12-1996

Transpiration of detached mycorrhizal and nonmycorrhizal leaves fed varying abscisic acid, pH, calcium and phosphorus

Craig Dale. Green

To the Graduate Council:

I am submitting herewith a thesis written by Craig Dale. Green entitled "Transpiration of detached mycorrhizal and nonmycorrhizal leaves fed varying abscisic acid, pH, calcium and phosphorus." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Landscape Architecture.

Robert M. Augé, Major Professor

We have read this thesis and recommend its acceptance:

Tim Tschaplinski, Bonnie Ownley

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

I am submitting herewith a thesis written by Craig D. Green entitled "Transpiration of Detached Mycorrhizal and Nonmycorrhizal Leaves Fed Varying Abscisic Acid, pH, Calcium and Phosphorus". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ornamental Horticultural and Landscape Design.

Robert M. Augé, Major Professor

We have read this thesis
and recommend its acceptance:

Bonnie H. Ownley

Jim J. Zachaplewski

Accepted for the Council:

Associate Vice Chancellor and

Dean of The Graduate School

TRANSPIRATION OF DETACHED MYCORRHIZAL AND NONMYCORRHIZAL LEAVES FED VARYING ABSCISIC ACID, pH, CALCIUM AND PHOSPHORUS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Craig D. Green

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DEDICATION

This thesis is dedicated to my beloved wife Jill. Her love and support made it possible for me to finish this.

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ABSTRACT

Mycorrhizal colonization can alter stomatal behavior of host leaves during drought without obvious changes in plant size or nutrition. This may be related to an altered production or reception of a chemical signal of soil drying. I tested whether intact root systems were required to observe a mycorrhizal effect on leaf transpiration (*E*), or whether some residual mycorrhizal influence on leaves could affect *E* of foliage detached from root systems. Transpiration assays were performed in the presence of several possible candidates for a chemical signal of soil drying. Although colonization alone did not alter *E* of detached leaves of *Vigna unguiculata* (cowpea), colonization interacted significantly with ABA and pH in regulating transpiration. Colonization affected *E* of detached *Rosa hybrida* (rose) leaves but had no effect on *E* of detached leaves of *Pelargonium hortorum* (geranium). In each species tested, increasing the ABA concentration decreased *E*. In cowpea, calcium appeared to alter stomatal sensitivity to ABA, as well as regulate stomatal activity directly. The pH of the feeding solution affected *E* in rose, but did not change *E* independently in cowpea or geranium. Adding phosphorus to the feeding solution did not alter *E*, but did change the apparent sensitivity of cowpea stomata to ABA. Colonization of roots by mycorrhizal fungi can result in residual effects in detached leaves, that can alter the stomatal reception of chemical signals in both rose and cowpea.

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Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi play important roles in the plant community, colonizing the roots of most plants, and markedly affecting their physiology. One example is their ability to relieve certain stresses imposed on a plant. AM fungi probably have their greatest impact when plants are exposed to "growth-limiting environmental stress" (Sylvia & Williams 1992) and are most often found in soils of low productivity, especially where water and nutrient stress prevail (Gerdemann 1976, Hayman *et al.* 1976).

Water is the single most important element limiting plant productivity throughout the world (Kreeb *et al.* 1989). Influences by AM fungi change a plant's ability to absorb and conduct water, and should thus be examined. Plants take up water from the soil through roots and deliver it through xylem to leaves where it exits leaves via transpiration. It is widely accepted that one of the greatest benefits to the plant from AM fungi is increased phosphorus nutrition, and most early studies attributed influences on water relations to such (Safir *et al.* 1971, Safir *et al.* 1972, Graham & Syvertsen 1985). Because of this, it became important to design experiments so that uncolonized plants would have adequate phosphorus to attain similar growth rates as AM plants. Differences in water relations between colonized and uncolonized plants appearing in light of these controls suggested an even

more complex effect on plants infected with AM fungi (Augé *et al.* 1986b, Augé 1989). Now there is conclusive evidence that AM fungi have both nutritional and non-nutritional effects on plants.

