Mycorrhiza (1998) 8:93–99

ORIGINAL PAPER

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Transpiration of detached leaves from mycorrhizal and nonmycorrhizal cowpea and rose plants given varying abscisic acid, pH, calcium, and phosphorus

Accepted: 22 June 1998

Abstract Vesicular-arbuscular mycorrhizal (VAM) colonization can alter transpiration of host leaves, but scientists remain unclear about the mechanisms involved. We tested whether intact root systems were required to observe effects of root colonization by *Glomus intraradices* on leaf transpiration, or whether some VAM influence resided in leaves even after they were detached from root systems. We measured the transpiration of detached leaves of VAM and nonmycorrhizal plants exposed to different levels of several substances known to influence stomata locally or act in whole-plant regulation of transpiration: abscisic acid, calcium, phosphorus, and hydrogen ions. In rose, some VAM influence on transpiration resided in leaves, even after they had been separated from their root systems. However, removing leaves from their root systems eliminated the VAM influence on stomatal behavior of cowpeas.

Key words *Glomus intraradices* · *Rosa hybrida* · Stomatal behavior · Vesicular-arbuscular mycorrhiza · *Vigna unguiculata*

Introduction

Vesicular-arbuscular mycorrhizal (VAM) fungi can affect the water balance of their hosts. Transpiration, in particular, is often higher in both unstressed and droughted mycorrhizal plants than in nonmycorrhizal counterparts, even when care is taken to compare plants having similar shoot size and/or phosphorus nutrition (e.g., Allen and Allen 1986; Augé et al. 1986b; Bethlenfalvay et al. 1987; Henderson and Davies 1990) and exposed to similar degrees of soil drying (e.g., Duan et al. 1996; Ebel et al. 1997). Questions remain, however, regarding mechanisms of mycorrhizal influence and where they reside.

This study tested whether intact root systems are required to observe an effect of *Glomus intraradices* on transpiration, or whether there is some residual VAM influence on foliage that continues to affect transpiration of leaves detached from root systems. This information will assist investigators in localizing the mycorrhizal influence on host water balance. To thoroughly explore the question, we conducted these tests on two host species, in the presence of xylem-fed substances known or suspected to (1) affect stomata directly, (2) affect stomatal sensitivity to abscisic acid (ABA), and (3) be affected by VAM symbiosis: ABA (Allen et al. 1982; Davies et al. 1994), calcium (Atkinson et al. 1990; Augé et al. 1992), phosphorus (Radin 1984) and pH (Jia and Zhang 1997). We examined cowpea and rose because their transpiration and stomatal conductance have frequently been modified by VAM symbiosis (e.g., Augé and Duan 1991; Augé et al. 1986a,b, 1992; Duan et al. 1996; Ebel et al. 1997; Faber et al. 1991).

Materials and methods

Plant materials and culture

Seeds of *Vigna unguiculata* (L.) Walp. (cowpea) were planted and grown in 1-l pots. *Rosa hybrida* L. cv. Proud Land (rose) plants, donated by Jackson and Perkins (Medford, OR, USA), were grown in 5.8-l pots. Plants of each species were grown in a medium composed of two parts autoclaved silica sand, one part calcined montmorillonite clay (Turface) (v:v). This medium was chosen because it promotes VAM colonization, it can be readily removed from roots, and its soil moisture characteristics are known (Augé et al. 1994). To this mixture was added pot culture which contained medium and roots from either *V. unguiculata* plants that were colonized by *G. intraradices* Schenck & Smith isolate WV114 or uncolonized *V. unguiculata* plants (1 pot culture:3 sand/Turface mixture; v:v). Pot cultures were grown in the

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sand/Turface medium previously described. All plants for a particular experiment were grown either on a greenhouse bench under natural light, or in a controlled growth chamber (M75, Environmental Growth Chambers, Chagrin Falls, OH) with irradiance for a 14-h photoperiod provided with an equal mix of 400-W high-pressure sodium and metal halide lamps, with photosynthetic photon flux density (PPFD) ranging from 750 to 900 μmol m^{-2} s^{-1} at leaf height. Growth room day temperature was set at 25 °C and night temperatures at 18–20 °C, and greenhouse temperatures typically ranged from 27–35 °C during the day and 16–22 °C during the night. Daytime humidity levels were maintained near 65% in the growth room and ranged from 30 to 55% in the greenhouse. Plants were fully watered throughout the experiments.

