



Comparing contributions of soil versus root colonization to variations in stomatal behavior and soil drying in mycorrhizal *Sorghum bicolor* and *Cucurbita pepo*

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Received 22 June 2006; accepted 18 August 2006

KEYWORDS

Drought;
Mycorrhiza;
Path analysis;
Soil water potential;
Stomatal conductance

Summary

In prior studies we learned that colonization of soil can be as important as colonization of roots in determining mycorrhizal influence on the water relations of host plants. Here we use a path analysis modeling approach to test (a) whether quantity of hyphae in soil contributes to variations in stomatal behavior and soil drying, and (b) whether soil colonization or root colonization has a stronger influence on these stomatal and soil drying responses. Experiments were performed on *Sorghum bicolor* and *Cucurbita pepo*, with soils and roots colonized by a mixture of *Glomus intraradices* and *Gigaspora margarita*. Soil colonization generally made more significant contributions to stomatal conductance than did root colonization. Soil colonization did not make significant direct contributions to soil water potential measures (soil water potential at stomatal closure or soil drying rate), whereas root colonization did contribute a potentially important path to each. The findings further support a role for mycorrhization of the soil itself in contributing to the regulation of stomatal behavior of host plants.

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Abbreviations: AM, arbuscular mycorrhizal; Ψ_{π} , osmotic potential; PGFI, Parsimonious Goodness of Fit Index; [P], phosphorus concentration; g_s , stomatal conductance; Y, water potential

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Introduction

Arbuscular mycorrhizal (AM) plants often fare better during drought than their non-AM counterparts (Augé and Moore, 2005), and the intensity of mycorrhizal effect can increase with the intensity of drought (e.g. Subramanian et al., 2006). AM fungi invade and inhabit living roots, and investigators have focused on how colonization of roots may be affecting plant behavior during drought: e.g. increased root hydraulic conductance (Kyllo et al., 2003), water uptake (Marulanda et al., 2003) and water use efficiency (Querejeta et al., 2003); increased antioxidant activity (Goicoechea et al., 2005); altered aquaporin expression (Porcel et al., 2006), osmotic adjustment (Wu and Xia, 2006) and hormone relations (Estrada-Luna and Davies, 2003); more effective biocontrol of pathogens (Garmendia et al., 2005); and enhanced foliar nutrition (Al-Karaki et al., 2004).

AM fungi also colonize soils, changing chemical and physical soil properties (Jastrow et al., 1998). These properties can affect plant response to drought (Augé, 2001). Therefore, in addition to influencing plants directly by colonizing plant tissues, AM symbiosis has the potential to affect drought response by changing the soils in which plants are growing. In fact, merely growing in a soil that had previously been mycorrhized resulted in higher stomatal conductance (g_s) of non-AM bean plants, under both amply watered and drought conditions (Augé et al., 2004). Colonization of soil by AM fungi can change soil moisture retention properties, in concert with changes in soil hyphal density and associated soil characters (Augé et al., 2001; Bearden, 2001).

In a test of whether colonization of soil or colonization of roots by AM fungi would contribute a stronger path to resistance to severe drought, variation in dehydration tolerance was best explained by soil hyphal density, among a suite of plant and soil characteristics (Augé et al., 2003). Soil hyphal colonization had larger direct and total effects on the leaf water potential (Ψ) and soil Ψ at which drought killed foliage than did root hyphal colonization, root density, soil aggregation, soil glomalin concentration, leaf phosphorus concentration ([P]) or leaf osmotic potential (Ψ_π). Others have noted that varying effectiveness of AM fungal species for enhancing plant water uptake from soil was related to the amount of external mycelium produced by each AM fungus as well as to the frequency of root colonization (Marulanda et al., 2003).

Soil hyphae appear to play a role in mycorrhizal influence on the water relations of host plants.

Here we summarize further tests of the relative influence of AM colonization of soil versus AM colonization of roots on host behavior before and during drought. We previously documented the importance of soil hyphae on plant response to a lethal drought (Augé et al., 2003). Our objectives here were (a) to learn if the amount of soil hyphae would also contribute a strong path to sub-lethal water relations variables during a more moderate drought; (b) to compare the effect of soil versus root colonization on stomatal behavior, the water relations parameter most studied in the mycorrhizal literature (Augé, 2001) and considered critical to the long-term performance of host plants in semiarid environments (Querejeta et al., 2006); and (c) to further test the path model of Augé et al. (2003) on another dicot and on a monocot. We tested two hypotheses in each of two experiments: (1) quantity of hyphae in soil will make a significant contribution to stomatal and soil drying variables, and (2) variations in these response variables will be more strongly associated with quantity of hyphae in soil than with quantity of hyphae in roots.

Materials and methods

In an effort to broaden the range of soil and root hyphal colonization among individuals and increase the generality and power of the modeling analysis, we produced experimental systems using varied inoculation rates, two host species, and about twice as many replications for each experiment as our previous effort (Augé et al., 2003). Stomatal behavior of host plants was studied in separate experiments, with path diagrams developed for three response (dependent) variables in each experiment: g_s integrated over the first 9 d of a drought episode, soil Ψ after 9 d of drought or at stomatal closure and g_s of amply watered plants integrated over several days prior to drought.