Transpiration (*E*) can be affected by AM colonization (Drüge and Schönbeck 1992). When compared, mycorrhizal rose plants had transpiration rates 12.2 mg m^{-2} s^{-1} to 14 mg m^{-2} s^{-1} higher than nonmycorrhizal rose plants of similar size and phosphorus content (Augé 1989), under similar conditions in either a greenhouse or growth room. Such a change may have been related to osmotic adjustments occurring in the leaf upon colonization. This idea has been explored (Allen & Boosalis 1983, Augé *et al.* 1986b) and it was found that mycorrhizal leaves had decreased osmotic potential (Ψ_π) at full turgor and at the turgor loss point. This was associated with a corresponding increase in the pressure potential (Ψ_p) at full turgor. Colonization apparently enabled plants to maintain leaf turgor at larger tissue water deficits, and lower leaf and soil water potentials (Ψ) compared to uncolonized plants. Mycorrhizae also influence photosynthesis, by increasing CO_2 assimilation rates (Drüge and Schönbeck 1992).

Additionally, the uptake of water by the root can be altered by colonization. Mycorrhizae have hyphae, some of which extend away from the root, penetrating into and between soil aggregates. Besides greatly increasing the absorptive surface area (Gerdemann 1975), these may

increase the root hydraulic conductivity of mycorrhizal plants (Hardie and Leyton 1981), with substantial increases in water uptake being associated with decreased root resistance (Faber *et al.* 1991). These extraradical hyphae may be acting as tiny conduits, exploring and scavenging water from portions of the soil environment where root hairs cannot reach. Mycorrhizal roots are able to maintain greater turgor across a range of tissue hydration, following drought, although under well-watered conditions mycorrhizal roots had lower solute concentrations in root symplasm than nonmycorrhizal roots (Augé and Stodola 1990).

Stomatal conductance (g_s), the inverse of the resistance of water loss from a leaf, can also be affected by mycorrhizal colonization (Levy and Krikun 1979, Hardie and Leyton 1981, Allen and Boosalis 1983, Augé *et al.* 1986a, Augé *et al.* 1987, Augé *et al.* 1991, Ebel *et al.* 1996). Usually, mycorrhizal symbiosis increases g_s. Early studies attributed changes in g_s to increased root length, surface area and lower root resistances in mycorrhizal plants (Hardie and Leyton 1981), or alternatively to altered stomatal regulation independent of root resistance (Levy and Krikun 1979). Higher g_s has been demonstrated in both wet and dry conditions, where stomatal closure occurred at lower leaf water potentials in mycorrhizal plants (Allen and Boosalis 1983). Well-watered rose plants colonized by AM fungi, and given low rates of P, had higher g_s even when there was no apparent difference in leaf water potential of colonized and uncolonized plants (Augé

et al. 1986a). Foliar leaf P levels did not appear to be the controlling factor. When plants were artificially manipulated by changing the soil Ψ_π to achieve similar low levels, AM plants also had higher g_s, suggesting that the colonized root systems either scavenged water of low activity more effectively or influenced nonhydraulic root-to-shoot communication differently than the non-infected root systems (Augé *et al.* 1992).

AM influence on "nonhydrualic", chemical signals of soil drying has been examined. Currently, the idea is that such a signal originates in roots in response to soil drying and is transported to the leaves. Infection of roots by mycorrhizal fungi has led to increases in gibberellin-like substances and decreases in ABA-like substances, both plant hormones with signal characteristics (Allen *et al.* 1981). In-split root experiments, mycorrhizal plants with one side of the root system dried displayed declines in g_s before any change was observed in leaf water potential, possibly due to altered chemical signal production associated with a change in the rate of turgor decline (Augé and Duan 1991). However, quantitative studies with an indirect ELISA have shown a considerably higher level of free ABA in AM infected plants (Danneberg *et al.* 1992). Zeatin riboside, another possible signal, did not differ with colonization (Danneberg *et al.* 1992). AM fungi may also eliminate the growth inhibition to chemical signals communicating dry soil conditions (Augé *et al.* 1994).