With every watering, plants of each species received 15-0-15 fertilizer (Peters Fertilizer Products, W.R. Grace, Fogelsville, PA) at 10.7 mM N. All plants also received 1 mM magnesium as $MgCl_2$ with each watering. Soluble trace elements were supplied once a week at 1 mM Mn (STEM, Peters Fertilizer Products). Iron was provided weekly at 0.1 mM as Sprint (Ciba-Geigy, Greensboro, NC). Phosphorus was applied as K_2HPO_4 once a week with VAM cowpeas receiving 1 mM P, VAM roses 0.5 mM P, nonmycorrhizal (NM) cowpeas 3.0 mM P and NM roses 2.5 mM P. Phosphorus fertilization was adjusted, in accordance with findings from several previous experiments, in an attempt to produce VAM and NM plants of similar size within each species. Rose bushes were 1–2 years old and of similar size when inoculated. The woody rose stems probably had sufficient nutrient reserves that a growth or phosphorus effect would not have been observed 3 months after inoculation, when the transpiration experiments with rose were performed. Furthermore, in each experiment we grew more plants of each species than necessary so that we could visually select VAM and NM plants of similar size from a larger population. Twenty-five VAM and 25 NM plants were grown for each cowpea experiment, and 20 VAM and 20 NM roses. Experiments described below were begun only after checks had shown that VAM plants were extensively colonized and NM plants uncolonized.

Stomatal conductance of intact cowpeas

Just prior to the ABA/pH transpiration experiment with cowpeas, we measured the stomatal conductance of these plants, to determine if VAM colonization had an effect on the stomatal behavior of intact leaves. Abaxial stomatal conductance of four unshaded lateral leaflets of eight VAM and eight NM plants was measured midway between midrib and margin with a diffusion porometer (AP4, Delta-T Devices, Cambridge, UK), calibrated immediately before each sampling. Measurements were made in the greenhouse at ambient CO_2, PPFD and vapor pressure deficit between 0900 and 1500 hours, a time period during which previous tests had revealed no consistent, significant diurnal changes in stomatal conductance.

Transpiration assays

Four factorial experiments evaluated the influence of VAM fungi on transpiration of detached leaves exposed to different ABA, pH, Ca, and PO_4: three with cowpea and one with rose. We examined whole-leaf transpiration, adapting the protocols of Trejo et al. (1993a); gravimetric measurements are generally more robust than other methods of estimating transpiration because they are direct, reliable, continuous and relatively free of instrument error and artifact. The influence of differing pH and concentrations of xylem-fed ABA on transpiration was examined in each species, and the influences of different concentrations of calcium and phosphorus were examined in cowpea.

For each plant species, leaves were excised or recut under distilled water (to allow refilling of cut xylem vessels with water rather than air). For cowpea, the terminal leaflet of the third-youngest leaf of 6-week-old (Ca experiments) or 9- to 12-week-old (pH and PO_4 experiments) VAM and NM plants was examined. Leaf age affects stomatal behavior of intact cowpea leaves (e.g., Table 2) and so leaves of the same age were used in all cowpea transpiration experiments. For rose, we used terminal leaflets of recently matured leaves from plants that had been inoculated when brought in as sizeable bare-root bushes 3 months previously. After rehydrating detached leaves or leaflets for 1 h in disH_2O (lower part of cut leaflet blade submerged for both cowpea and rose, in covered vials in the dark), leaves were transferred to 10-ml vials containing treatment solutions and placed in random order on a laboratory bench beneath two 400-W sodium vapor lamps. After a 30-min adjustment period, vials were quickly weighed, then replaced beneath lamps. Vials were weighed every 30 min for 3 h. Vials were sealed at the top with aluminum foil, so that only water loss from leaves was accounted for in calculating transpiration. Forty-eight leaves were assayed each assay day: two mycorrhizal treatments × three levels of either pH, PO_4 or Ca × four levels of ABA × two replicates of each treatment. We repeated these assays four times for each experiment, for a total of eight replicates per treatment. In each assay, replicates of treatments were completely randomized. After the transpiration assay, the area of the transpiring part of the leaf (above the foil) was measured with a leaf area meter (Li-Cor LI-3000a, Lincoln, NE). Transpiration rate was calculated as $(W_1–W_2)/(\text{leaf area} \times t \times M)$, where W_1 and W_2 were the initial and final weights in grams of each leaf + vial + foil + solution at the beginning and end of each transpiration assay, respectively, t was time in seconds, and M is the molecular weight of water (18 g mol^{-1}). Transpiration generally reached a plateau within 30–90 min; the average transpiration of each species and treatment combination depicted in Figs. 2 and 3 represents the average transpiration of the last 90 min of each 3-h assay for each leaf. Across all transpiration assays on all days on which we performed this work, PPFD reaching leaves ranged from 200 to 400 μmol m^{-2} s^{-1}, laboratory air temperatures remained near 27 °C, and laboratory relative humidity ranged from 26% to 62%. During each particular assay day, relative humidity was similar across treatments.