Sorghum experiment

Plant materials and culture

Tests were conducted on sorghum (*Sorghum bicolor* L.) cv. Dekalb DK40Y. Inoculum was a mixture of *Glomus intraradices* Schenck and Smith (isolate WV114, INVAM, West Virginia University, Morgantown, WV, USA) and *Gigaspora margarita* Gerdemann and Trappe (isolate S3000, University of Florida, Gainesville, FL, USA). Colonization by *Gl. intraradices* and *Gi. margarita* previously have been shown to affect host and soil water balance

(e.g. Duan et al., 1996; Augé et al., 2001, 2003). Inoculum consisted of pot cultures of each mycobiont, soil plus roots of 3-month-old sorghum plants grown on the experimental soil/sand potting medium described below. *Gl. intraradices* allocates substantial carbon into intraradical vesicles and might be expected to produce relatively less extraradical hyphae, whereas *Gi. margarita*, which does not form vesicles, tends to allocate more carbon into extraradical hyphae (e.g. Augé et al., 2003).

One hundred 2.8 L pots containing *S. bicolor* were grown from seed in an autoclaved soil/sand potting medium (v/v): 1 part soil (Sequatchie, fine-loamy, siliceous, thermic Humic Hapudults, sieved at 8 mm before mixing)/2 parts silica sand (medium-to-coarse, mined, sieved). Seven seeds were planted per pot. At planting, 1.4 L of autoclaved medium ($2 \times$ in 24 h at 121 °C, 60 min each time) was placed into each pot. Mycorrhizal inoculum was then placed into pots at rates of 0, 3, 10, 25, 100 mL per pot (20 pots of each rate) and adjusted with nonmycorrhizal inoculum to bring the total inoculum volume to 100 mL per pot (e.g. 3 mL AM culture/97 mL non-AM culture, etc.). The inoculum was mixed into the soil, and then pots were filled with additional autoclaved medium. Twenty-five milliliters of both AM and non-AM inoculum filtrates were added to each pot to encourage similar microflora between the 0 mL mycorrhizal inoculum treatment and other treatments.

Weekly, all plants received a liquid macro- and micronutrient fertilizer at a rate of 150 ppm nitrogen (Champion 15N-0P-15K Alkaline Plus, Chilean Nitrate Co., Norfolk, VA, USA). All plants received 0.8 mM P weekly as KH_2PO_4 . The experiment was conducted under ambient light in a glasshouse in Knoxville, TN, USA, and plants were adequately watered until the drying treatment began. Glasshouse temperatures, measured each hour during the experiment using shaded thermocouples placed near the canopy, averaged 27.6 °C during the day and 23.9 °C during the night. PPFD, measured each hour during the drying period with a quantum sensor (LiCor, Lincoln, NE, USA), averaged $\sim 96 \mu\text{mol m}^{-2} \text{s}^{-1}$ from dawn to dusk with daily maxima averaging $\sim 300 \mu\text{mol m}^{-2} \text{s}^{-1}$ over the course of the experiment. Seven weeks after planting, foliage of all plants was removed and allowed to regrow.

Drought treatment and water relations measurements

Fourteen weeks after planting, before soil volume was fully ramified by roots, all plants were watered to field capacity and then subjected to a

continuous soil drying episode by withholding water from pots. Beginning on the day water was first withheld (subsequently referred to as day 0), g_s at the tips of two leaves of each plant was measured daily with a diffusion porometer (AP4, Delta-T Devices, Cambridge, UK) between 1000 and 1230 h. Preliminary tests comparing mid-leaf, leaf tip and mid-way between tip and mid-leaf positions indicated that g_s was highest near leaf tips. Bulk foliar stomatal closure was defined as the point at which the average g_s for a plant had declined to below $10 \text{ mmol m}^{-2} \text{ s}^{-1}$. Stomatal conductance was also measured on 6 d during the 2 weeks prior to initiating drought.

Soil Ψ was measured for each pot at the point of stomatal closure, on one sample per pot (~ 2.5 mL). Samples were removed from portals cut halfway down the side of the pot and immediately sealed in a sample cup for measurement in a chilled-mirror dewpoint potentiometer (WP4, Decagon Devices, Pullman, WA, USA), calibrated with a graded series of NaCl solutions.

Leaf Ψ_π was measured for each pot on day 0 between 1000 and 1200 h. Leaves were placed in a syringe, frozen in liquid N_2 and placed in a freezer at -80 °C pending later analysis. Syringes were thawed until samples reached room temperature, and the Ψ_π of expressed sap was measured with a vapor pressure osmometer (model 5500XR, Wescor, Inc. Logan, UT, USA) calibrated with a graded series of NaCl solutions.

