explained (r^2) in response surface analyses for plant response variables.

Source of variation*	Uptake for leaves (g·month^{-1})		
	P ($n = 221$)	Zn ($n = 222$)	Fe ($n = 221$)
Model (F ratio)	36.65 (<0.0001)	40.03 (<0.0001)	23.62 (<0.0001)
Glomus intraradices (G) infectivity	1.77 (0.0775)	1.85 (0.0651)	0.67 (0.5024)
Scutellospora heterogama (S) infectivity	–2.02 (0.0444)	–1.51 (0.1320)	–2.51 (0.0129)
Olsen's available P (P)	11.74 (<0.0001)	13.87 (<0.0001)	10.29 (<0.0001)
G × G	–4.40 (<0.0001)	–3.37 (0.0009)	–3.72 (0.0003)
G × S	–3.22 (0.0015)	–2.84 (0.0050)	–3.21 (0.0015)
G × P	—	1.29 (0.1989)	1.70 (0.0909)
P × P	–5.52 (<0.0001)	–6.32 (<0.0001)	–4.66 (<0.0001)
Error (MS)	0.02	23.91	179 093.00
r^2	0.51	0.57	0.44

*Predictor variables shown are those revealed to contribute to explaining variation in the data by a forward stepwise regression followed by fitting the data to the model using a standard least squares regression.

S. heterogama infectivity was most strongly correlated with plant growth, where P was least limiting (Figs. 2*b*, 2*d*, 2*f*), whereas *G. intraradices* was more consistently effective in improving plant growth over the available P gradient.

When controlling for available P, there was a negative and significant interaction between *G. intraradices* and *S. heterogama* inocula on plant growth (G × S: t ratio = –2.79, $P = 0.006$), whereas no significant interaction was detected for G × S and % leaf P. The quadratic effect of *G. intraradices* and available P on plant biomass is negative (significantly convex curve), reflecting a peak in the efficiency with which *G. intraradices* and P improved plant growth at intermediate levels of infectivity and available P (G × G: t ratio = –2.33, $P = 0.021$; P × P: t ratio = –3.11, $P = 0.002$; Figs. 2*b*, 2*d*, 2*f*). ANOVA and Tukey HSD tests results reinforce this interpretation, as 50:0 monocultures of *G. intraradices* and 0:50 monocultures of *S. heterogama* are associated with plant growth rates that are significantly different from controls that received no AMF inoculum, whereas the 50:50 mixture of *G. intraradices* – *S. heterogama* does not differ from the control (Fig. 1*b*).

Glomalin production

Scutellospora heterogama propagule density was positively and linearly correlated with glomalin concentration (model $F_{8, 209} = 6.38$, $P < 0.0001$; *S. heterogama*: t ratio = 2.29, $P = 0.023$). The linear effect of *G. intraradices* density alone was not important in predicting concentrations of glomalin, but *G. intraradices* significantly interacted with % leaf P, such that glomalin reached its highest concentrations where *G. intraradices* infectivity was high and P was at intermediate concentrations (G × P: t ratio = 2.06, $P = 0.041$) (Fig. 3). The 75:25 *G. intraradices* – *S. heterogama* inoculum treatment was associated with significantly greater glomalin concentrations than were the *G. intraradices* monocultures, 25:75 *G. intraradices* – *S. heterogama* inoculum treatment and the control (Fig. 1*c*). Plant growth rate also positively and significantly affected glomalin production (plant growth rate in g·month^{-1}: t ratio = 4.63, $P < 0.0001$).

Plant nutrition

Rates of Zn, Fe, and P uptake (μg·month^{-1} for dry leaf mass) were affected similarly by experimental treatments (Table 2; Figs. 1*d*, 1*f*). Uptake of all three nutrients was greater under high P availability (Table 2). Nutrient uptake peaked at intermediate levels of available P and *G. intraradices* infectivity (Table 2). The significant negative interaction of *G. intraradices* and *S. heterogama* for all nutrients tested can be attributed to greater nutrient uptake rates associated with plants inoculated with *G. intraradices* alone relative to those associated with inocula containing *S. heterogama* (Table 2; Figs. 2*d*–2*f*). Infectivity of *S. heterogama* was significantly negatively correlated with P and Fe but not Zn uptake (Table 2).