Transpiration assays were performed for each plant species using feeding solutions consisting of physiologically active (Trejo et al. 1993a,b) ABA concentrations of 10^{-7} M, 10^{-6} M, 10^{-5} M and 0 M, in distilled water, standardized to a pH of 6.0 for P and Ca assays. 10^{-7} M and 10^{-6} M are fairly representative of in situ ABA concentrations in xylem fluid of droughted plants, and pH 6.0 is representative of the xylem pH of undroughted plants. Transpiration assays were also performed for each plant species with feeding solutions having pH of 5.5 and 6.5, with the highest pH representative of droughted plants. The effects on transpiration of different concentrations of calcium and phosphorus in the xylem feeding solution, alone and in concert with ABA, were further examined in cowpea, to test both for effects of each ion as well as the ion effect on transpirational response to ABA. Calcium was supplied in concentrations of 0.0 mM, 1.0 mM, and 5.0 mM, and phosphorus in concentrations of 0.0 mM, 0.8 mM, and 4.0 mM. These concentrations, as well as those used for ABA, span ranges previously found in xylem sap of cowpea and other species (e.g., Duan et al. 1996; Gollan et al. 1992; Ruiz et al. 1993).

Shoot and root attributes

Hyphal, arbuscular, and vesicular colonization of roots was determined at the end of each experiment on four VAM and four NM plants from the population of plants used in that experiment, on 50 1-cm root pieces from each plant, after clearing with 10% KOH in an autoclave at 121 °C for 15 min, staining with trypan blue for 1 h, and destaining. The phosphorus concentration of recently matured leaves of each plant of each species was determined spectrophotometrically using the vanadate–molybdate–yellow method on samples dry–ashed with magnesium nitrate at 700 °C for 2 h and digested in nitric acid (Chapman and Pratt 1961). Whole-shoot dry weights (oven-dried at 80 °C for at least 48 h) and leaf areas were recorded following the experiments.

Statistical analysis

We examined transpiration of eight replicates of each treatment for all transpiration experiments. Data were analyzed within species as completely randomized designs, using the ANOVA procedure of the Statistical Analytical Services (SAS) programs. Treatment main effects consisted of mycorrhizae, ABA, pH, calcium, phosphorus, depending upon the experiment.

Results

Shoot and root attributes

Mycorrhizal roots of plants of each species in all experiments were well colonized (Table 1). Hyphal colonization was consistently quite high, and considerable numbers of vesicles formed in all roots. Arbuscular colonization was very high in cowpea and low in rose (although the greater difficulty of visualizing arbuscules in the coarser rose roots may have artificially depressed arbuscular colonization numbers). No colonization was observed in NM plants used in any of the experiments. Whole-plant leaf areas were statistically similar in VAM and NM plants in each experiment. Shoot dry weights were similar in VAM and NM plants in each experiment except the cowpea/pH experiment, where the average dry weight of NM shoots was 13% higher than that of VAM plants ($P=0.02$). Phosphorus concentrations were similar in leaves of VAM and NM in all experiments except the cowpea/phosphorus experiment, where NM leaves had almost twice as much phosphorus as VAM leaves. With few exceptions, we produced VAM and NM plants of statistically similar size and P nutrition. Areas of detached leaves assayed were similar in VAM and NM treatments of all experiments except the cowpea/pH experiment. There, differences in VAM and NM leaf areas were statistically but probably not biologically significant.