Other plant and soil measurements

Shoots were excised in each pot following soil Ψ measurement on the day that the plants in that pot reached stomatal closure, and dry weight determined. Leaf [P] was determined spectrophotometrically using the vanadate-molybdate-yellow method on samples ground and dry-ashed with magnesium nitrate at 700 °C for 2 h and digested in nitric acid (Chapman and Pratt, 1961). Soil was then removed from the pot, placed in a tray and the soil volume split in two longitudinally. One half of the soil volume was sealed immediately in a plastic bag and frozen for several subsequent root and soil measurements. The other half of the soil volume was allowed to air dry on the greenhouse bench, for measurement of water-stable aggregation. Hyphal, arbuscular and vesicular colonization of roots was determined for roots of each pot, on one grid intersection on each of 100 ~ 0.5 -cm root pieces, after clearing with boiling 10% KOH for 10 min, acidifying with 2% HCl for 1.5 h, staining with 0.05% Trypan blue for 1 h, and destaining in a lactoglycerol solution.

Soil hyphal density was measured based on the protocols of Miller et al. (1995). After thoroughly mixing soil from each pot, a 10 g sample was removed and suspended in a glycerol/HCl destaining solution. The suspension was agitated in a blender, sieved to remove silt particles (45 mm sieve), then levelled to 200 mL with distilled water. After stirring slowly, 10 mL of suspension was placed on a membrane filter (GN6, 0.45 μm , grid line interval 3 mm, Gelman Sci., Ann Arbor, MI, USA) which was sprayed first with 10% ethanol and attached to a vacuum apparatus. After vacuuming, the filter was covered with a trypan blue staining solution for 10 min. After rinsing stain, hyphae were resuspended in water to ensure a homogeneous distribution on the membrane. Water was filtered off and the membrane placed in a covered foil weighing pan with ~ 1 mL of water (to prevent dessication of hyphae). Hyphal segments were quantified using a dissecting microscope and hyphal density calculated as described before (Augé et al., 2003). No attempt was made to distinguish AM hyphae and non-AM hyphae of similar diameter. Gravimetric measurements of soil water content were made at the same time when soil was sampled, so that soil hyphal density could be computed on a soil dry weight basis.

Roots were carefully excavated from another 25 g of soil of each replicate, for measurement of root length, using scanning equipment and imaging software (WinRhizo, Regent Instruments Inc., Quebec City, Canada). Water-stable aggregation of air-dried soil was determined as described before (Augé et al., 2001, 2003), for the 0.25–0.5, 0.5–1.0, 1.0–2.0 and 2.0–8.0 mm size classes.

Squash experiment

Plant materials and culture

Inoculum was comprised of the same mixture of *Gl. intraradices* and *Gi. margarita* isolates and the same soil mix described above for the sorghum experiment. Sorghum pot cultures were 9 months old at the time of use. Ninety-six 4 L pots containing squash (*Cucurbita pepo* L.) cv White Bush Scallop were grown from seed in the soil/sand potting medium described for the sorghum experiment. Procedures for inoculation, water filtrates, fertilization, irrigation and measurement of glasshouse temperatures and PPFD were as described above for the sorghum experiment, with the exception that four rather than five rates of inoculum were used (0, 4, 16 and 64 ml per pot, brought to 100 mL with nonmycorrhizal inoculum). Three seeds were planted per pot and thinned to

one plant per pot after germination. Air temperatures averaged 24.3 $^{\circ}\text{C}$ during the day and 21.1 $^{\circ}\text{C}$ during the night. PPFD averaged $\sim 57 \mu\text{mol m}^{-2} \text{s}^{-1}$ from dawn to dusk with daily maxima averaging $\sim 350 \mu\text{mol m}^{-2} \text{s}^{-1}$ during the course of the experiment.

Drought treatment and water relations measurements

Nine weeks after germination, before soil volume was fully ramified by roots, all plants were watered to field capacity and then subjected to a continuous soil drying episode by withholding water from pots. Beginning on the day water was first withheld (subsequently referred to as day 0) and continuing until day 9 of the drying episode, g_s of three leaves of each plant was measured every 3 d with a diffusion porometer (AP4, Delta-T Devices) between 1000 and 1230 h. Stomatal conductance was also measured on 4 d during the 2 weeks prior to initiating drought.

Osmotic potential, soil Ψ , shoot biomass, shoot P, water-stable aggregation, mycorrhizal colonization of roots, soil hyphal density and root length density were measured as described in the sorghum experiment.

Experimental design and statistical analysis

Each experiment was arranged as a completely randomized block design with blocking on pot location in the greenhouse. For sorghum experiment, there were 20 replicates of each of the five inoculation rates (total of 100 plants). For squash experiment, there were 24 replicates of each of the four inoculation rates (total of 96 plants). Correlations among water relations parameters and the root and soil variables were tested by computing Pearson correlation coefficients.