Although overall P uptake was improved by AMF, *G. intraradices* and *S. heterogama* interacted with available P to maintain % leaf P concentrations below that of controls under moderate and high available P (G × P: t ratio = –2.82, $P = 0.005$; S × P: t ratio = –2.39, $P = 0.018$; Figs. 2*a*, 2*c*, and 2*e*). Similar to the relationships with available P, Zn, and Fe concentrations in plant leaves were both positively correlated with % leaf P (t ratio = 5.94, $P \leq 0.0001$; t ratio = 10.52, $P \leq 0.0001$, respectively).

When normalizing plants for % leaf P, functional differences between AMF were elucidated. The significant negative linear and quadratic effects of *G. intraradices* on leaf Zn could be attributed to the lower contribution of *G. intraradices* to leaf Zn than *S. heterogama* monocultures of intermediate infectivity (G: t ratio = –3.35, $P = 0.001$; G × G: t ratio = 2.71, $P = 0.007$; Fig. 2*g*). Inoculum infectivity for both *G. intraradices* and *S. heterogama* were negatively associated with leaf Fe concentrations when comparing plants of similar P status (% leaf P) (G: t ratio = –2.63, $P = 0.009$; S: t ratio = –2.71, $P = 0.007$). However, when comparing residuals of leaf Fe on % leaf P, only controls had significantly higher concentrations of Fe than inoculum mixtures of 50:50 *G. intraradices* – *S. heterogama* infectivity (Fig. 2*h*).

Sporulation

For both AMF tested, each fungus was positively impacted by its own initial infectivity and that of the other fungi (*S. heterogama* sporulation model details below, otherwise see Table 3). There was no detectable effect of avail-

Fig. 2. The relationship between inoculum type (composed of *Glomus intraradices* and (or) *Scutellospora heterogama* propagules), % leaf P for three concentrations of available P (*a*, *c*, and *e*; model $F_{[7, 213]}$ = 15.16, $P < 0.0001$, r^2 = 0.33) and the concurrent relationship between inoculum type and plant growth rate for three levels of available P (*b*, *d*, and *f*; model $F_{[8, 213]}$ = 20.93, $P < 0.0001$, r^2 = 0.44). (Note that for *a*, *c*, and *e*, z-axes are not to scale.) Concentrations of P shown were selected from the continuous range of concentrations tested (3–60 µg·g^{-1} sand) and are 3, 30, and 60 µg·g^{-1} Olsen's P, as measured at the beginning of the experiment.
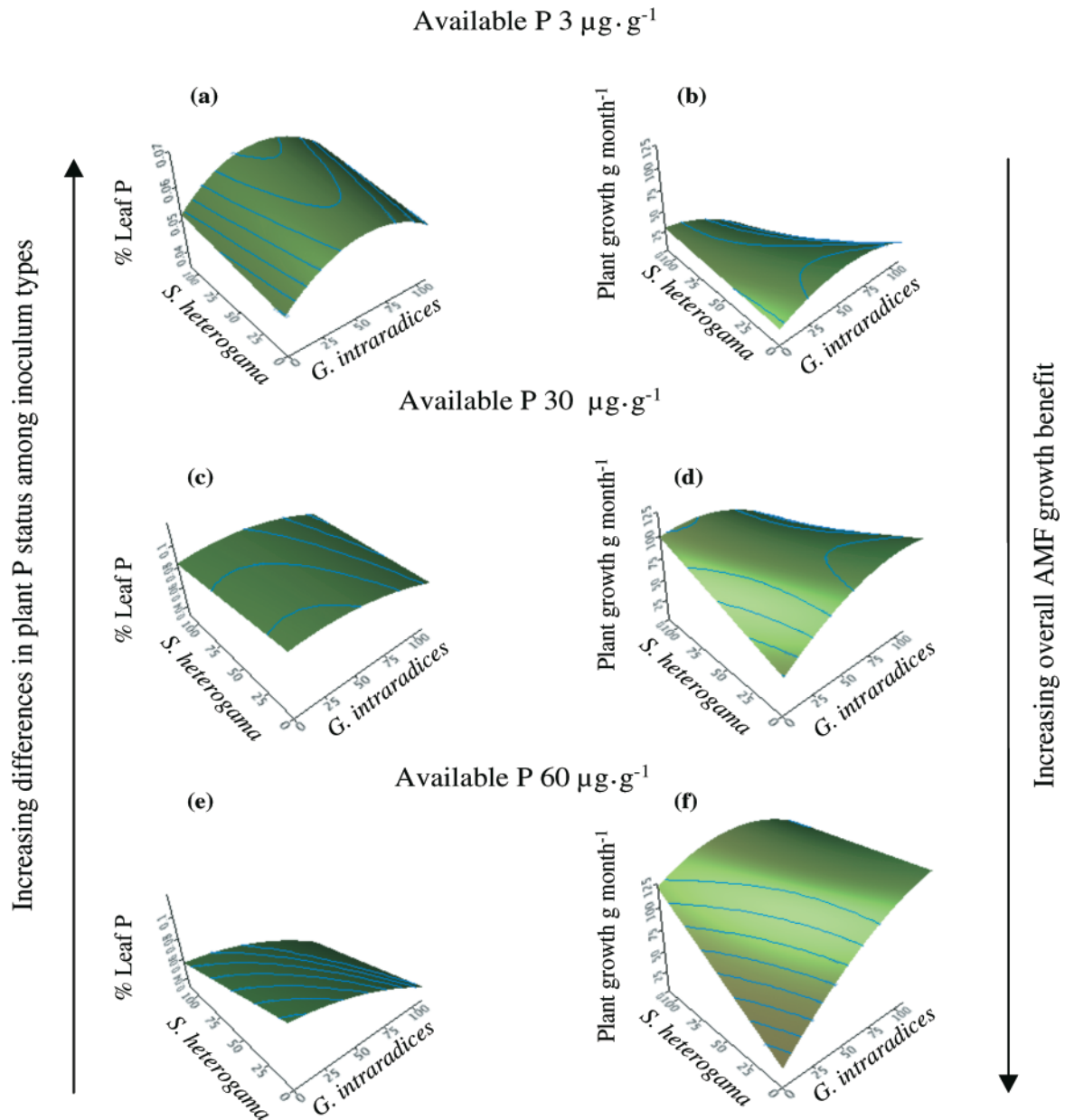

