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Plant growth, phosphorus nutrition, and root morphological responses to arbuscular mycorrhizas, phosphorus fertilization, and intraspecific density

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Abstract We examined the effects of arbuscular mycorrhizas (AM), phosphorus fertilization, intraspecific density, and their interaction, on the growth, phosphorus uptake, and root morphology of three facultative mycotrophic crops (*Capsicum annuum*, *Zea mays*, and *Cucurbita pepo*). Plants were grown in pots with or without AM at three densities and four phosphorus availabilities for 10 weeks. AM colonization, plant weight, and shoot phosphorus concentration were measured at harvest. Root morphology was assessed for *C. annuum* and *Z. mays*. Phosphorus fertilization reduced but did not eliminate AM colonization of all species. AM, phosphorus, and density interacted significantly to modify growth of *C. annuum* and *C. pepo* such that increased density and phosphorus diminished beneficial effects of AM. Increased density reduced positive effects of AM on *C. annuum* and *C. pepo* shoot phosphorus concentrations. AM altered both *Z. mays* and *C. annuum* root morphology in ways that complemented potential phosphorus uptake by mycorrhizas, but increased density and phosphorus diminished these effects. We infer that increased density predominantly influenced plant responses by affecting whether or not carbon (photosynthate) or phosphorus limited plant growth. By exacerbating carbon limitation, high density reduced the benefit/cost ratio of mycorrhizas and minimized their effects.

Keywords Facultative mycotrophs · Intraspecific competition · Phosphorus-immobilizing soil · Row crops · Specific root length

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Introduction

Arbuscular mycorrhizas (AM, used to refer to all glomeromycotan mycorrhizas) are widely recognized to improve plant acquisition of sparsely available, poorly mobile mineral nutrients such as phosphorus, but this view is based largely upon studies of single plants grown in pots. Relatively little attention has been paid to how plant density influences the effects of mycorrhizas on their hosts. Moreover, the effects of augmentation of limiting soil resources on plant responses to density are difficult to predict. Wilson (1988) reviewed several studies of root and shoot competition and found there were similar numbers of reports of fertilizer addition increasing competition as there were of fertilization decreasing it. Most such studies have ignored mycorrhizas. Notwithstanding the ubiquity of AM and their acknowledged effects on nutrient uptake, the joint effects of plant density, soil fertility, and mycorrhizas have seldom been documented.

We know of only three plant growth studies that have demonstrated interactions among plant density, phosphorus availability, and mycorrhizas. The first two of these, by Hartnett et al. (1993) and Hetrick et al. (1994), are similar in that both examined responses to intraspecific density by *Andropogon gerardii* Vitman and *Elymus canadensis* L., inoculated with AM or not at the same two phosphorus levels in the same soil. Hetrick et al. (1994) additionally included *Koeleria pyramidata* (Lam.) Beauv. Neither study found effects of mycorrhizal inoculation or phosphorus fertilization on *E. canadensis*, although its growth was affected by density. In contrast, both studies found that at low phosphorus, non-inoculated *A. gerardii* failed to grow, but AM-inoculated plants did grow and compete. Thus, at low phosphorus, increased density diminished the size advantage of mycorrhizal *A. gerardii* plants over non-inoculated plants. *K. pyramidata* responded similarly (Hetrick et al. 1994). At high phosphorus availability, Hartnett et al. (1993) found that increased density did not affect mycorrhizal *A. gerardii* but did reduce the growth of non-inoculated plants. Consequently, at elevated phosphorus, increasing intraspecific density increased the growth

benefit of AM to *A. gerardii*. Hetrick et al. (1994) failed to find a significant effect of AM on either *A. gerardii* or *K. pyramidata* at high phosphorus, although growth of both species was diminished by increased density.

In the third study, Schroeder and Janos (2004) examined intraspecific density responses of four facultatively mycotrophic species with or without AM under nine different phosphorus additions. Mycorrhizas, phosphorus, and their interaction, significantly altered dry weight responses of *Capsicum annuum* L., *Coriandrum sativum* L., and *Lycopersicon esculentum* Mill., but not *Z. mays*, to intraspecific density. For *C. sativum* at low phosphorus availability, an unusual facilitative effect of density was observed. Increased density increased the growth only of mycorrhizal plants, consequently its net effect was to increase AM benefit. Schroeder and Janos (2004) attributed this to enhancement of AM colonization. At low phosphorus availability, increased density did not alter the other three species responses to AM. In contrast, at high phosphorus availability, increased density diminished the effects of AM on plant growth, reducing AM benefit to *C. annuum* and *L. esculentum*, and reducing AM detriment to *C. sativum*. A diminishing negative effect of AM on *C. sativum* growth with increased plant density may be similar to the increasing benefit of AM with density that Hartnett et al. (1993) found for *A. gerardii* at high phosphorus. Both are a consequence of density having a stronger effect on non-inoculated plants than on mycorrhizal plants.

Diminishing plant responses to AM as density increases have been attributed to the overlap of root and AM hypha phosphorus depletion zones (Hayman 1983). Inorganic phosphorus often occurs in low concentrations in soil and primarily moves to roots by diffusion. As phosphorus is absorbed rapidly, phosphorus depletion zones can form around roots and hyphae (Vance et al. 2003). Provided that root density is low, extraradical AM fungus hyphae can extend beyond root phosphorus depletion zones, thereby improving phosphorus uptake by mycorrhizal plants. Additionally, Howeler and Sieverding (1987) have suggested that AM hyphae may have a lower threshold for uptake of phosphorus than that of non-colonized plant roots, which would allow mycorrhizal plants access to phosphorus concentrations not available to non-colonized plants. Increased density of plants and of their roots, however, will increase overlap of root and AM hypha depletion zones, thus diminishing the benefit received from AM (Hayman 1983).

Positive growth effects of AM occur when the benefits of mycorrhizas exceed their carbon cost (Fitter 1991). If mycorrhiza carbon costs predominate when root and hypha phosphorus depletion zones overlap, growth depression may result. Moreover, effects of carbon drain on a host plant will be exacerbated if crown competition at high density reduces photosynthesis. In contrast to the situation at low phosphorus, at high phosphorus availability there may be little phosphorus uptake enhancement by AM, and if colonization is not eliminated by high phosphorus, the carbon cost of AM remains and may cause growth depression. Increased root growth at high phosphorus availability may increase the overlap of non-colonized plant root phosphorus depletion zones to a greater extent than those of mycorrhizal plants. If AM hyphae can avoid root phosphorus depletion zones or absorb phosphorus from lower solution concentrations that can non-colonized roots, they may thereby increase the supply of phosphorus to the host. That may increase the net growth benefit from AM, or diminish their detrimental effects.

Because of the importance of root and hypha phosphorus depletion zone overlap to mycorrhiza functioning, both phosphorus availability and density may indirectly influence the benefit to be gained from mycorrhizas by altering host root morphology. Added phosphorus has been shown to increase root diameter and, consequently, to reduce specific root length (SRL) (Powell 1974). Root density can modify root morphology, either through interference by space fragmentation (McConnaughay and Bazzaz 1992), or through reduced nutrient uptake (Caldwell and Richards 1986).

AM alter root morphology primarily when phosphorus is limiting (Berta et al. 1993), but the ways in which root morphology changes are not consistent. At low phosphorus availability, AM have decreased SRL, increased average root diameter (Price et al. 1989; Berta et al. 1993), increased branching in some cases (Berta et al. 1993), and decreased branching in others (Price et al. 1989). Mycorrhiza effects on root morphology have commonly been attributed to improvement of phosphorus uptake by AM, but AM effects on hormone production may also be responsible (Berta et al. 1993).