Path analysis is a statistical method for modeling the correlation structure among variables, allowing complex patterns of inter-relationships to be tested (Shipley, 2002). It was used to test relationships among variables hypothesized to be involved in stomatal behavior and in the ability to withstand drought. Although correlation and regression analyses were also conducted, the advantages of path analysis are in examining relationships among explanatory (independent) variables and allowing intermediate dependent variables to act as explanatory variables for later steps in the hypothesized path (e.g. Mitchell, 1992; Jastrow et al., 1998). Path analysis measures the ability of explanatory variables to explain the ultimate variables of interest, such as g_s during a particular

time frame or soil Ψ at stomatal closure. Direct and indirect paths connecting variables in the model provide a basis for deeper understanding of the entire system. Path modeling techniques do not allow testing of causality among variables. Rather, a priori knowledge of the system or theoretical reasoning is used to construct a conceptual model (path diagram) of the causal and noncausal relationships among the measured variables. Observed data are then used to evaluate the model (Jastrow et al., 1998).

Separate path diagrams for prestress g_s , g_s during the first 9 d of drought, and soil Ψ were initially proposed based on previous knowledge. Variables involved were checked for normality and path analysis run. Preliminary analyses resulted in minor modifications to the path diagrams. Parsimonious Goodness of Fit Index (PGFI; Mulaik et al., 1989) and chi-square (χ^2) were used to assess model fit, and residuals were examined. Path coefficients, indirect effects, standard errors and coefficients of determination were calculated with Proc Calis in SAS (SAS Institute Inc., Cary, NC, USA).

Results

Path models

In a prior work, we demonstrated that soil colonization by AM fungi had stronger contributions than root colonization to ability of the host plant to withstand a lethal drought (Augé et al., 2003). Here we use the same path analysis modeling approach to compare the effects of soil hyphae and root hyphae on plant response before and during a more moderate drought.

Our conceptual model for evaluating the contribution of soil and root hyphae to variations in g_s and soil Ψ included several other soil and plant characteristics that are often affected by AM symbiosis and that have been postulated to affect plant or soil water relations in a direct or indirect way (Figs. 1–4). In the path diagrams, a straight single-headed arrow indicates a direct causal path. Indirect causal effects are connoted by a variable being linked to a given dependent variable via one or more intermediary variables. The path coefficients (numbers on lines) indicate the relative magnitudes of the direct effects of the explanatory variables linked to each dependent variable. Proportion of total variance explained by the model was computed for each dependent variable (italicized numbers above boxes).

Rationale for including each variable in the model is summarized in Augé et al. (2003). Among

arbuscular, vesicular and total hyphal colonization of roots, arbuscular colonization was selected for further analysis because it tended to give the strongest contributions among the three colonization variables in the path analyses of both sorghum and squash. Among the water-stable aggregate size classes tested, the 0.5–1.0 mm class provided the strongest path to the water relations variables and therefore was used to represent water-stable aggregation in the analysis. Ranges and means for each variable included in the models are given in Table 1.

For all path diagrams, path values ≥ 0.20 represent significant contributions. For clarity's sake, paths having values less than 0.10 are not shown. Paths having values between 0.10 and 0.20 are shown, as these are potentially important in portraying causal relationships in the model.

Sorghum

Several of the path model's variables contributed to variations in g_s during drought (average g_s measured on each of the first 9 d of the drying period) (Fig. 1). The strongest contributions were made by soil hyphae, leaf [P] and soil aggregation. Higher values of each of these three explanatory variables were associated with lower values of g_s . Root colonization did not contribute to g_s during drought. In this analysis, as in the others (Figs. 2–4), the model explained only a small proportion of total variance for this response variable (0.20).

Several explanatory variables also contributed to variations in soil Ψ at stomatal closure, a measure of the extent to which soil had to dry to cause stomata to close (Fig. 2). Days of drying to reach stomatal closure ranged from 9 to 14. The strongest contributions to soil Ψ at stomatal closure were made by root colonization and leaf [P], with smaller contributions by soil aggregation and shoot size. The direct effect of each of these explanatory variables on soil Ψ was positive; more root colonization or higher leaf [P] was associated with higher soil Ψ at the point of stomatal closure.

A path model was also derived for prestress g_s (average g_s measured on 6 d prior to initiating drought) to assess the strength of the contributions of the various explanatory variables to stomatal behavior of amply watered plants (diagram not shown, because almost all path coefficients were similar to those depicted in Fig. 1 for g_s during drought). The model fit the observed data (PGFI = 0.35, $\chi^2 = 13.15$; df = 10; $P = 0.22$). As for g_s during drought, soil hyphae made a significant contribution to prestress g_s (path coefficient = -0.21), while root colonization had no effect on prestress g_s (path coefficient = -0.04). Although