able P on sporulation of either fungus. *Scutellospora heterogama* sporulation rates (r = ln(spores·mL^{-1})$_{t1}$ – ln(spores·mL^{-1})$_{t0}$) were highly variable, and mean rates per inoculum treatment were low (r ranged between 0.004 and 0.015). Thus, only the probability of *S. heterogama* sporulation was examined and not the sporulation rate. For *S. heterogama* (model: χ^2 = 58, df = 6, $P < 0.0001$), not only did *G. intraradices* infectivity increase the probability of *S. heterogama* sporulation (*G. intraradices*: χ^2 = 10.356, $P = 0.001$), but the interaction between the two fungi was also significant (G × S: χ^2 = 13.525, $P < 0.0001$). For inocula containing 25:75 or 50:50 ratios of *G. intraradices* –

S. heterogama, *S. heterogama* spores were detected in 50% of the pots. Sporulation occurred in 37.5% of the cases where plants were inoculated with 75:25 *G. intraradices* – *S. heterogama*. Although the presence of *S. heterogama* in inoculum increased the probability of its own sporulation (*S. heterogama*: χ^2 = 33.517, $P < 0.0001$), the incidence of sporulation was lower for the 0:100 *S. heterogama* monocultures (37.5%) than it was for the 0:50 *S. heterogama* monocultures (43.75%).

In contrast, *G. intraradices* sporulation rate (r = ln(spores·mL^{-1})$_{t1}$ – ln(spores·mL^{-1})$_{t0}$) was highest for the highest starting infectivity, and the G × S interaction did not affect