In this study, we examined the effects of phosphorus availability, intraspecific density, AM inoculation, and their interaction, on the growth, phosphorus uptake, and root morphology of three facultatively mycotrophic plant species. Only facultative mycotrophs can grow without AM, so only such species can be used to examine the effects of mycorrhizas on plant competition. We used three different species representing various growth forms (shrub, grass, and vine) from three different families to survey a potentially broad range of responses to AM and density. In contrast to our prior work, which investigated multiple levels of phosphorus addition (Schroeder and Janos 2004), the balanced design of this study (four phosphorus treatments coupled with three intraspecific densities applied to plants with and without AM inoculation) facilitated the examination of interactions among treatments. The principal questions we addressed are: (1) what levels of intraspecific density and available phosphorus maximize plant growth or phosphorus uptake benefits from AM, and (2) how do plant density, phosphorus availability, mycorrhizal inoculation, and their interaction affect root morphology.

Materials and methods

Experiment design

Capsicum annuum L. var. CATIE-8093 (Chile; Solanaceae), *Zea mays* L. var. Maizena (Corn; Poaceae), and *C. pepo* L. (Zucchini; Cucurbitaceae) were examined separately in fully factorial, three-factor pot experiments comprising: AM inoculation or none, four levels of applied phosphorus [low (P1), medium (P2), medium-high (P3), or high (P4)], and three levels of plant intraspecific density [low (D1), medium (D2), and high (D3)]. Each of the 24 combinations was replicated nine times resulting in 216 pots per species. The low density treatment was one individual per pot for all species, but medium and high density treatments differed among species depending upon their individual growth characteristics. *Capsicum annuum* D2 and D3 comprised 7 and 12 individuals per pot, *Z. mays* D2 and D3 5 and 15 individuals per pot, and *C. pepo* D2 and D3 5 and 12 individuals per pot, respectively. All experiments were conducted in greenhouses at the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE) in Turrialba, Cartago, Costa Rica from 6 July through 9 December 2001. During this time, mean maximum and minimum temperatures were 29.1 and 18.4°C, respectively.

Capsicum annuum seeds were provided by CATIE, *Z. mays* seeds were donated by local farmers in Agua Buena, Coto Brus, Costa Rica, and *C. pepo* seeds (produced by Ohlsens-Enke, Tastrup, Denmark) were purchased from a seed store in Turrialba, Costa Rica. Seeds of all species were surface-disinfected (10% NaOCl) for 2 min and germinated on moist filter paper in Petri dishes in a lighted chamber at 25°C for 3–7 days. Germinated seeds of each species were planted in a regular arrangement in 3.79 l (1 gallon), 16 cm diameter, black plastic pots that contained 1,472 g (dry weight) of a 3:1 (v/v) soil/sand mixture.

The soil for all experiments, a tropical Umbric Andosol (Donald Kass, CATIE, personal communication), was collected to a depth of 60 cm from San Juan Sur (10° 52′ 50″ N, 83° 41′50″ W). Soil was homogenized after collection, and then, to improve drainage, was mixed 3:1 (v/v) with medium-to-coarse sand collected from a nearby riverbed. The soil/sand mixture had very low available phosphorus (2.9 mg l^{-1} Olsen extractable P) and a high phosphorus retention capacity (97.5%) as determined by the New Zealand method (Saunders 1965). The soil/sand mixture was autoclaved at 118°C at 1 kg cm^{-2} for 60 min, held at room temperature for 24 h, and autoclaved again for 60 min.

All plants were watered daily except on days that fertilizers were applied. For each species, pots were fully randomized, spaced approximately 25 cm apart atop benches. Because of the rapid growth of *C. pepo* vines, 3 weeks after planting, stakes were placed in pots at frequencies of one for D1, two for D2, and four for D3 to prevent vines entangling with neighboring pots. At that time, Rally 40 WP fungicide (manufactured by Rohm and Haas, Philadelphia, Pa.) was applied once to *C. pepo* leaves to combat a powdery mildew that infected all treatments. *Cucurbita pepo* flushed new leaves the following week.

Mycorrhiza inoculation

Inoculated and non-inoculated treatments received either live or autoclaved AM inoculum, respectively, half of which was mixed thoroughly with the soil and half placed in planting holes. Pots were capped with 150 ml sterilized sand to prevent splash contamination. *Capsicum annuum* received 4 g total fresh weight of inoculum, and the other two species received 10 g total. The AM inoculum was a mixture of soil, chopped roots, and spores derived from pot cultures of mixed *Glomus* spp. raised on *Bromus* sp. for several months under greenhouse conditions at CATIE. This inoculum originated from collections at the Centro International de Agricultura Tropical (CIAT, Cali, Colombia). Prior examination (G. Rivas-Platero, unpublished) of these pot cultures found more than 200 spores of four to five *Glomus* spp. per 100 g. A microbial filtrate, excluding AM fungus propagules, was prepared by soaking 100 g fresh weight AM inoculum in 7 l water for 3 days and then filtering the resulting infusion through Whatman no. 4 filter paper. Non-inoculated pots of all species were prepared in the same manner as the AM inoculated pots, the only difference being that each non-inoculated pot received autoclaved AM inoculum and 100 ml of this microbial filtrate approximately 1 week after planting. At the same time, 100 ml sterilized water was added to each AM-inoculated pot.

Phosphorus and base nutrient addition

Phosphorus treatments were applied as 150 ml per pot of soluble $NaH_2PO_4{\cdot}H_2O$ in four different concentrations each week beginning 1 week after planting. Because *C. annuum* is known to have a high P requirement (Schroeder and Janos 2004), 100, 200, 400, and 800 mg P l^{-1} solutions were applied. For *Z. mays* and *C. pepo*, phosphorus was applied as 25, 50, 100, and 200 mg P l^{-1} solutions. Base nutrients were supplied each week as 200 ml per pot of modified Long Ashton solution lacking P with the composition (g l^{-1}): 1.01 g KNO_3, 1.574 g $Ca(NO_3)_2{\cdot}4H_2O$, 0.738 g $MgSO_4{\cdot}7H_2O$, 0.049 g ferric citrate, 0.0034 g $MnSO_4{\cdot}7H_2O$, 0.0005 g $CuSO_4{\cdot}5H_2O$, 0.0006 g $ZnSO_4{\cdot}7H_2O$, 0.0037 g H_3BO_3, and 0.0001 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$.

Harvest

All plants were harvested 10 weeks after planting, which was before flowering by *C. annuum* and *Z. mays*. *Cucurbita pepo* began producing flower buds 3 weeks after planting, so all flower buds were excised repeatedly as needed to maintain vegetative growth. For each species, fine roots were carefully washed free from the soil/sand mixture above a 250 μm sieve. Because fine roots were distributed relatively uniformly within pots, a sample (ca. 20% of root fresh weight) was removed by haphazard clipping throughout the extracted root systems. Root samples were weighed and stored in 50% alcohol for subsequent assessment of

AM colonization and root morphology. All shoots were separated, dried, and then weighed individually. Because individual root systems were impossible to separate, total roots per pot were dried at 60°C for 96 h and then weighed. Mean shoot and root dry weights per pot were calculated by total shoot or root dry weights per pot divided by plant density per pot. All shoots were ground and homogenized per pot and the three pots of *Z. mays* and *C. pepo*, and the four pots of *C. annuum* that had the highest total dry biomass were selected from each treatment for determination of shoot phosphorus concentrations at the CATIE Nutrient Analysis Laboratory. Resources did not permit analysis of all pots, and so those with the largest plants were used for comparability and to represent a bounding condition. Ground tissue samples were ashed in a muffle furnace at 550°C for 4 h, dissolved in HCl, and phosphorus concentrations were determined by colorimetric analysis.

Soil samples (200 ml) were collected from each pot and homogenized within each phosphorus treatment per species for analysis of available phosphorus and phosphorus retention. Sub-samples were extracted in modified (by addition of EDTA) Olsen solution, and available P concentrations were determined by colorimetric analysis (Olsen and Sommers 1982). Soil retention of phosphate was determined by the New Zealand method (Saunders 1965).

Assessment of AM colonization and root morphology

Root samples were examined for AM colonization after clearing in 10% KOH, acidifying in HCl, and staining with 0.05% Trypan Blue in acid glycerol. The proportion of root length internally colonized by AM fungi was determined by the gridline intersection method (Giovannetti and Mosse 1980) with a dissecting microscope (20–40×) using 200 intersections per sample. Selected colonized root segments were mounted on slides for confirmation of their AM status by examination with a compound microscope. Before staining, *C. annuum* and *Z. mays* root sample total length, average diameter, and number of forks (root branch points) were measured with a WinRHIZO (version 3.9e, Régent Instruments, Québec, Canada) image analysis system (scanned at 600 dpi). SRL was calculated as sample total root length divided by sample dry weight.