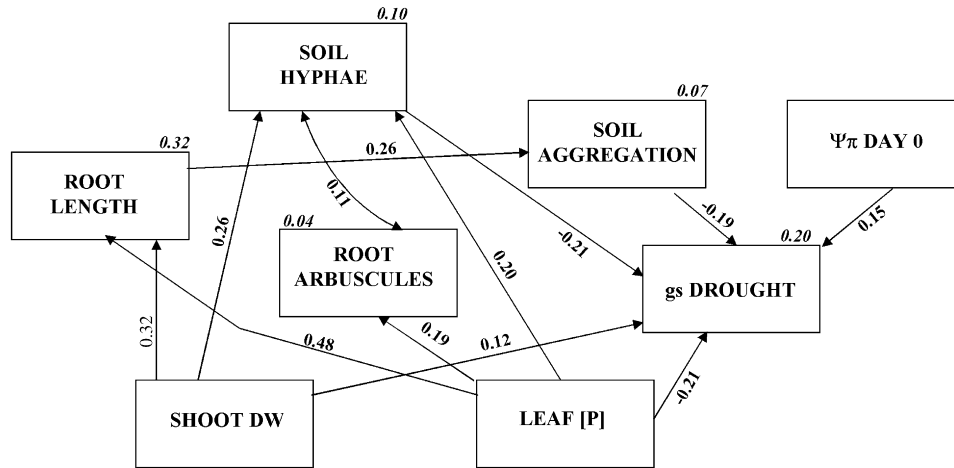


Figure 1. *Sorghum*. Path diagram depicting the hypothesized causal relationships among independent and dependent variables, for g_s during the first 9 d of drought. Each single-headed arrow signifies a direct causal relationship in the direction of the arrow. Double-headed arrows indicate a correlation between two variables. Indirect causal effects occur if one variable is linked to another via other, intermediate variables. Numbers on arrows are path coefficients (standardized partial regression coefficients derived from the regression of each response variable on those variables directly linked to it) indicating the relative strength of each path leading to a given response variable. Values ≥ 0.20 represent significant contributions; for clarity's sake, arrows representing direct paths and correlations having values < 0.10 were deleted from the diagram. Italicized numbers above variable boxes are estimates of the proportion of total variance explained (squared multiple correlations) for each dependent variable. Full variable names and their units are given in Table 1. The model fit the data (PGFI = 0.33, $\chi^2 = 15.06$; df = 10; $P = 0.13$).

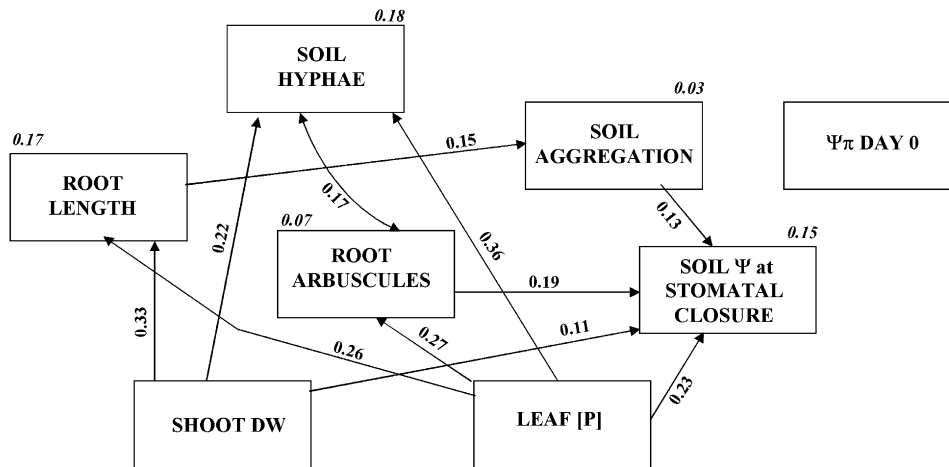


Figure 2. *Sorghum*. Path model depicting the hypothesized causal relationships among independent and dependent variables, for soil water potential at stomatal closure. See Fig. 1 legend for explanation of diagram components. The model fit the data (PGFI = 0.35, $\chi^2 = 12.62$; df = 10; $P = 0.25$).

average prestress and drought g_s were similar (Table 1), g_s did decline considerably during the drying episode. Some cloudy weather during the 2 weeks preceding day 0 diminished g_s somewhat during that period. On day 0 and day 1, g_s averaged 227 and 243 $\text{mmol m}^{-2} \text{s}^{-1}$, respectively, and on day 8 and day 9 averaged 70 and 26 $\text{mmol m}^{-2} \text{s}^{-1}$, respectively.

Leaf [P] had relatively high path coefficients viewed over the three sorghum analyses, denoting

its influence (sometimes positive, sometimes negative) on several of the plant and soil variables in the model. Leaf $\Psi\pi$ contributed a positive path to g_s during drought as well as to prestress g_s ; higher g_s values were associated with fewer leaf solutes.

Table 2A summarizes Pearson correlation analysis among experimental variables for the sorghum experiment. For the three measures of stomatal behavior being modeled, correlation coefficients were higher for soil hyphal density than for root colonization