Mycorrhizal fungi historically have been shown to increase plant uptake of phosphorus and calcium (Gerdemann 1978). Both ions have been implicated in drought-induced chemical signaling as has the pH change potentially stimulated in the xylem by this increased accumulation (Davies *et al.* 1990, Hartung & Radin 1989).

Chemical signals and stomatal behavior

The idea of chemical signals affecting plant water relations can be best described by comparing it to the traditional idea of hydraulic limitation. Hydraulic limitation occurs when soil water content drops, causing root dehydration. Eventually, water loss rate from leaves exceeds replacement rate by root water absorption. This is followed by declines in leaf Ψ, Ψ_p, and relative water content (RWC). Stomata then close, which minimizes further leaf dehydration (Figure 1). The nonhydraulic, root-to-shoot signaling hypothesis asserts that a chemical(s) alerts the leaf to changing soil conditions. This can occur before soil is dry enough for leaf water relations to have changed. Upon reception of the signal, stomata can close, reducing further water loss and potential damage (Figure 2) (Blackman and Davies 1985, Saab and Sharp 1989, Zhang and Davies 1989, Zhang and Davies 1990).

Researchers have used various procedures to test for the presence of chemical signals. One involves splitting the root system of one plant

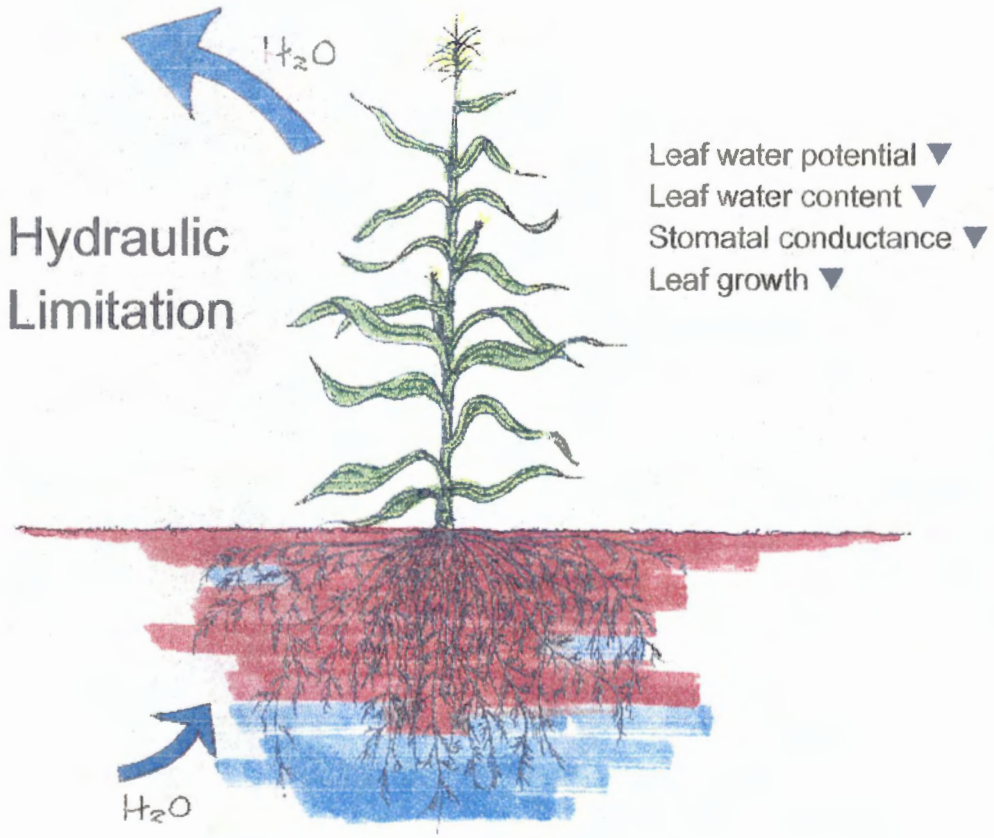

Figure 1. Illustration of hydraulic limitation of stomatal behavior. Arrow size represents relative water movement. Brown areas are dryer soil, blue areas are moister soil.