Data analysis

For each species, percent AM colonized root length of inoculated plants was analyzed by two-way analysis of variance (ANOVA) using applied phosphorus and density as treatment factors. Mean individual root, shoot, and total dry weights, and root-to-shoot ratios per pot for each species were analyzed for treatment effects by three-way multivariate analysis of variance (MANOVA) with AM inoculation, applied phosphorus, and density as between-subject factors. For each species, shoot phosphorus concentrations and total shoot phosphorus contents were analyzed by three-way ANOVA with AM inoculation, applied phosphorus, and density as treatment factors. SRL, average root diameter, and number of forks per sample dry weight were analyzed for each species assessed by three-way MANOVA using the same factors as for biomass analyses. Homogeneity of variance was tested with Levene's Test for MANOVA or Bartlett's Test for ANOVA. If necessary, data were transformed (arcsine square root transformation for colonization, square root transformation for *C. annuum* biomass, $\log_{10}$ transformation for *Z. mays* and *C. pepo* biomass, and $\log_{10}$ transformation for SRLs and number of forks per dry weight). Significant MANOVA results were subsequently examined with univariate tests (three-way ANOVAs) to determine which of the dependent variables contributed significantly to differences. For each MANOVA, a Type III sum of squares was used in SPSS v. 10.1.0 (SPSS 2000). All other analyses were performed with Statistix v. 7.0 (Statistix 2000).

Results

AM colonization

Fine roots of all non-inoculated plants showed negligible AM colonization (n=105, mean =1.7%, ±0.39 SE) with just one non-inoculated *C. annuum* plant (P1-D1) having a colonization level (12%) similar to those of inoculated plants. That plant was excluded from analyses. Inoculation produced AM on all species, and colonization ranged from 1.5% to 20.4% in *C. annuum* roots, from 8% to 48.3% in *Z. mays* roots, and from 4.4% to 38.7% in *C. pepo* roots.

At harvest, soil bulked from the *Z. mays* and *C. pepo* experiments within each phosphorus addition of 25, 50, 100, and 200 mg P l^{-1} showed that 3.6, 3.2, 5.7, and 10.8 mg l^{-1} Olsen P remained available in the soil, respectively. Soil bulked from the *C. annuum* experiment within each phosphorus addition of 100, 200, 400, and 800 mg P l^{-1} showed that 6.9, 10.9, 17.4, and 35.2 mg l^{-1} Olsen P remained available in the soil, respectively. Two-way ANOVAs revealed that *C. annuum* AM colonization at harvest was reduced by soluble P addition ($F_{3,92}$=5.28, P=0.002) and by increased density ($F_{2,92}$=5.19, P=0.007), but that these factors did not interact ($F_{6,92}$=1.91, P=0.088) (Fig. 1). Phosphorus availability and density interacted significantly to affect the colonization of *Z. mays* roots ($F_{6,96}$=2.29, P=0.041), such that increased density decreased AM colonization only at phosphorus additions of 50 and 200 mg P l^{-1} (Fig. 1). *Cucurbita pepo* root colonization by AM at harvest significantly decreased with increased phosphorus ($F_{3,96}$=13.2, P<0.0001), but was affected neither by increased density ($F_{2,96}$=1.95, P=0.149) nor by the interaction of phosphorus and density ($F_{6,96}$=1.07, P=0.388) (Fig. 1).

Plant growth

Both MANOVA (Table 1) and separate ANOVA analyses (Table 2) revealed that all *C. annuum* dependent growth

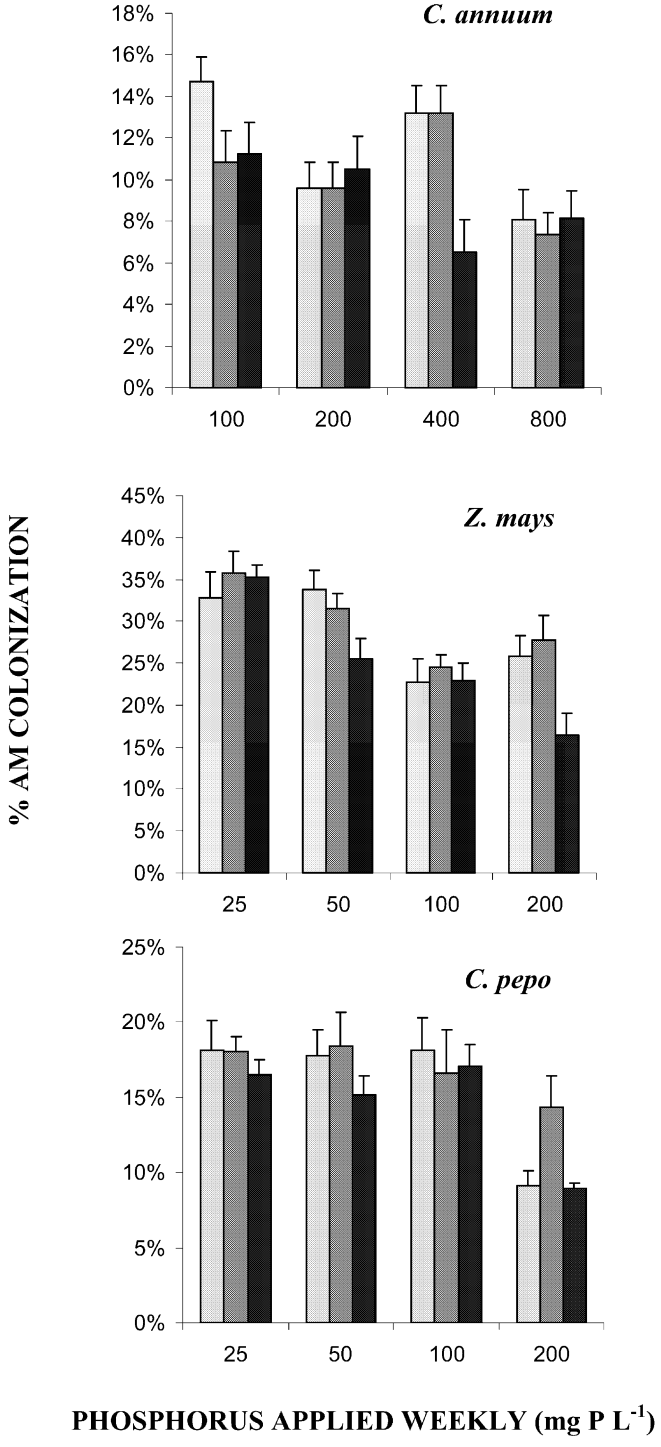

Fig. 1 Mean (+1 SE; *n*=9) arbuscular mycorrhizal (AM) colonization per inoculated pot versus concentration of phosphorus in 150 ml water applied weekly to *C. annuum*, *Z. mays*, and *C. pepo*. *Light*, *medium*, and *dark shaded bars* represent low density, medium density, and high density treatments, respectively

variables (total, shoot, root, and root:shoot dry weights) were significantly affected by the interaction of AM inoculation, phosphorus, and density. At low phosphorus and low density, AM inoculation significantly increased *C. annuum* total dry weight. At low density, benefit from AM became a detriment with increased phosphorus (Fig. 2; Table 2). At medium and high density, AM became neutral (no net effect of AM, Fig. 2). Although MANOVA results suggested an overall effect of AM and density on *Z. mays* (Table 1), the results of separate ANOVA analyses showed neither a main effect of inoculation nor an interaction between AM and density for any dry weight variable (Fig. 2; Table 2). For both *C. annuum* and *Z. mays*, phosphorus and density significantly interacted (Table 2), such that these species had the greatest total dry weight response to phosphorus addition at low density (Fig. 2). When dependent variables were analyzed separately for *C. pepo*, only root-to-shoot ratio was significantly affected by the interaction of AM inoculation, phosphorus, and density (Table 2). AM inoculation and phosphorus significantly interacted such that at low phosphorus, AM inoculation decreased *C. pepo* total dry weight and shoot dry weight and this AM detriment diminished or reversed with increased phosphorus (Fig. 2; Table 2). AM inoculation and density interacted significantly such that AM inoculation reduced *C. pepo* root dry weight as density increased (Fig. 2; Table 2).