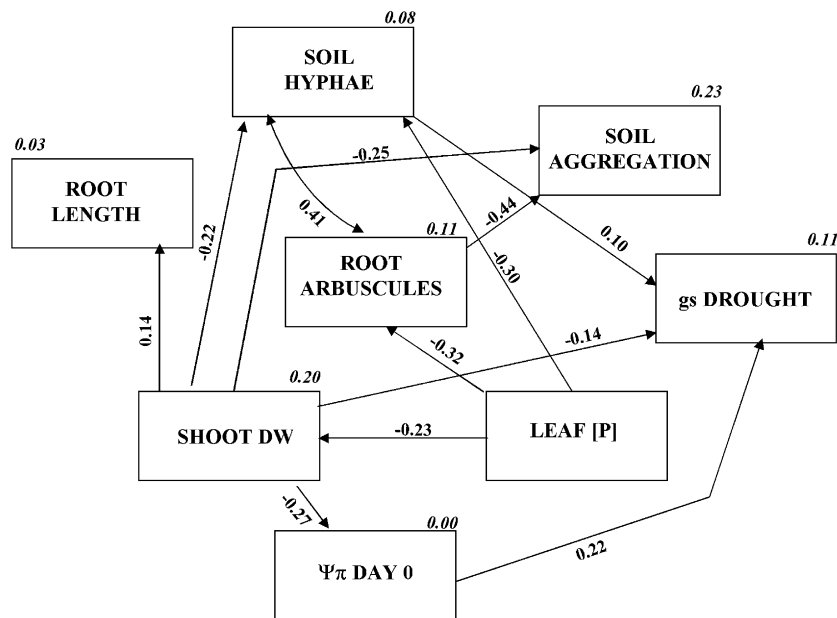


Figure 3. *Squash*. Path model depicting the hypothesized causal relationships among independent and dependent variables, for g_s during the 9 d of drought. See Fig. 1 legend for explanation of diagram components. The model fit the data (PGFI = 0.28, $\chi^2 = 4.67$; df = 10; $P = 0.79$).

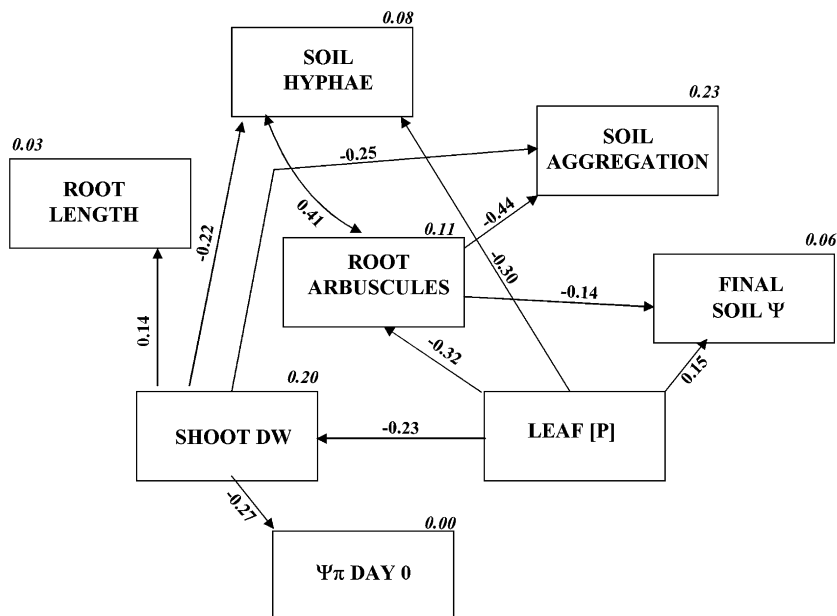


Figure 4. *Squash*. Path model depicting the hypothesized causal relationships among independent and dependent variables, for final (day 9) soil water potential. See Fig. 1 legend for explanation of diagram components. The model fit the data (PGFI = 0.28, $\chi^2 = 9.69$; df = 10; $P = 0.29$).

(characterized as root arbuscular colonization). The correlation was significant at $P \leq 0.05$ for soil hyphal density and soil Ψ at stomatal closure. Soil hyphae were significantly correlated with soil Ψ at stomatal closure in sorghum (Table 2, Pearson correlation efficient of 0.33). And yet in the path diagram (Fig. 2), soil hyphae show no direct effect on soil Ψ

at stomatal closure. This indicates that the apparent correlation measured by Pearson is due to indirect effects. This is a benefit of path analysis; it allows separation of direct and indirect effects.

Root colonization and shoot size were not correlated with any of the water relations variables, nor with any of the explanatory variables.

Table 1. Ranges and means \pm SD for plant and soil variables for the sorghum and squash experiments

	Sorghum	Squash
Prestress g_s ($\text{mmol m}^{-2} \text{s}^{-1}$)	68–303 160 \pm 50	288–705 481 \pm 78
Drought g_s ($\text{mmol m}^{-2} \text{s}^{-1}$) (days 1–9 of drying)	84–331 146 \pm 52	135–439 288 \pm 59
Soil ψ at stomatal closure (MPa)	–4.42 to –0.07 –1.77 \pm 0.79	
Final soil ψ (day 9) (MPa)		–30 to 0 –2.73 \pm 6.46
Initial leaf Ψ_π (day 0) (MPa)	–1.11 to –0.49 –0.82 \pm 0.09	–1.00 to –0.63 –0.76 \pm 0.05
Shoot dry weight (g)	1.2–3.9 2.7 \pm 0.6	1.5–5.0 3.4 \pm 0.7
Leaf [P] (mg g^{-1})	1.77–4.33 3.17 \pm 0.54	2.5–10.5 4.6 \pm 1.7
Arbuscular root colonization (%)	0–75 34 \pm 16	0–62 20 \pm 18
Soil hyphal density (cm g^{-1} dry soil)	0–56 21 \pm 13	0.1–55 18 \pm 11
Root length density (cm g^{-1} dry soil)	11.1–55.6 26.3 \pm 8.9	3.9–19.8 8.1 \pm 2.5
Water-stable aggregation (mg g^{-1} soil DW)	36–76 52 \pm 5	11–20 14.9 \pm 2.1

Variables were averaged across inoculation rates: sorghum: $n = 100$; squash: $n = 96$.