Fig. 3. Interactions between arbuscular mycorrhizal fungal infectivity, glomalin, and plant P status. The effect of *Glomus intraradices* infectivity and % leaf P on the production of glomalin (immunoreactive protein to antibody MAb32B11 expressed in $\mu g \cdot g^{-1}$ sand$\cdot$month^{-1}).
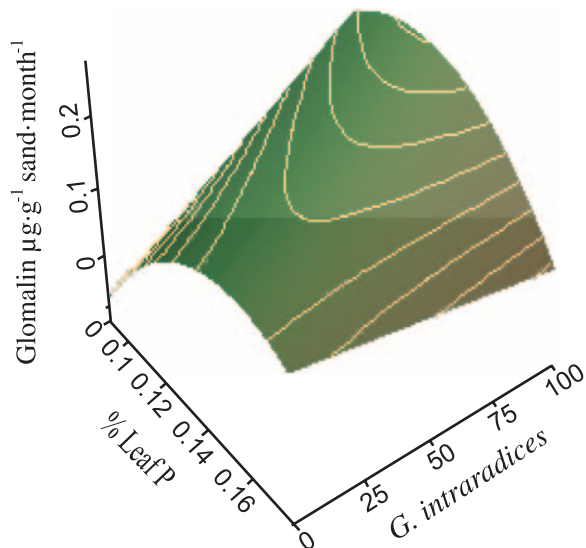
sporulation rate (Table 3). The mean (±1 SE) *G. intraradices* sporulation rate for the highest infectivity monocultures (100:0) was 1.485 ± 0.210, whereas the rate decreased proportionately with monoculture infectivity to 0.748 ± 0.090 for the 50:0 *G. intraradices* inoculum types. For a given *G. intraradices* infectivity, disproportionately higher sporulation rates were estimated for the 25:75 and the 50:50 *G. intraradices* – *S. heterogama* inoculum compositions. Rates were 1.039 ± 0.202 and 0.784 ± 0.146 for each inoculum, respectively. For the 75:25 *G. intraradices* – *S. heterogama* inoculum, the mean sporulation rate was 1.018 ± 0.188.

Discussion

The hypothesis that the benefits of inoculating plants with mixtures of AMF would be a simple sum of the benefits provided by each AMF at its respective infectivity in monoculture was rejected for *G. intraradices* and *S. heterogama* in *P. americana*. Instead, over the total range of available P concentrations tested, interspecific AMF density-dependent interactions negatively impacted plant growth such that for a given *G. intraradices* infectivity (propagule density at the start of the experiments), *G. intraradices* was associated with a greater plant growth benefit in monocultures than when plants were co-inoculated with both *G. intraradices* and *S. heterogama*. The presence of *S. heterogama* also reduced the *G. intraradices*-associated leaf P, Zn, and Fe uptake benefit. Thus, our data provide no evidence to support AMF functional complementarity for nutrient acquisition or plant growth benefit in *P. americana*.

Interestingly, the negative impact imposed on plants supporting mixtures of AMF was reduced over the available P gradient. At higher P availability, differences among AMF species were reduced, although the overall difference between nonmycorrhizal and AMF plants increased. Also at

high available P, little difference could be detected in % leaf P or in total leaf P uptake among AMF inoculum treatments. Our results were not predicted but are consistent with findings from N–P–K nutrient addition studies performed in serpentine grasslands where AMF-related plant growth benefit increased with fertilization (Gustafson and Casper 2004).

Both AMF tested interacted with available P to improve plant growth, but the relationship among AMF infectivity, plant P status (% leaf P), (or P uptake for dry leaf mass), and growth benefit differed for inoculum composition. *Scutellospora heterogama* infectivity was negatively related to overall plant growth, % leaf P, and P and Fe uptake where P was limiting. The significant interaction between *S. heterogama* and available P is attributed to the plant growth benefit associated with *S. heterogama* only at high available P. In contrast, *G. intraradices* did not negatively impact plant growth at any available P concentration or negatively affect the uptake of any nutrient tested.

Unlike *S. heterogama*, the plant benefit received from *G. intraradices* was positively related to infectivity, but the relationship was asymptotic, meaning that intraspecific density dependence for *G. intraradices* inoculum influenced plants such that growth, % leaf P, and P uptake ($\mu g \cdot month^{-1}$ for dry leaf mass) peaked at intermediate levels of infectivity. Several authors (Sanders et al. 1977; Menge et al. 1980) have proposed that initial AMF propagule density or infectivity may alter AMF nutrient absorption abilities owing to density dependence. Density dependence might be responsible for inconsistencies among studies conducted to determine if there are AMF functional differences, and it could be an important consideration in designing future experiments.