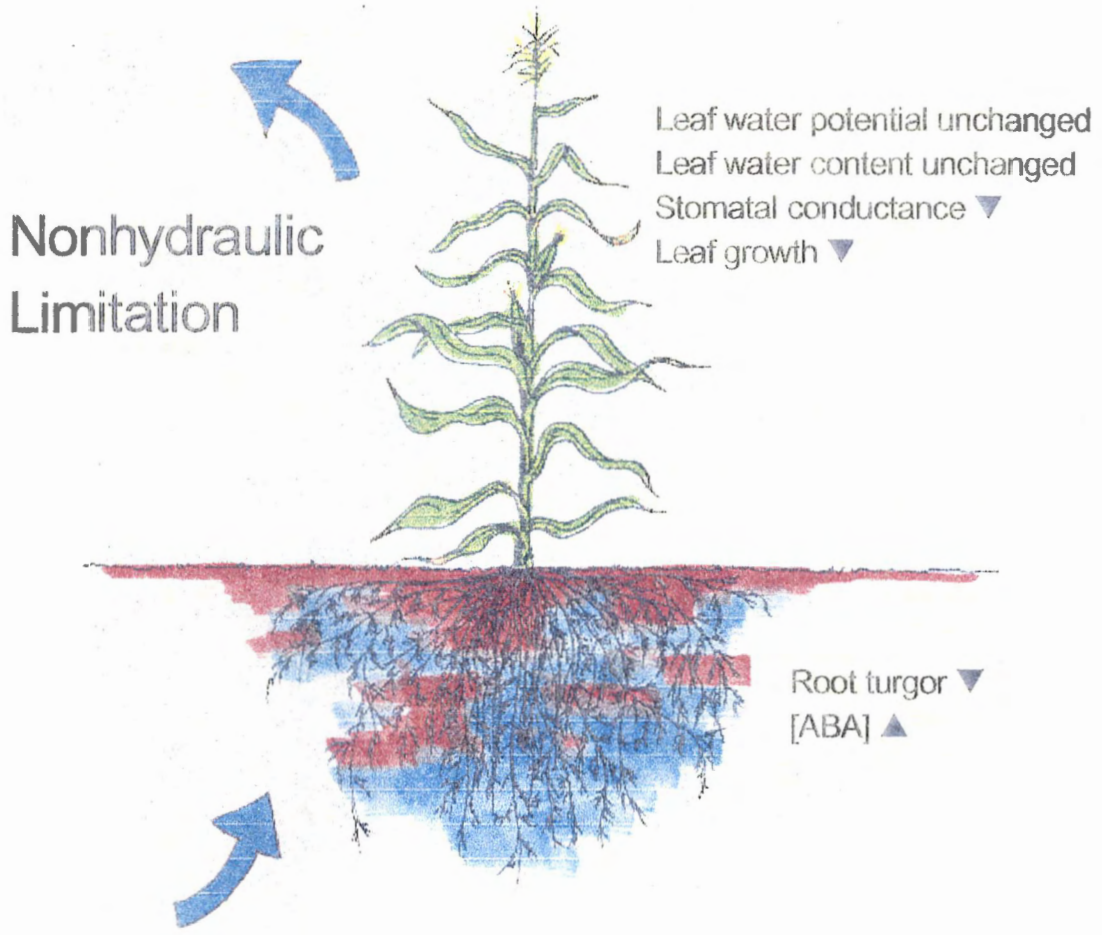

Figure 2. Illutration of nonhydraulic limitation of stomatal behavior. Arrow size represents relative water movement, blue areas are moist soil and brown areas are dry soil.

between two pots (Zhang *et al.* 1987, Saab and Sharp 1989, Gowing and Davies 1990, Zhang and Davies 1990). One pot would be dried and the other watered regularly, providing enough moisture to shoots to maintain normal leaf water status (Zhang *et al.* 1987, Zhang and Davies 1989), while simultaneously drying part of the root system sufficiently to produce the chemical signal. Severing the dry side would result in restored g_s due to a decreased signal concentration. To further test for the signal, plants were grown with roots under pressure to force normal hydration (Gollan *et al.* 1986, Passioura 1988), or in deep containers so that only a portion of the root system dried (Zhang and Davies 1989, Blum *et al.* 1990, Zhang and Davies1990). In both situations, where normal or control leaf hydration was maintained, g_s declined relative to controls, supporting the existence of a chemical signal affecting water relations of a plant.