Among all explanatory variables, leaf [P] was correlated with the most response variables (drought g_s and soil Ψ at stomatal closure) and the most other explanatory variables (initial leaf Ψ_π , root length density and water-stable aggregation). Sorghum plants with highest prestress g_s were able to maintain g_s to the lowest soil Ψ .

Squash

Three explanatory variables contributed to variations in g_s of squash during drought (average g_s measured on days 3, 6 and 9 of the drying period) (Fig. 3). Leaf Ψ_π contributed the strongest path. Shoot size and soil hyphae were of potential importance in explaining changes in g_s during drought. Neither root colonization nor leaf [P] contributed to g_s during drought.

Only two variables had potentially important paths to final soil Ψ (Ψ after 9 d of drying; Fig. 4). Root colonization had a negative direct effect – more arbuscules were associated with lower soil Ψ – and leaf [P] a positive effect. Soil hyphae did not have a direct effect on final soil Ψ .

A path model was also derived for prestress g_s of squash plants (average g_s measured on 4 d prior to initiating drought) (diagram not shown; almost all path coefficients were similar to those depicted in Fig. 3 for g_s during drought). The model fit the data (PGFI = 0.28, $\chi^2 = 4.42$; df = 10; $P = 0.82$). Soil

colonization and root colonization made equal and significant contributions to prestress g_s (path coefficients were –0.28 and 0.28, respectively).

Viewed over each of the three squash analyses, no one explanatory variable contributed the strongest path to the g_s measures or to soil Ψ . Soil hyphae and shoot size were important or potentially important for explaining prestress g_s and g_s during drought but not final soil Ψ . Root colonization was important or potentially important for explaining prestress g_s and final soil Ψ but not g_s during drought.

Table 2B summarizes correlation analysis among experimental variables for the squash experiment. Neither prestress g_s nor g_s during drought was significantly correlated with any of the plant or soil variables measured in the study. This indicates that other variables were responsible for variations in g_s . Stomatal conductance responds to many external and internal factors, especially to light, humidity and temperature, and it is not surprising that these controlling factors swamped out the more subtle influences of leaf [P] and osmotic status, root ramification of the soil volume, shoot size and the mycorrhizal colonization variables. The purpose of the work was to test the relative contributions of soil and root colonization using path analysis, which we were able to do. In terms of correlation analysis, however, neither appeared to

Table 2. Pearson's product-moment correlations (*r*) between variables included in the path models (Figs. 1–4)

A	Prestress g_s	Drought g_s	Soil ψ at closure	Initial leaf Ψ_π	Leaf [P]	Soil hyphae	Root length	Root-arbuscules	WSA	Shoot biomass
Sorghum										
Prestress g_s		-0.20	-0.43**	-0.01	-0.23	-0.10	-0.28*	-0.02	0.04	-0.23
Drought g_s	-0.20		-0.18	0.24	-0.39**	-0.24	-0.13	-0.15	-0.22	0.04
Soil ψ at stomatal closure	-0.43**	-0.18		-0.22	0.36*	0.33*	0.14	0.24	0.10	-0.08
Initial leaf Ψ_π	-0.01	0.24	-0.22		-0.37**	-0.10	-0.10	-0.08	0.05	-0.10
Leaf [P]	-0.23	-0.39**	0.36*	-0.37**		0.19	0.41**	0.17	0.29*	-0.01
Soil hyphal density	-0.10	-0.24	0.33*	-0.10	0.19		0.17	0.09	0.00	0.23
Root length density	-0.28*	-0.13	0.14	-0.10	0.41**	0.17		0.04	0.24	0.25
Arbuscular colonization	-0.02	-0.15	0.24	-0.08	0.17	0.09	0.04		-0.09	-0.19
Water-stable aggregation	0.04	-0.22	0.10	0.05	0.29*	0.00	0.24	-0.09		0.16
Shoot biomass	-0.23	0.04	-0.08	-0.10	-0.01	0.23	0.25	-0.19	0.16	
B	Prestress g_s	Drought g_s	Soil ψ day 9	Initial leaf Ψ_π	Leaf [P]	Soil hyphae	Root length	Root-arbuscules	WSA	Shoot biomass
Squash										
Prestress g_s		0.13	0.03	0.05	0.07	-0.15	-0.02	0.15	-0.13	-0.05
Drought g_s	0.13		0.02	0.15	-0.16	0.14	-0.02	0.15	-0.17	-0.14
Soil ψ on day 9	0.03	0.02		0.04	0.20*	-0.08	-0.24*	-0.19	0.11	-0.09
Initial leaf Ψ_π	0.05	0.15	0.04		0.10	-0.02	-0.09	-0.16	0.13	-0.32**
Leaf [P]	0.07	-0.16	0.20*	0.10		-0.23*	-0.08	-0.32**	0.25*	-0.32**
Soil hyphal density	-0.15	0.14	-0.08	-0.02	-0.23*		0.05	0.42***	-0.22*	-0.04
Root length density	-0.02	-0.02	-0.24*	-0.09	-0.08	0.05		0.06	-0.14	0.18
Arbuscular colonization	0.15	0.15	-0.19	-0.16	-0.32**	0.42***	0.06		-0.47***	0.27**
Water-stable aggregation	0.13	-0.17	0.11	0.13	0.25*	-0.22*	-0.14	-0.47***		0.33**
Shoot biomass	-0.05	-0.14	-0.09	-0.32**	-0.32**	-0.04	0.18	0.27**	0.33**	