In summary, our hypothesis that function will change over the P gradient tested was correct, but the prediction that more mutualistic AMF associations would be found under low available P was false. In terms of plant growth benefit, *G. intraradices* was a superior mutualist compared with *S. heterogama* at low concentrations of available P and when comparing plants of similar P status. These results sharply contrast with those demonstrating that AMF interactions decreased the growth of *Panicum virginatum* at high concentrations of available P (Johnson 1998), but they support the idea that some AMF are more mutualistic under low P and others are more mutualistic under high P (Graham and Abbott 2000). We did not predict the negative intraspecific interaction of the poor AMF mutualist and plant growth, the lack of a positive intraspecific interaction for the superior mutualist and plant growth, or the impact of available P on intraspecific density-dependent plant benefits.

A possible explanation for the variation in plant growth benefit is that the C cost of attaining P in mycorrhizal plants differed over the available P gradient and for the two fungi. Different C costs of maintaining the AMF symbiosis were detected for mycorrhizal citrus trees of similar size and P status (Graham et al. 1997). Similar responses have been documented in other studies (Mosse 1973; Helgason et al. 2002), and these responses imply that at low P concentrations plants may be more C limited than P limited because of the cost of the symbiosis (Stribley et al. 1980; Jones and Smith 2004). This suggests that plants hosting *S. heterogama* under severely P-limiting conditions may be

Table 3. Results of standard least squares fit showing t ratios and significance levels (in parentheses, $P \leq 0.05$) and total percentage of variance explained (r^2) in response surface analyses for *Glomus intraradices* sporulation rate.

Source of variation*	Sporulation rate[†]
	t ratio
Model (F ratio)	17.452 (<0.0001)
Glomus intraradices (G) infectivity	7.213 (<0.0001)
Scutellospora heterogama (S) infectivity	2.091 (0.038)
Olsen's available P (P)	0.601 (0.549)
G × S	1.355 (0.177)
P × P	−1.796 (0.074)
Error (MS)	0.683
r^2	0.29

*Predictor variables shown are those revealed to contribute to explaining variation in the data by a forward stepwise regression followed by fitting the data to the model using a standard least squares regression.

[†]*Glomus intraradices* sporulation rate was calculated as $\ln(\text{spores·mL}^{-1})_{t1} - \ln(\text{spores·mL}^{-1})_{t0}$ ($n = 224$).

expending C below ground without a P gain commensurate with that of plants hosting *G. intraradices*. Arbuscular mycorrhizal fungi have been found to possess different mutualistic capabilities in this respect in other studies (Abbott and Robson 1981; Graham et al. 1997). Graham and Abbott (2000) attribute these findings to differences in AMF colonization rates. Consistent with their hypothesis, fast colonizing AMF, such as *G. intraradices*, are more efficacious mutualists under low P availability than slow colonizers like *S. heterogama*. The precise mechanisms responsible for these relationships are unknown.

Glomalin production

Marked differences existed between the effects of *S. heterogama* and *G. intraradices* on glomalin production. *Scutellospora heterogama* propagule density was positively correlated with glomalin production but also with reduced plant growth rates relative to plants inoculated with *G. intraradices*. The positive effect of *S. heterogama* on glomalin production provided evidence of a tradeoff between the ability of AMF to contribute to glomalin production and promote plant growth. Yet the positive relationship between plant growth and glomalin production rates that can be attributable to plants inoculated with both fungi suggests that despite the greater cost of being associated with *S. heterogama,* plants inoculated with mixtures of both fungi reaped both the benefits of enhanced growth relative to non-mycorrhizal plants and higher glomalin production compared with plants inoculated with *G. intraradices* alone or no AMF.

Neither *G. intraradices* infectivity, nor P status alone significantly impacted glomalin production, but *G. intraradices* did interact with P to increase glomalin. For inocula containing *G. intraradices,* it appears that glomalin is highest where *G. intraradices* infectivity is highest and at intermediate levels of % leaf P, meaning that plant P status and *G. intraradices* infectivity appear to be regulating glomalin production. No such relationship appeared to exist for *S. heterogama* and % leaf P, implying that *S. heterogama* was able to contribute proportionally more to glomalin production per plant C gain regardless of plant P status.