Plant phosphorus

Phosphorus addition significantly increased shoot phosphorus concentrations of *C. annuum* and *C. pepo*, but not *Z. mays* (Fig. 3; Table 3). Increased density significantly decreased shoot phosphorus concentrations of *C. annuum* and *Z. mays*, but increased *C. pepo* shoot phosphorus concentration (Fig. 3; Table 3). For *C. annuum*, AM inoculation significantly increased shoot phosphorus concentration at low density and this AM benefit was reduced as density increased (Fig. 3), accounting for the significant interaction of AM inoculation and density (Table 3). For *Z. mays*, AM generally had no effect on shoot phosphorus concentration, but AM marginally significantly (P=0.067 for MYC×DEN) increased shoot phosphorus concentration at high density (Fig. 3; Table 3). At low density, AM inoculation significantly increased *C. pepo* shoot phosphorus concentration, and increased density diminished this effect (Fig. 3; Table 3).

Total shoot phosphorus contents (not shown) were significantly increased by phosphorus addition for *C. annuum* ($F_{3,71}$=24.99, P<0.0001), *Z. mays* ($F_{3,47}$=35.51, P<0.0001), and *C. pepo* ($F_{3,48}$=20.81, P<0.0001). In contrast, increased density significantly decreased total shoot phosphorus contents (*C. annuum*: $F_{2,71}$=158.16, P=<0.0001; *Z. mays*: $F_{2,47}$=218.70, P<0.0001; and *C. pepo*: $F_{2,48}$=580.71, P<0.0001). Density had its largest effect at high phosphorus for all species as demonstrated by significant interactions between phosphorus and density (*C. annuum* $F_{6,71}$=14.09, P=<0.0001; *Z. mays* $F_{6,47}$=21.69, P<0.0001, *C. pepo* $F_{6,48}$=7.16, P<0.0001). For *C. annuum* and *Z. mays*, AM inoculation did not significantly affect total shoot phosphorus content (*C. annuum* $F_{1,71}$=0.83, P=0.364; *Z. mays* $F_{1,47}$=1.57, P=0.216). For *C. pepo*, AM inoculation

Table 1 Results from three-way MANOVAs of dry weight data (total, shoot, root, and root: shoot) for *C. annuum*, *Z. mays*, and *C. pepo*. AM inoculation, phosphorus addition, and density are abbreviated as *MYC*, *P* and *DEN*, respectively. Degrees of freedom (*df*) shown as hypothesis *df*, and error *df*. Significant *P* values ($P<0.05$) in bold. See Table 2 for subsequent three-way univariate analyses

Species	Factor	Wilk's λ	*df*	*F*	*P*
C. annuum	MYC	0.946	4, 183	2.593	**0.038**
	P	0.657	12, 484	6.944	**<0.0001**
	DEN	0.299	8, 366	37.849	**<0.0001**
	MYC×P	0.796	12, 484	3.637	**<0.0001**
	MYC×DEN	0.904	8, 366	2.356	**0.018**
	P×DEN	0.720	24, 640	2.627	**<0.0001**
	MYC×P×DEN	0.724	24, 640	2.582	**<0.0001**
Z. mays	MYC	0.933	4, 186	3.341	**0.011**
	P	0.421	12, 492	15.849	**<0.0001**
	DEN	0.196	8, 372	58.607	**<0.0001**
	MYC×P	0.968	12, 492	0.502	0.914
	MYC×DEN	0.903	8, 372	2.432	**0.014**
	P×DEN	0.566	24, 650	4.805	**<0.0001**
	MYC×P×DEN	0.886	24, 650	0.959	0.521
C. pepo	MYC	0.880	4, 189	6.420	**<0.0001**
	P	0.906	12, 500	1.592	0.090
	DEN	0.049	8, 378	167.09	**<0.0001**
	MYC×P	0.830	12, 500	3.050	**<0.0001**
	MYC×DEN	0.906	8, 378	2.393	**0.016**
	P×DEN	0.768	24, 660	2.158	**0.001**
	MYC×P×DEN	0.794	24, 660	1.876	**0.007**

significantly increased total shoot phosphorus contents at low density, but increased density diminished this AM benefit as revealed by a significant interaction between AM and density ($F_{2,48}$=7.55, P=0.001).

Root morphology

Three-way interactions of AM, phosphorus, and density were significant for both *C. annuum* and *Z. mays* (Table 4), but root morphological responses differed between these

Table 2 *P* values from univariate analyses of dry weights (total, shoot, root, and root: shoot) for *C. annuum*, *Z. mays*, and *C. pepo*. Factors as in Table 1. Significant *P* values ($P<0.05$) in bold

Species	Factor	*df*	Total dry weight	Shoot dry weight	Root dry weight	Root: Shoot
C. annuum	MYC	1	0.229	0.209	0.486	0.800
	P	3	**<0.0001**	**<0.0001**	**<0.0001**	**0.002**
	DEN	2	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	MYC×P	3	**0.030**	**0.033**	**0.044**	0.489
	MYC×DEN	2	0.285	0.207	0.988	**0.027**
	P×DEN	6	**<0.0001**	**<0.0001**	**0.018**	**0.001**
	MYC×P×DEN	6	**0.026**	**0.033**	**0.002**	**0.022**
	ResiduaL	186				
Z. mays	MYC	1	0.780	0.612	0.426	0.166
	P	3	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	DEN	2	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	MYC×P	3	0.468	0.485	0.517	0.769
	MYC×DEN	2	0.773	0.814	0.452	0.072
	P×DEN	6	**<0.0001**	**<0.0001**	**0.009**	0.052
	MYC×P×DEN	6	0.768	0.785	0.760	0.542
	Residual	189				
C. pepo	MYC	1	0.083	0.194	**0.005**	**<0.001**
	P	3	0.808	0.842	0.288	0.071
	DEN	2	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	MYC×P	3	**0.044**	**0.020**	0.901	0.279
	MYC×DEN	2	0.082	0.170	**0.002**	**0.001**
	P×DEN	6	0.383	0.268	0.356	**0.047**
	MYC×P×DEN	6	0.971	0.887	0.440	**0.015**
	Residual	192				