*, ** and *** indicate that correlations were significant at $P \leq 0.05$, 0.01 or 0.001, respectively. Correlation coefficients not followed by asterisk(s) indicate correlation was not significant. WSA = water-stable aggregation.

be closely linked to g_s . Final soil Ψ was correlated with both leaf [P] and root length density. Plants having more roots dried soils more during the first 9 d of drying. Plants having higher leaf [P], which tended to be smaller plants (negative leaf [P]/shoot size correlation for squash), dried soil less during these 9 d.

Discussion

Our objective was to test two hypotheses regarding relative contributions of root and soil colonization to host behavior before and during a

sub-lethal drought: that soil colonization (characterized as soil hyphal density) would make significant contributions to stomatal behavior and extent of soil drying, and that soil colonization would make stronger contributions than root colonization to these variables. Both hypotheses were true. Viewed over all models (all variables and each species), soil colonization did contribute direct paths to g_s , having significant path coefficients to three of the six response variables. Root colonization had a significant path coefficient to just one of the six response variables.

Each of the significant soil hyphae path coefficients occurred in a g_s model, and each was

negative, indicating that less hyphae in soil were related to higher g_s (perhaps the opposite relationship we would have postulated, given the increased g_s associated with nonmycorrhizal beans grown in a mycorrhized soil, Augé et al., 2004). Davies et al. (1992) observed that extraradical hyphal development in AM soils was associated with greater drought resistance of plants growing in those soils. Drought resistance can be associated both with maintenance of g_s (tolerance) and early stomatal closure (avoidance; more sensitive stomates) in drying soils (e.g. Ludlow, 1989). Root colonization had less influence on g_s than did soil colonization. Conversely, although the path coefficients were not significant (<0.20), root colonization had more influence on soil Ψ than did soil colonization. As opposed to a prior work in which soil hyphae contributed a strong path to lethal soil Ψ (Augé et al., 2003), soil hyphae had a much smaller direct effect on soil Ψ at stomatal closure and essentially no effect on soil drying rate integrated over the first 9 d of the drought episode.

As is well known, mycorrhizal symbiosis can dramatically influence plant phosphorus nutrition, which in turn can influence many aspects of plant physiology and plant response to its environment. Leaf [P], which can influence stomatal behavior (e.g. Radin, 1984), generally made stronger contributions to the stomatal measures in our experiments than did soil or root colonization, underscoring how this mycorrhiza-influenced character plays a role in plant water relations and in plant response to drought.

We conducted this study not because we necessarily predicted a direct mechanistic relation of soil hyphal density to leaf g_s . Rather, we selected and examined soil hyphal density as a measure of extent to which AM symbiosis developed in the soil and hence potentially affected various soil characteristics which could have some impact on a plant's physiology. We previously observed that simply growing in a mycorrhizal soil changed the stomatal behavior of nonmycorrhizal plants (Augé et al., 2003), and we wanted to test for a relationship between the degree of soil mycorrhization and variations in g_s . Soil hyphal density is a simple means of quantifying overall soil mycorrhization. That we observed any direct influence at all in the path diagram of soil hyphae on g_s could be considered surprising, given the large number of environmental and internal factors that can greatly influence g_s in the short- and long-term, e.g. light, vapor pressure deficit, leaf orientation, leaf age and size, leaf and air temperature, wind speed, diurnal fluctuations and nutrition. We attempted to standardize and control for as many of these factors as

we could. It is gratifying to have teased out a direct influence of soil colonization on a complex behavior such as g_s , despite the fact that the contributions of the variables in the path models, though often significant, were mostly small, as were estimates of the proportion of total variance explained for each of the stomatal variables.

Investigators, including us, typically provide some measure of root colonization as a means of substantiating that the symbiosis occurred in an experiment and quantifying the extent to which it occurred. This work demonstrates that it may be important to consider the changes wrought by mycorrhizal symbiosis on soils, when attempting to understand or predict how AM symbiosis affects plant water relations and drought response.

Acknowledgments

This manuscript is based upon work supported by the US Department of Agriculture under Award no. 00-35100-9238 and by the Tennessee Agricultural Experiment Station. The initial inoculum for *Gi. maragarita* cultures was kindly provided by Dr. David Sylvania.

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