Table 1 Average root colonization, shoot and leaf sizes, and leaf phosphorus concentrations of plants used in each of the four transpiration experiments with detached leaves ($n=4$ for colonization values, $n=8$ for other values). The pH, Ca, and PO_4 designation refer to experiments in which those constituents were altered in the assay solutions, in combination with changing ABA. Mycorrhizal (*VAM*) and nonmycorrhizal (*NM*) values were compared within each experiment by ANOVA: *italicized type* indicates that VAM and NM values were significantly different at $P \leq 0.05$. *Plant leaf area* is average total leaf area (all leaves on the plant) for the plants grown for each experiment. *Assay leaf area* refers to the average area of detached leaves used in the transpiration assays (unsubmerged part of the leaflet lamina above the foil). Plant leaf area and *shoot dry weight* were not measured for rose plants, because stems of spent rose blooms of these sizeable bushes had been pruned consistently for the 3 months of mycorrhizal culture. Root colonization is given only for VAM plants; NM plants of each host for each experiment remained uncolonized

Shoot and root attributes	Experiment			
	Cowpea			Rose
	pH	Ca	PO_4	pH
VAM root colonization (%)				
Vesicles	60	57	54	79
Arbuscules	85	84	78	7
Hyphae	95	98	96	68
Plant leaf area (cm^2)				
VAM	3072	183	955	–
NM	3287	224	1246	–
Shoot dry weight (g)				
VAM	*27.1*	2.1	6.4	–
NM	*30.7*	2.0	9.3	–
Leaf phosphorus ($mg\ g^{-1}$)				
VAM	3.5	0.8	*2.3*	1.4
NM	4.3	1.1	*4.3*	1.2
Assay leaf area (cm^2)				
VAM	*40.4*	10.0	35.4	15.5
NM	*42.9*	9.5	35.8	16.1

Stomatal conductance of intact cowpea leaves

VAM colonization significantly increased the stomatal conductance of intact leaves of adequately watered cowpea plants (Fig. 1, Table 2). Vapor pressure deficits, wind speeds, leaf sizes, and shoot sizes were similar in and around VAM and NM plants, which were completely randomized on the greenhouse bench. Therefore, transpiration rates of intact leaves were also higher in VAM than in NM plants. These plants were subsequently used for the ABA/pH transpiration experiment. The stomatal conductance and transpiration of intact rose leaves was not measured but has previously

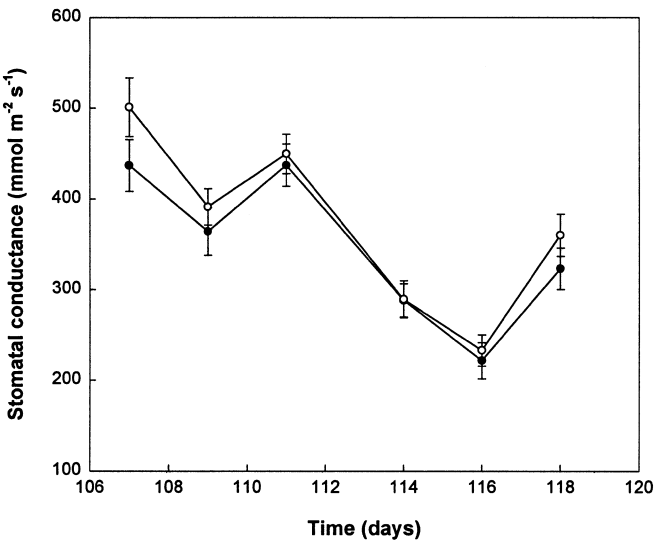

Fig. 1 Stomatal conductance of intact vesicular-arbuscular mycorrhizal (VAM) and nonmycorrhizal (NM) leaves of cowpea plants used subsequently in the ABA/pH experiment. *Each point* represents the average of eight plants, and four leaves per plant. *Closed circles* represent NM plants, *open circles* represent VAM plants. Results of ANOVA are shown in Table 2

Table 2 Summary of analyses of variance for the experiments. Significant probability values ($P \leq 0.05$) are indicated in *italics*