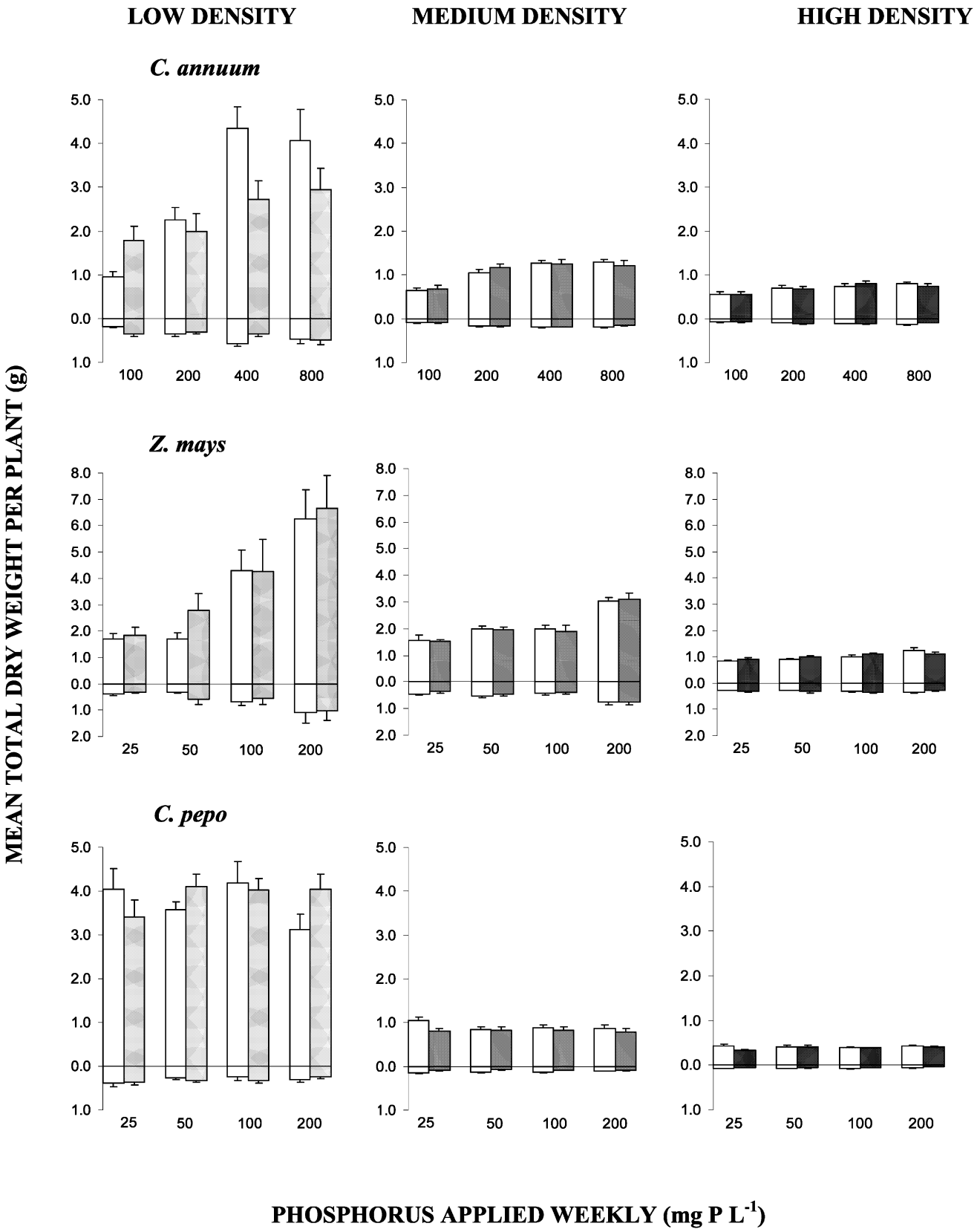

Fig. 2 Mean (+1 SE; *n*=9) shoot (above the x-axis) and root (positive values below the x-axis) dry weights per plant at harvest of non-inoculated (*open bars*) and inoculated (*solid bars*) *C. annuum*, *Z. mays*, and *C. pepo* versus phosphorus applied weekly at low (*light shading*), medium (*medium shading*), and high density (*dark shading*)

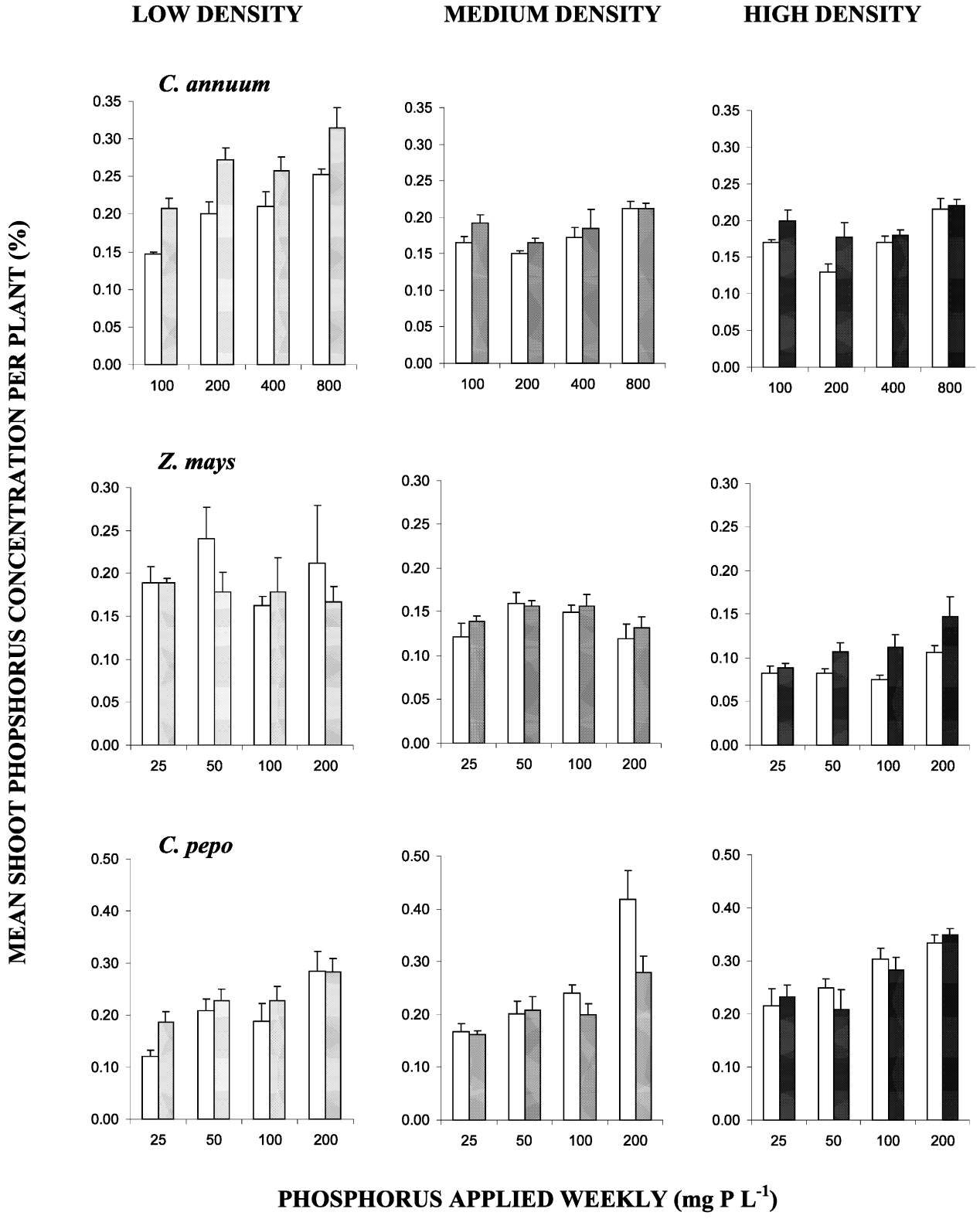

Fig. 3 Mean (+1 SE; *C. annuum* *n*=4, *Z. mays* and *C. pepo* *n*=3) shoot phosphorus concentrations (%) per plant at harvest of non-inoculated (*open bars*) and inoculated (*solid bars*) *C. annuum*, *Z. mays*, and *C. pepo* versus phosphorus applied weekly at low (*light shading*), medium (*medium shading*), and high (*dark shading*) density

Table 3 Results from three-way ANOVAs for mean shoot P concentration per plant for *C. annuum*, *Z. mays*, and *C. pepo*. Factors as in Table 1. Significant *P* values ($P<0.05$) in bold