Sporulation

Our results demonstrated that a negative AMF feedback on plants can occur in the absence of an associated shift in propagule composition towards the inferior AM fungal mutualist. Although interspecific AMF interactions increased the probability of sporulation for the inferior mutualist (*S. heterogama*), the sporulation rate of the superior mutualist (*G. intraradices*) was also positively impacted by the presence of the inferior mutualist (relative to *G. intraradices* sporulation in monoculture). In a mixture, spore composition clearly shifted to favor *G. intraradices*. The difference in spore composition is great enough that *G. intraradices* infectivity likely increased to surpass that of *S. heterogama* for all mixtures at the end of the experiments. This strongly contrasts results found for *Plantago lanceolata*, where the inferior AMF mutualist accumulated in the rhizosphere to a greater extent than the superior AMF mutualist (Bever 2002).

In this AMF–host complex, maximum host benefit was received at intermediate levels of *G. intraradices* infectivity. *Scutellospora heterogama* was capable of reducing the *G. intraradices* – plant benefit even at low *S. heterogama* infectivity. Thus, although in a mixture the sporulation of the superior AMF mutualist was disproportionate to that of the inferior AMF mutualist, it is plausible that the resulting AMF community had the potential to negatively impact plants relative to monocultures of the superior AMF mutualist.

Previous work demonstrated that many AMF reach some minimum amount of root infection before they sporulate (Gazey et al. 1992) and that plant P status can interfere with root infection (Menge et al. 1978). Thus, it is logical to predict that *S. heterogama* is more likely to sporulate in the presence of *G. intraradices* because of differences in plant size or P status that better enable *S. heterogama* to reach this critical infection level. However, plants inoculated with mixtures of the AMF were of similar size and P status as those inoculated with *S. heterogama* alone. Further, contrary to the hypothesis that less mutualistic AMF will dominate the spore com-

munity under high available P (Johnson 1993), P had no detectable impact on the outcome of AMF interspecific interactions.

A more plausible hypothesis is that the positive interspecific AMF interactions on sporulation are a reaction to competition between the AMF that hastens fungal sporulation. For different fungi sharing space and a common substrate, competition has frequently been observed to result in higher sporulation rates relative to those observed for the fungi in monocultures (e.g., Newton et al. 1999). Higher sporulation rates in a mixture can provide a competitive advantage in situations where early sporulation is important to root infection during favorable periods (i.e., before AMF competition becomes severe). If the cost of early sporulation is passed onto plants, then this could serve as a mechanism by which interspecific AMF interactions reduce the AMF-related plant growth benefit.

Conclusions

The results of this research demonstrate that the nature of the association between *P. americana* and AMF may vary over a narrow range of available P and AMF infectivities. Interspecific and intraspecific AMF density dependence affect AMF ability to promote *P. americana* growth. For *S. heterogama*, intraspecific density dependence was important for determining belowground glomalin production. Our data suggest that when plants are infected by inferior AMF mutualists (in monoculture or mixture with superior mutualists) there is (*i*) greater glomalin production and (*ii*) lower plant biomass per amount of % leaf P gained at low concentrations of P, relative to plants that are infected by a superior mutualistic AMF alone.

Our results reinforce models that propose negative AMF community feedbacks on plant growth (Bever 2003). Specifically, for negative AMF–plant feedbacks to occur under field conditions, an inferior AMF mutualist must have the capacity to limit plant growth in the presence of a superior AMF mutualist. However, in a mixture AMF propagule composition clearly shifted to favor the superior mutualist and not the inferior AMF mutualist as predicted. Overall, interspecific AMF interactions positively impacted sporulation of both AMF and were associated with a reduced AMF-related plant growth benefit.

Despite the greater costs to plants of supporting multiple AMF relative to monocultures of the superior AMF mutualist reported herein, at moderate concentrations of available P, the growth of plants supporting two fungi was greater than that of nonmycorrhizal plants. If plants cannot discriminate among inferior and superior AMF mutualists, support of multiple AMF may be the only alternative to being nonmycorrhizal. Such a scenario could account for the occurrence of multiple AMF in single root systems. Although this study provides no evidence that AMF functional complementarity leads to increased nutrient uptake or plant growth for individual plants co-inoculated with different AMF, it does provide some support with regard to glomalin production.

Acknowledgements

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