Source	*df*	*F*-value	Probabilities of significance
Intact leaves cowpea			
Colonization	1	4.28	*0.04*
Leaf age	3	24.5	*0.0001*
Day	5	40.6	*0.0001*
Colonization × leaf age	3	1.42	0.24
Colonization × day	5	0.73	0.60
Leaf age × day	15	3.9	*0.0001*
Colonization × leaf age × day	15	0.90	0.57
pH experiment: cowpea			
Colonization	1	0.27	0.60
pH	2	1.36	0.26
ABA	3	150.84	*0.0001*
Colonization × pH	2	10.66	*0.0001*
Colonization × ABA	3	4.35	*0.006*
pH × ABA	6	0.53	0.79
Colonization × pH × ABA	6	0.21	0.97
pH experiment: rose			
Colonization	1	5.35	*0.02*
pH	2	29.64	*0.0001*
ABA	3	70.30	*0.0001*
Colonization × pH	2	3.57	*0.02*
Colonization × ABA	3	1.62	0.19
pH × ABA	6	1.14	0.34
Colonization × pH × ABA	6	1.02	0.41
Calcium experiment: cowpea			
Colonization	1	0.42	0.52
Ca	2	5.05	*0.007*
ABA	3	36.89	*0.0001*
Colonization × Ca	2	0.71	0.49
Colonization × ABA	3	0.05	0.99
Ca × ABA	6	2.44	*0.03*
Colonization × Ca × ABA	6	0.38	0.89
Phosphorus experiment: cowpea			
Colonization	1	1.93	0.17
PO_4	2	0.34	0.71
ABA	3	356.94	*0.0001*
Colonization × PO_4	2	1.47	0.23
Colonization × ABA	3	0.63	0.60
PO_4 × ABA	6	2.54	*0.02*
Colonization × PO_4 × ABA	6	0.41	0.87

been observed on several occasions to be higher in VAM than in NM roses (Augé 1989; Augé et al. 1986a,b, 1987a; Henderson and Davies 1990).

Transpiration of detached leaves

VAM colonization resulted in statistically significant effects on stomatal response to ABA in both cowpea and rose (Fig. 2, Table 2). This is indicated by the significant main effect of colonization in rose and, in cowpea, significant colonization × pH and colonization × ABA interactions, suggesting that stomata of leaves of VAM and NM cowpeas reacted differently as pH and ABA levels changed.

Although VAM influence on transpiration was not predictable across the pH treatments, the pH-induced differences in transpiration displayed by VAM and NM leaves tended to be consistent in cowpea and rose (Fig. 2). As in cowpea, transpiration tended to be higher in NM than in VAM leaves of rose plants at pH 5.5, whereas at pH 6.0, VAM transpiration was higher than NM transpiration across the four ABA concentrations. At the lower two pHs, differences in transpiration in VAM and NM leaves of cowpea and rose tended to be more evident at lower ABA concentrations (10^{-7} and 10^{-6}). In each host species, VAM and NM differences in transpiration disappeared at ABA concentrations of 10^{-5} M.

VAM colonization did not affect the transpirational response of cowpea leaves to changes in calcium and phosphorus concentrations, nor did VAM colonization affect transpirational response to ABA in these two experiments (Fig. 3). Calcium affected transpiration, regardless of mycorrhizal treatments. Significant calcium × ABA and phosphorus × ABA interactions revealed the influence of these ions on sensitivity of transpiration of both hosts to ABA concentrations; in the absence of calcium or phosphorus, cowpea stomata were unresponsive to the addition of 10^{-7} M ABA to the feeding solution (Fig. 3, 0.0 mM Ca and 0.0 mM $P0_4$ graphs). Increasing ABA concentrations led to decreasing transpiration in each species (Figs. 2, 3).

Discussion

VAM symbiosis can change leaf biochemistry, in terms of nutrient status, overall turgor, and solute concentrations (Augé et al. 1986b), and carbon assimilation (Bethlenfalvay et al. 1990; Wang et al. 1989). Hence, it seems theoretically feasible that a leaf from a VAM plant might retain some VAM influence even after it has been separated from its root system and its below-ground source of substances and signals. It is also conceivable, although to date without experimental support, that VAM and NM leaves may develop hydraulic differences that would affect the rate at which they lose water, even in VAM and NM leaves of similar size and phosphorus concentration.

Our work demonstrates that there was a residual influence of mycorrhizal symbiosis in rose leaves, but not in cowpea leaves. That VAM and NM rose leaves had different transpiration rates is consistent with previous findings. In most studies of the water relations of mycorrhizal roses, VAM fungi have altered the water balance of rose plants, under adequate soil moisture (Augé et al. 1986a) or water limitation (Augé and Duan 1991; Augé and Stodola 1990; Augé et al. 1986b, 1987a,b, 1992; Henderson and Davies 1990). Given the significant colonization × pH and colonization × ABA interactions in cowpea leaves in the pH experiment, we could strain to make a case for a residual mycorrhizal influence in detached cowpea leaves. However, the pH difference inverted the effect of colonization on transpiration, and this inconsistency suggests that the statis-