Species	Factor	*df*	*SS*	*F*	*P*
C. annuum	MYC	1	0.0255	35.28	**<0.0001**
	P	3	0.0515	23.80	**<0.0001**
	DEN	2	0.0541	37.50	**<0.0001**
	MYC×P	3	0.0023	1.08	0.365
	MYC×DEN	2	0.0099	6.88	**0.002**
	P×DEN	6	0.0235	5.43	**0.001**
	MYC×P×DEN	6	0.0014	0.31	0.9289
	Residual	71	0.0512		
Z. mays	MYC	1	0.0003	0.24	0.623
	P	3	0.0038	0.95	0.426
	DEN	2	0.0954	35.31	**<0.0001**
	MYC×P	3	0.0026	0.63	0.599
	MYC×DEN	2	0.0076	2.82	0.067
	P×DEN	6	0.0112	1.38	0.241
	MYC×P×DEN	6	0.0049	0.61	0.722
	Residual	47	0.0635		
C. pepo	MYC	1	0.0007	0.33	0.567
	P	3	0.2025	33.40	**<0.0001**
	DEN	2	0.0389	9.62	**0.0003**
	MYC×P	3	0.0107	1.77	0.165
	MYC×DEN	2	0.0176	4.37	**0.018**
	P×DEN	6	0.0199	1.64	0.157
	MYC×P×DEN	6	0.0161	1.33	0.263
	Residual	48	0.09700		

two species (Figs. 4, 5). When root morphology dependent variables were analyzed separately for *C. annuum*, AM inoculation significantly increased SRL and increased phosphorus enhanced this AM effect while increased density diminished it (Fig. 4; Table 5). The average diameter of *C. annuum* roots was not significantly affected by AM inoculation as a main effect or by its interaction with other factors (Table 5), but increased density significantly increased *C. annuum* average root diameter at high phosphorus addition (Fig. 4; Table 5). AM inoculation, phosphorus, and density significantly interacted to affect *C. annuum* number of forks (root branch points per dry weight) such that at low phosphorus and low density, AM increased the number of forks of *C. annuum* roots but increased phosphorus and density diminished this effect (Fig. 4; Table 5).

For *Z. mays*, AM, phosphorus, and increased density interacted significantly for all root morphology variables (Table 5). Although AM did increase *Z. mays* SRL at particular low-to-medium phosphorus and density levels, increased phosphorus and density diminished these AM effects (Fig. 5). At low phosphorus, AM significantly decreased *Z. mays* average root diameter, while increased phosphorus and increased density diminished these AM effects (Fig. 5). AM significantly increased *Z. mays* number of forks and both increased phosphorus and density diminished this effect of AM (Fig. 5).

Discussion

AM colonization

As commonly observed for facultatively mycotrophic plant species (e.g., Amijee et al. 1989), phosphorus fertilization significantly decreased AM colonization of all three host species in our study (Fig. 1). AM colonization persisted in spite of high phosphorus additions, however, perhaps because of the high phosphorus immobilization capacity of the tropical soil that we used coupled with potential leaching loss of the soluble phosphorus that was applied (Schroeder and Janos 2004). Although elevated plant density might have been expected to increase AM colonization by reducing the amount of phosphorus available per individual, in this study increased density reduced AM colonization of *C. annuum* and *Z. mays* (Fig. 1), as has been observed for other species (Koide 1991; Facelli et al. 1999). High plant density might increase shoot competition, thereby diminishing photosynthate available to sustain

Table 4 Results from three-way MANOVAs of root morphological data (specific root length, average root diameter, and number of forks per gram dry weight) for *C. annuum* and *Z. mays*. Factors as in Table 1. Significant *P* values ($P<0.05$) in bold. See Table 5 for subsequent three-way univariate analyses

Species	Factor	Wilk's λ	*df*	*F*	*P*
C. annuum	MYC	0.971	3, 184	1.857	0.138
	P	0.886	9, 448	2.550	**0.007**
	DEN	0.894	6, 368	3.529	**0.002**
	MYC×P	0.880	9, 448	2.691	**0.005**
	MYC×DEN	0.951	6, 368	1.575	0.153
	P×DEN	0.744	18, 521	3.182	**<0.0001**
	MYC×P×DEN	0.778	18, 521	2.682	**<0.0001**
Z. mays	MYC	0.807	3, 187	14.936	**<0.0001**
	P	0.859	9, 455	3.254	**0.001**
	DEN	0.541	8, 374	22.386	**<0.0001**
	MYC×P	0.844	9, 455	3.655	**<0.0001**
	MYC×DEN	0.879	8, 374	4.136	**<0.0001**
	P×DEN	0.778	18, 529	2.733	**<0.0001**
	MYC×P×DEN	0.703	18, 529	3.910	**<0.0001**

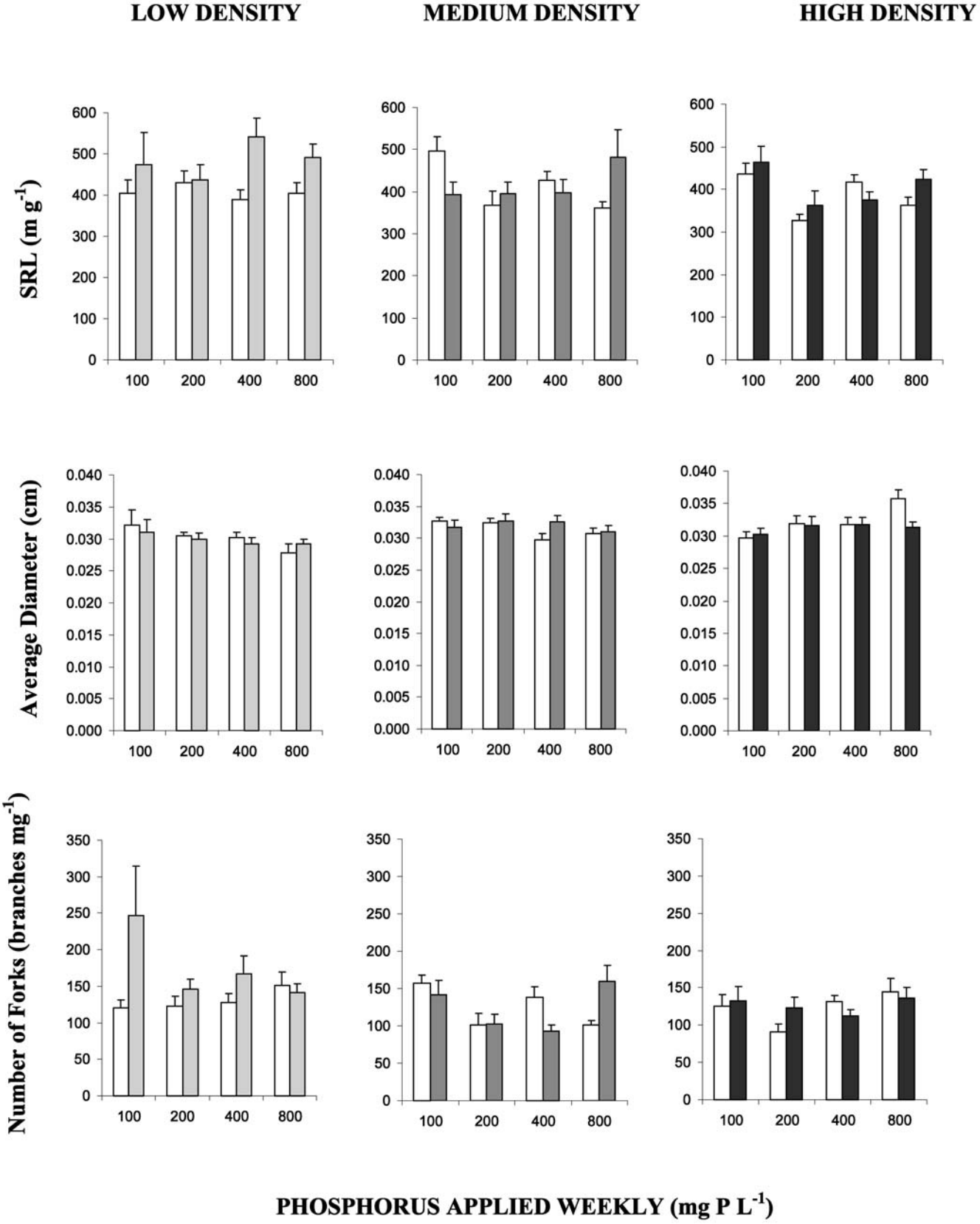

Fig. 4 Mean (+1 SE; n=9) specific root length (SRL) (mg^{-1}), average root diameter (cm), and number of forks (root branch points mg^{-1}) per plant at harvest of non-inoculated (*open bars*) and inoculated (*solid bars*) *C. annuum* versus phosphorus applied weekly at low (*light shading*), medium (*medium shading*), and high (*dark shading*) density