Several possibilities exist for the nature of the signal, but it is likely a positive inhibitor conveyed by hormonal, nutritional, or ionic means (Gowing *et al.* 1990). ABA is a positive inhibitor of g_s and is currently considered a chemical signal of soil drying (Davies *et al.* 1994). Other possible signals include calcium, phosphorus, or pH changes in the xylem sap (Radin 1984, Hartung and Radin 1988, Atkinson *et al.* 1990,). As mentioned previously, calcium and phosphorus are affected by AM colonization which could change xylem sap pH levels. The objective of my study was to examine the effect of AM fungi on the reception of these suspected signals at the

stomata when the roots are not connected to the plant. To do this, detached leaf transpiration assays were performed. This study was conducted to determine if AM fungi, upon colonization, cause metabolic or biochemical changes that alter the sensitivity of stomata to these chemical signals, changes that remain even after the fungi are separated from the leaf.

II. MATERIALS AND METHODS

In situ leaf conductance

Plant material and culture. Seeds of *Vigna unguiculata* (L.) Walp. 'White acre' (cowpea) were planted and grown in 1-liter pots, containing a medium composed of two parts autoclaved silica sand, and one part calcined montmorillonite clay (Turface) (v:v). This medium was chosen because it promotes extensive mycorrhizal colonization, it can be readily removed from the roots allowing quantification of the fungus, and the soil moisture characteristics are known (Augé *et al.* 1994). To this mixture was added pot culture which contained medium and roots from either cowpea plants whose roots were colonized by mycorrhizal fungi (*Glomus intraradices* Schenck & Smith) or cowpea plants whose roots were not colonized. The added medium was mixed one part pot culture to three parts of the sand/turface mixture (v:v). Work benches, utensils, and containers were sterilized with a 10% bleach mixture to prevent contamination of the nonmycorrhizal pot culture and newly planted plants. Plants were put under high intensity sodium lights on a greenhouse bench to grow until sufficiently colonized.

With every watering (usually every day), the plants received a 0.21 M concentration of Peter's (Grace-Sierra, Milpitas, CA, USA) 15-0-15

fertilizer and a 1 mM concentration of magnesium as $MgCl_2$. To adjust for similar size, mycorrhizal plants received less phosphorus, generally 1mM, with nonmycorrhizal plants receiving either 2, 3, or 4 mM P as K_2PO_4 once a week. Soluble trace elements were supplied once a week at 1 μM Mn (STEM, Peters Fertilizer Products, W.R. Grace, Fogelsville, PA, USA). Fe was provided at 1.0 mM as Sprint (Ciba-Geigy, Greensboro, NC, USA).

Between 2 April and 28 April, 1995 mycorrhizal cowpea plants with high colonization and nonmycorrhizal plants of similar size were tested for differences in abaxial g_s. Four to five leaves per plant were measured using a diffusion porometer (AP4, Delta-T Device, Cambridge, Great Britain) placed adjacent to the mid-vein. g_s readings were taken between 9:00 am and 3:00 pm for ten days. Leaf temperatures ranged from 16.6 °C to 32.6 °C. Relative humidities in the greenhouse ranged from 30 % to 55 %. This was a preliminary test to verify that AM fungi do in fact alter g_s. Photosynthetic flux density was recorded with each measurement using the light sensor on the porometer; values ranged from 10 to 2400 μmol m^{-2} s^{-1}. Upon completion of the experiment total leaf area was determined with a leaf area meter (Li-Cor LI-3000A, Lincoln, NE, USA). Plants were oven dried at 80 ° C and dry weight was determined.