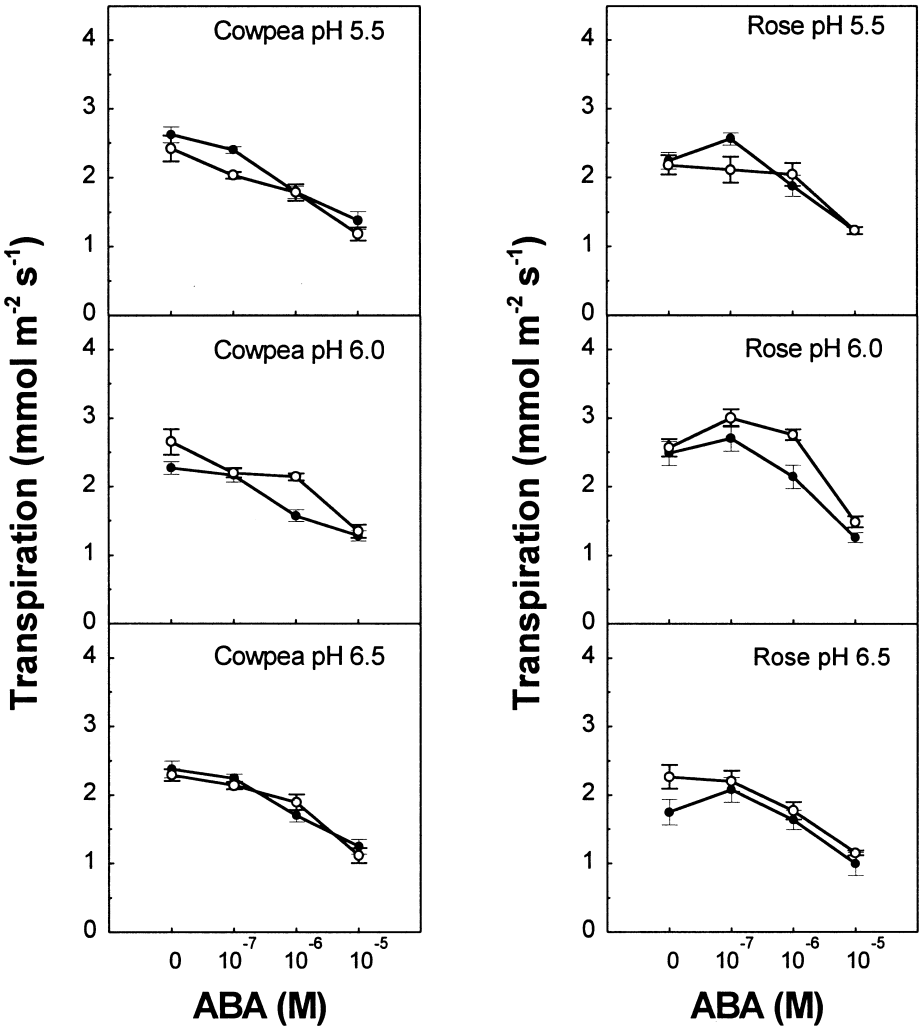

Fig. 2 Whole-leaf transpiration of detached leaves from VAM and NM plants of cowpea and rose, given solutions of different ABA and pH. *Each point* represents the average of eight plants. *Closed circles* represent NM plants, *open circles* represent VAM plants. Results of ANOVA are shown in Table 2

tical difference probably does not indicate biological significance. The lack of significant colonization main effects or interactions on cowpea leaves in the calcium and phosphorus experiments further supports this view. We usually find that VAM colonization modifies stomatal behavior of intact cowpea leaves (Augé et al. 1992; Duan et al. 1996; Ebel et al. 1996, 1997; Fig. 1, current work), but in the current work, mycorrhizal symbiosis did not cause a predictable or meaningful alteration of transpiration in detached leaves.