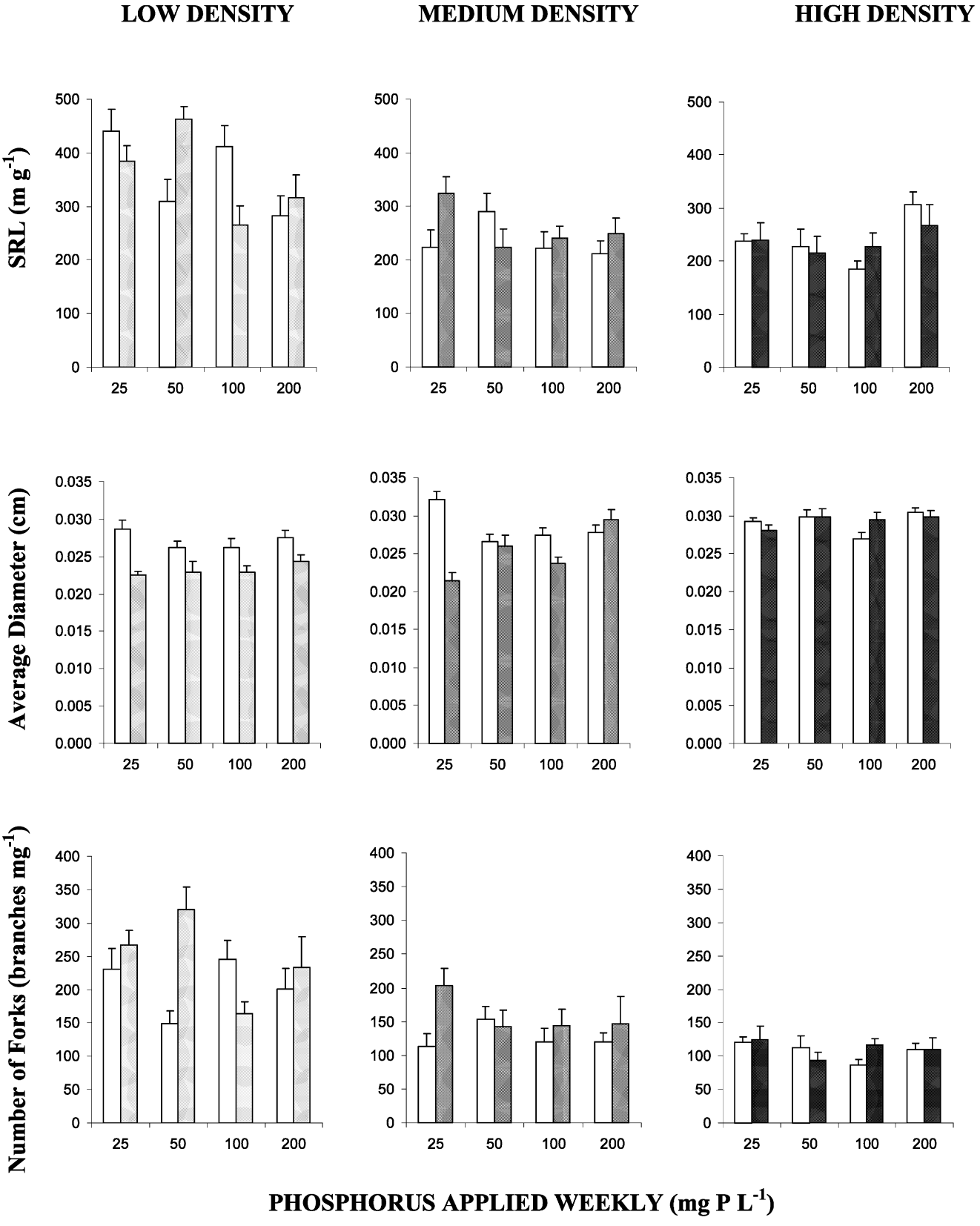

Fig. 5 Mean (+1 SE; n=9) SRL (mg^{-1}), average root diameter (cm), and number of forks (root branch points mg^{-1}) per plant at harvest of non-inoculated (*open bars*) and inoculated (*solid bars*) *Z. mays* versus phosphorus applied weekly at low (*light shading*), medium (*medium shading*), and high (*dark shading*) density

Table 5 *P* values for univariate analyses of specific root length (SRL), average root diameter, and number of forks per gram dry weight for *C. annuum* and *Z. mays*. Factors as in Table 1. Significant *P* values ($P<0.05$) in bold

Species	Factor	*df*	SRL	Diameter	No. forks
C. annuum	MYC	1	**0.019**	0.565	0.077
	P	3	**0.012**	0.773	**0.001**
	DEN	2	**0.009**	**0.003**	**0.002**
	MYC×P	3	0.059	0.656	0.136
	MYC×DEN	2	0.086	0.330	0.054
	P×DEN	6	0.206	**0.005**	0.543
	MYC×P×DEN	6	**0.025**	0.161	**0.004**
	Residual	186			
Z. mays	MYC	1	0.619	**<0.0001**	**0.033**
	P	3	0.082	**0.004**	0.101
	DEN	2	**<0.0001**	**<0.0001**	**<0.0001**
	MYC×P	3	0.847	**<0.0001**	0.480
	MYC×DEN	2	0.519	**<0.0001**	0.418
	P×DEN	6	**0.034**	0.636	0.955
	MYC×P×DEN	6	**<0.0001**	**0.001**	**<0.0001**
	Residual	189			

AM fungi, or inoculum might be diluted by high root density (Koide and Dickie 2002). Either or both effects might override contravening effects on per capita phosphorus availability. We supplied less inoculum to *C. annuum* than to other species, and *Z. mays* roots grew rapidly, both consistent with density reducing AM colonization by means of an inoculum dilution effect. Although increased density did reduce shoot phosphorus concentrations of both *C. annuum* and *Z. mays*, and diminished internal phosphorus concentrations may lead to higher AM colonization (Graham et al. 1981), our contrary findings of reduced AM colonization further suggest that this was a consequence of inoculum dilution during these relatively short experiments (ca. 10 weeks). Nevertheless, we cannot rule out a complementary effect of photosynthate limitation on AM colonization at high density. Regardless of its cause, reduction of AM colonization by increased phosphorus and density may have exacerbated diminution of mycorrhiza effects at high phosphorus and high density.

Plant growth

As we expected because of their taxonomic and growth form diversity, *C. annuum*, *Z. mays*, and *C. pepo* responded differently to phosphorus availability, intraspecific density, and AM. For all species, increased density significantly diminished all dry weight variables (Fig. 2; Table 1), demonstrating that intraspecific competition occurred. The plant densities we employed correspondingly diminished both positive and negative effects of mycorrhizas. Although MANOVA (Table 1) revealed that dry weights of all three species were affected by mycorrhizas, these AM effects significantly interacted with phosphorus and density for *C. annuum* and *C. pepo*, and significantly interacted with density for *Z. mays*. Species' growth responses to AM inoculation ranged from positive through neutral to negative depending upon phosphorus availability and/or intraspecific density. Understanding the conjoint effects of phosphorus availability and plant density may be fundamental to understanding the responses of facultatively mycotrophic plant species to mycorrhizas.

At low density, phosphorus fertilization considerably increased *C. annuum* and *Z. mays* growth, but did not affect *C. pepo* growth at any density (Fig. 2). The latter observation suggests that phosphorus availability did not limit the growth of *C. pepo* in our experiment. The highly significant main effect of phosphorus fertilization, increasing *C. pepo* shoot phosphorus concentrations but not increasing dry weights, further supports this conclusion. *Cucurbita pepo* growth was most likely limited by light because of being staked and thereby self-shading, besides having lost leaves to a powdery mildew. Phosphorus fertilization of *C. annuum* and *Z. mays* at high density was ineffective at stimulating growth, and similarly suggests light limitation of growth at high density. Because of the carbon cost of persistent mycorrhizas (Fitter 1991), high phosphorus can contribute to growth depression by AM. In the same way, when light availability and photosynthesis are limiting, AM can cause growth depression (Son and Smith 1988). At high phosphorus, however, light limitation with increased plant density may reduce the growth of non-colonized plants more than that of already carbon-limited AM plants, hence increased density at high phosphorus availability might reduce growth disadvantages of AM as we observed for *C. annuum* in this study and for *Coriandrum sativum* in our previous study (Schroeder and Janos 2004).