Preliminary assay to test transpiration assay procedure

Fifty mycorrhizal and 50 nonmycorrhizal cowpea (White acre) plants of similar size, grown and cultured as described in the *in situ* leaf conductance experiment, were used for a second preliminary test to verify the effectiveness of the detached leaf transpiration assay procedure used to follow stomatal activity. These tests were performed on 15 and 17 February, 1995. For each assay the terminal leaflet from the third leaf of each plant was excised under distilled water. Once detached, the leaf was trimmed and transferred to a storage vial containing only distilled water and allowed to rehydrate in the dark. When turgid, a leaf would be transferred into a treatment vial containing a concentration of abscisic acid (ABA) (10^{-7}, 10^{-6}, 10^{-5} M) or a control vial containing only distilled water. The vials were then moved to a benchtop space illuminated by high intensity sodium halide lamps (PPFD 200-400 µmol m^{-2} s^{-1}). The vials were weighed every 30 minutes on a Sartorius analytical balance, with the weight and time being recorded into a spreadsheet template by Sartowedge software and an RS232 interface. This continued for three hours. The area of the transpiring part of the leaf was measured using a leaf area meter and recorded. The transpiration rate (*E*) was calculated using the following formula:

$$E = \frac{\Delta W}{\text{leaf area} * \Delta t}$$

where W was weight of water in grams and t was time in seconds. Leaf areas were recorded as square meters. Light levels for this and following assays were maintained close to 325 μmol m^{-2} s^{-1} PPFD. Air temperatures for all assays were about 27 °C and relative humidity in the study area ranged from 26 % to 62 %.

Cowpea pH transpiration assay, mycorrhizal vs nonmycorrhizal

To test the effect of changing pH levels in the xylem sap as a possible chemical signal affecting stomatal opening, 50 mycorrhizal and 50 nonmycorrhizal cowpea (White acre) plants were grown and cultured similarly to those in the preliminary experiment. The exception was that these plants were transferred to a controlled growth chamber (M75, Environmental Growth Chambers, Inc., Chagrin Falls, OH, USA) shortly after germination to grow until the experiment was complete. The chamber was set on a 14 hour day/10 hour night program with day temperature set at 25 °C and night temperatures of 18-20 °C, with relative humidity levels set to 65 %. Light levels at bench height were approximately 400 mmol m^{-2} s^{-1} PPFD during the light cycle. Here also the terminal leaflet from the third leaf of each plant was excised under distilled water, trimmed and transferred to a storage vial containing only distilled water and allowed to rehydrate. Once turgid, the leaflet was transferred into a treatment vial containing a 10^{-7}, 10^{-6}, 10^{-5} M concentration of ABA or a control vial containing only distilled water.

Each solution had been adjusted to one of three predetermined pH values (5.5, 6.0, and 6.5). In one day, two complete replications could be done. To obtain eight replications of each treatment, the assay was repeated for four days. The assay was completed in 3 hours and the *E* was calculated. After the experiment, total leaf area, percent root colonization by AM fungi and shoot dry weight of ten mycorrhizal and ten nonmycorrhizal plants were determined.

Cowpea phosphorus transpiration assay, mycorrhizal vs nonmycorrhizal

Cowpea (White acre) plants planted 19 June, 1995 were grown and cultured as described in the cowpea pH experiment, fifty mycorrhizal and fifty nonmycorrhizal. For this assay, the terminal leaflet from the third leaf of each plant, after being excised under distilled water, trimmed, and transferred to a storage vial containing only distilled water, and allowed to rehydrate, was transferred into a treatment vial containing a concentration of ABA ranging from 10^{-7} to 10^{-5} M or a control vial containing only distilled water. Each vial contained one of three phosphorus concentrations (0 mM, 0.4 mM, and 8 mM). Phosphorus was added as KH_2PO_4. The pH of each solution was adjusted to 6.0. Two complete replications were completed each day and the assay was repeated for four days between 13-19 September, 1995 to obtain eight replications of each treatment. The assay was completed in 3 hours and the *E* was calculated. At the end of this

experiment, shoot dry weight, percent root colonization by AM fungi and total leaf area were measured.