The difference in transpiration of detached VAM and NM leaves from rose plants under standardized atmospheric conditions indicates that mycorrhizal symbiosis can cause changes in some intrinsic foliar factor, apart from the possibility of altered delivery of an outside (extrafoliar) chemical signal or hydraulic influence. The study was not designed to determine what that intrinsic foliar difference was, but we are able to rule out differences in leaf size or phosphorus concentration. This is significant, because there is apparently a close connection between the nutritional status of leaves and their stomatal responses to ABA (Schurr et al. 1992). For instance, leaves of cotton plants grown with different phosphorus fertilization have had stomates that respond differently to similar ABA (Radin 1984). Leaf size is also important. We conducted the transpiration assays under conditions where PPFD, leaf-to-air vapor pressure gradients, and air velocities were similar across treatments, precluding the possibility of confounding environmental influences on transpiration. But had leaf sizes differed between treatments, this could have affected boundary layer resistances, which can affect transpiration (Nobel 1991).

We included different constituents in the feeding solutions because these constituents are commonly found in xylem sap and have previously been implicated in affecting transpiration or stomatal conductance (Atkinson et al. 1990; De Silva et al. 1986; Gollan et al. 1992; Hartung and Radin 1989; Ruiz et al. 1993; Slovik and Hartung 1992a,b). With the possible exception of pH, we found little evidence that the presence of these xylem constituents caused marked transpirational differences in VAM versus NM leaves. Significant colonization × pH interactions in both cowpea and rose experiments suggest that VAM symbiosis may have affected stomatal sensitivity to xylem pH, a putative regulator of

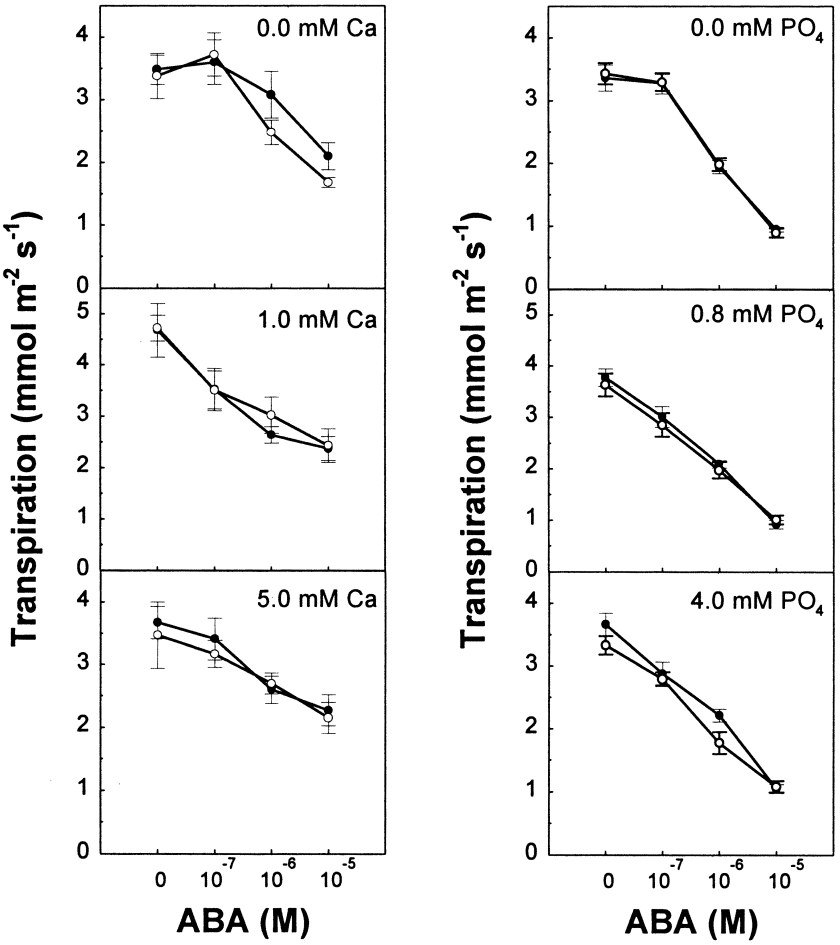

Fig. 3 Whole-leaf transpiration of detached leaves from VAM and NM plants of cowpea, given solutions of different ABA, calcium, and phosphorus. *Each point* represents the average of eight plants. *Closed circles* represent NM plants, *open circles* represent VAM plants. Results of ANOVA are shown in Table 2

stomatal response to leaf water status (Wilkinson and Davies 1997; Wilkinson et al. 1998).

Acknowledgements We thank the reviewers for their critical reading of the manuscript. This work was supported in part by U.S. Department of Agriculture Competitive Research Grant 91-37100-6723 to R.M.A. and by a University of Tennessee Professional Development Research Award to R.M.A.

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