Prior studies of intraspecific density effects on plant responses to AM have found the benefits of mycorrhizas to diminish as density increases (Koide 1991; Allsopp and Stock 1992; Hartnett et al. 1993; Hetrick et al. 1994; Facelli et al. 1999; Schroeder and Janos 2004), as we found for *C. annuum* at low phosphorus. Although diminution of mycorrhiza benefit might be caused at least in part by reduced AM colonization, increased plant density does not always significantly reduce colonization (e.g., Schroeder and Janos 2004) although diminishing the benefit of mycorrhizas. Consequently, diminution of AM enhancement of plant growth with increasing plant density is most often attributed to increased overlap of root and hypha phosphorus depletion zones, which greatly reduce or eliminate phosphorus uptake benefits of mycorrhizas without reducing their carbon cost to the host (e.g., Hayman 1983; Koide and Dickie 2002).

In this study, no host species responded to phosphorus fertilization at high density (Fig. 2), suggesting that carbon was limiting. Hence, neither phosphorus fertilization nor mycorrhizas could compensate for increased plant density. In contrast, Hartnett et al. (1993) found that at high phosphorus, increased density amplified the growth differential between mycorrhizal and non-colonized *A. gerardii* by reducing the growth of only the non-colonized plants. In that case, it is likely that phosphorus, not light, limited the

growth of those plants. Mycorrhizas were able to overcome the phosphorus limitation. Notwithstanding the potential for increased density to expand the overlap of root and hypha phosphorus depletion zones, at elevated phosphorus availability mycorrhizal fungus hyphae must have been able to access more phosphorus than was available to non-colonized roots over the duration of the Hartnett et al. (1993) experiment. AM fungal hyphae may increase phosphorus uptake as far as 11 cm from roots (Li et al. 1991), and hence may quickly exploit a larger soil volume than roots. Moreover, Howeler and Sieverding (1987) have suggested that AM fungus hyphae may be able to absorb phosphorus from lower solution concentrations than can roots.

Plant phosphorus

In our experiments, mycorrhizas had a significant beneficial main effect on shoot phosphorus concentration of *C. annuum*, and mycorrhizas interacted significantly with density for *C. annuum*, *C. pepo*, and marginally significantly for *Z. mays* shoot phosphorus concentrations (Table 3). Increased shoot phosphorus concentrations of mycorrhizal *C. annuum* and *Z. mays* at high density (Fig. 3) in the absence of a mycorrhiza effect on growth (Fig. 2) supports our contention that phosphorus did not limit growth at high density. Total shoot phosphorus content of these species was not significantly affected by mycorrhizas. The latter result suggests that elevation by mycorrhizas of the shoot phosphorus concentrations of *C. annuum* at low density and medium and high phosphorus was a consequence of the carbon cost of mycorrhizas retarding growth (see Stribley et al. 1980), and was not a consequence of mycorrhizas improving phosphorus acquisition.

Main effects of phosphorus fertilization on both *C. pepo* shoot phosphorus concentration (Fig. 3; Table 3) and total shoot phosphorus content in the absence of a main effect of phosphorus fertilization on *C. pepo* growth (Table 1) affirms that phosphorus was not limiting to *C. pepo* in our experiment. Density increased *C. pepo* shoot phosphorus concentrations (Fig. 3) even though diminishing total shoot phosphorus contents, probably because carbon limitation hampered plant growth more than any phosphorus uptake limitation caused by overlap of root phosphorus depletion zones. Although mycorrhizas increased both shoot phosphorus concentration and content of *C. pepo* at low density and low phosphorus availability, this was insufficient to enhance *C. pepo* growth. Nevertheless, in consequence of their improved phosphorus status, mycorrhizal individuals of other plant species have had greater reproductive yields (Bryla and Koide 1990), increased seed phosphorus contents (Koide and Lu 1992), and greater offspring survival (Heppell et al. 1998) than non-colonized individuals. Therefore, even in the absence of growth improvement by mycorrhizas of *C. pepo* and *Z. mays* in this study, their AM fungus symbionts may be mutualists.

Root morphology

Besides significantly improving the growth of *C. annuum* and the phosphorus content of *C. pepo*, mycorrhizas had a significant main effect on *Z. mays* root morphology and significant interactions with phosphorus and density for *Z. mays* and *C. annuum* root morphology (Table 4). Mycorrhizas increased SRL of *Z. mays* and *C. annuum* at some phosphorus applications at both low and medium plant densities, but not at high density (Figs. 4, 5; Table 5). Concomitantly, the average diameter of *Z. mays* fine roots was diminished by mycorrhizas although that of *C. annuum* was not affected significantly. Our results differ from those of other studies that found mycorrhizas decreased SRL while increasing diameters, especially under conditions of low phosphorus availability (Price et al. 1989; Berta et al. 1993). In our study, root branching of both hosts was increased by mycorrhizas at some phosphorus applications except at high density. Berta et al. (1993) similarly reported that AM increased branching of *Allium porrum* L. roots, but Price et al. (1989) found that AM decreased *Gossypium hirsutum* L. root branching. Such seemingly contradictory results among studies of effects of mycorrhizas on root morphology may arise because of different limitations on plant growth that affect allocation of energy and materials between roots and shoots. In our experiments, plant density had significant main effects of reduction of root branching for both hosts examined, which might be a direct consequence of carbon limitation at elevated density. In contrast, mycorrhizas increased root branching of both hosts under phosphorus-limited, low density conditions.

Although Hetrick (1991) viewed root morphology changes that can enhance phosphorus uptake as alternatives to mycorrhizas, it is possible that some of these may complement mycorrhizas under conditions of phosphorus limitation. For example, we found mycorrhizas to increase SRL of both *C. annuum* and *Z. mays* (Figs. 4, 5; Table 5). Because there was no main effect of mycorrhizas on root dry weight of either species (Table 2), those mycorrhizal plants with increased SRL likely had greater total lengths of fine roots than their corresponding control plants. Mycorrhizas did reduce the average diameter of *Z. mays* fine roots, however, which diminishes root surface area and thereby may reduce phosphorus absorption and exaggerate reliance on AM for phosphorus uptake (Hetrick 1991). Nevertheless, because root length is far more important for phosphorus uptake than root diameter (Casper and Jackson 1997), it is probable that the net effects of AM on root morphology in our experiments were complementary to mycorrhizal enhancement of phosphorus uptake.

Mycorrhizas might affect root morphology by several different mechanisms (Berta et al. 1993). Mycorrhizas themselves can improve mineral nutrient uptake, especially that of phosphorus, thereby reducing root hair length and number (see Vance et al. 2003), or they might compete with root growth centers for carbon. Mycorrhizal fungi might produce plant hormones (Allen et al. 1980), or might stimulate host hormone production (Edriss et al. 1984). In our experiments, increased phosphorus availability at low and medium

density had opposite effects on mycorrhizal versus control plant SRL and root branching of *C. annuum*, and on average root diameter of *Z. mays*. This suggests that the effects of mycorrhizas on these morphological attributes was not mediated by improved phosphorus uptake (Berta et al. 1993).

Conclusions

With respect to our main questions, our results demonstrate that the benefits of AM for *C. annuum* growth are maximized by low density and relatively low phosphorus availability, just as they are for phosphorus content of *C. pepo*. Our work underscores the importance of the interaction of phosphorus availability with plant density, especially because their balance may influence whether or not plant growth is limited by phosphorus or carbon. In turn, which of these factors limits plant growth may affect whether or not root morphology alterations induced by mycorrhizas complementarily augment phosphorus uptake or are alternative to mycorrhizas. Although we worked with crop species, if their range of responses is broadly indicative of that of other facultatively mycotrophic plant species, then understanding the impacts of AM on the many woodland herbaceous species that recruit at high densities will require consideration of the interaction of mycorrhizas, available phosphorus, and plant density.

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