Cowpea calcium transpiration assay, mycorrhizal vs nonmycorrhizal

Fifty mycorrhizal and 50 nonmycorrhizal cowpea (White acre) plants planted 1 November, 1995 were grown and cultured as described in the cowpea pH experiment. The assay solutions in which the terminal leaflets were place in after rehydration contained three levels of calcium (0 mM, 1 mM and 5 mM) and either ABA at a concentration of 10^{-7}, 10^{-6} or 10^{-5} M or only distilled water as a control. The calcium was supplied as $CaCl_2$. The pH of each solution was adjusted to 6.0. There were eight replications of each treatment, requiring four days (20 December, 1995 to 3 January, 1996) to complete the experiment. At the end of the experiment, plant dry weight, percent colonization and total leaf area were measured.

Rose pH transpiration assay, mycorrhizal vs nonmycorrhizal

Plant material and culture. Rose plants (*Rosa hybirda*) were donated by Jackson and Perkins (California). One hundred plants were transplanted and grown in 5.8-liter pots, containing a medium composed of two parts autoclaved silica sand, and one part calcined montmorillonite clay (Turface) (v:v) on 10 January, 1995. To this mixture was added pot culture which contained medium and roots from either cowpea plants whose roots were

colonized by mycorrhizal fungi (*Glomus intratadices* Schenck & Smith) or cowpea plants whose roots were not colonized (50 of each). The added medium was mixed one part pot culture to three parts of the sand/turface mixture (v:v). Special precautions were made to prevent contamination of the nonmycorrhizal pot culture and newly planted plants. Plants were allowed to grow on a greenhouse bench until the experiment was completed.

With every watering (usually every day), the plants received a 0.21 M concentration of Peter's (Grace-Sierra, Milpitas, CA, USA) 15-0-15 fertilizer and a 1 mM concentration of magnesium as $MgCl_2$. To adjust for similar size, mycorrhizal plants received less phosphorus, generally 1 mM, with nonmycorrhizal receiving 2, 3, or 4 mM depending on the particular experimental conditions. Phosphorus was applied as K_2PO_4 once a week. Soluble trace elements were supplied once a week at 1 μM Mn (STEM, Peters Fertilizer Products, W.R. Grace, Fogelsville, PA, USA). Fe was provided at 1.0 mM as Sprint (Ciba-Geigy, Greensboro, NC, USA) once a week.

To test the effectiveness of pH as a chemical signal, leaves from each plant were excised under distilled water and transferred to a storage vial containing only distilled water and allowed to rehydrate. Turgid leaves were transferred into a treatment vial containing a concentration of ABA ranging from 10^{-7} to 10^{-5} M or only distilled water that had been adjusted to

one of three predetermined pH values (5.5, 6.0, and 6.5). The assay was completed in three hours and the *E* was calculated. There were eight replications of each treatment, requiring four days between 23 June, 1995 and 30 June, 1995 to complete the experiment. After the experiment total leaf area, percent root colonization by AM fungi and shoot dry weight were determined.

Geranium pH transpiration assay, mycorrhizal vs nonmycorrhizal

Plant material and culture. Geranium, *Pelargonium hortorum* (Designer Scarlet) stem cuttings were taken from the trial garden located at the University of Tennessee Agricultural campus. One hundred cuttings were started under mist in nursery trays in low light. Later the cuttings were transplanted and grown in 1-liter pots, containing a medium composed of two parts autoclaved silica sand, and one part calcined montmorillonite clay (Turface) (v:v). To this mixture was added pot culture which contained medium and roots from either cowpea plants whose roots were colonized by mycorrhizal fungi (*Glomus intraradices* Schenck & Smith) or plants whose roots were not colonized (50 of each). The added medium was mixed one part pot culture to three parts of the sand/turface mixture (v:v). Precautions were made to prevent contamination of the nonmycorrhizal pot culture and newly planted plants. Plants were allowed to grow on a greenhouse bench until the experiment was completed on 21 August, 1995.

With every watering (usually every day), the plants received a 0.21 M concentration of Peter's (Grace-Sierra, Milpitas, CA, USA) 15-5-15 fertilizer and a 1 mM concentration of magnesium as $MgCl_2$. Phosphorus was applied as K_2PO_4 once per week. Soluble trace elements were supplied once per week at 1 μM Mn (STEM, Peters Fertilizer Products, W.R. Grace, Fogelsville, PA, USA). Fe was provided at 1.0 mM as Sprint (Ciba-Geigy, Greensboro, NC, USA) weekly.

To test the effectiveness of pH as a chemical signal, leaves from each plant were excised at the petiole under distilled water, transferred to a storage vial containing only distilled water and allowed to rehydrate. When turgid, the leaf's petiole was placed into a treatment vial containing a concentration of ABA ranging from 10^{-7} to 10^{-5} M (or distilled water) that had been adjusted to one of three predetermined pH values (5.5, 6.0, and 6.5). This assay also was completed in three hours and the *E* was calculated. There were eight replications of each treatment, requiring four days between 14 August, 1995 and 21 August, 1995 to complete the experiment. After the experiment total leaf area, percent root colonization by AM fungi and shoot dry weight were determined.

Shoot dry weight, leaf phosphorus concentration, and root colonization

Degree of colonization of root systems by AM fungi was determined at the end of each experiment with eight mycorrhizal and nonmycorrhizal

plants from each experiment. A clearing and staining procedure was used as described by Brundrett *et al.* (1983). The percentage of root colonization was determined using 50 pieces of stained root segments per treatment. The pieces were mounted onto a slide and the mycorrhizal vesicles, arbuscules and hyphae intersecting the line were counted. The scope was set to 100x magnification. Colonization was calculated by dividing colonized roots by total roots examined and expressed as a percentage. Phosphorus concentration of oven-dried (70 °C) leaves was assayed using the vanadate-molybdate-yellow method (Chapman & Pratt, 1961). Samples of leaves were dry-ashed with magnesium nitrate at 700 °C for 2 hours, then digested in nitric acid. Shoots were oven-dried at 80 °C for at least 48 hours and the dry weight measured.

Statistical analysis

Data was analyzed as a completely randomized design using the Analysis-of-Variance (ANOVA) procedure of the Statistical Analytical Services (SAS) programs.

III. RESULTS

In situ leaf conductance

Stomatal conductance of two groups of well-watered cowpea plants were compared, one extensively colonized by AM fungi and one not colonized (Figure 3). Mean g_s for these two group differed significantly after the 10 days of sampling (Table 1). Leaf age also affected g_s, (Table 1), and so I used only the 3rd leaflet throughout all cowpea experiments. The day the sample was taken also changed g_s, thus each replication was always completed in one day. These data, proving AM fungi alter g_s of plants, allowed the experiments to proceed: if I was going to test for a residual effect of mycorrhizal colonization on leaves, first I had to demonstrate that the mycorrhizal effect was present in intact leaves.

Preliminary assay to test assay procedure

In this preliminary assay, mycorrhizal and nonmycorrhizal cowpea leaves did not have different E (P = 0.1305) (Figure 4). Likewise, the sensitivity of the stomata to ABA supplied to the mycorrhizal leaves in the assay solution did not differ from the nonmycorrhizal leaves (P = 0.3962), as monitored by E. Increasing the concentration of ABA supplied in the assay solution from 10^{-7} to 10^{-5} µM caused a decrease in E of 53 % for nonmycorrhizal and 47 % for mycorrhizal cowpea plants. ABA did have a

Table 1. *In situ* conductance of intact cowpea plants. Significant differences in bold.

Source	PR > F
DAY	**0.0001**
LEAF AGE	**0.0001**
COLONIZATION	**0.0393**
DAY*LEAF AGE	**0.0001**
DAY*COLONIZATION	0.3028
LEAF AGE*COLONIZATION	0.2363
DAY*LEAF AGE*COLONIZATION	0.5679

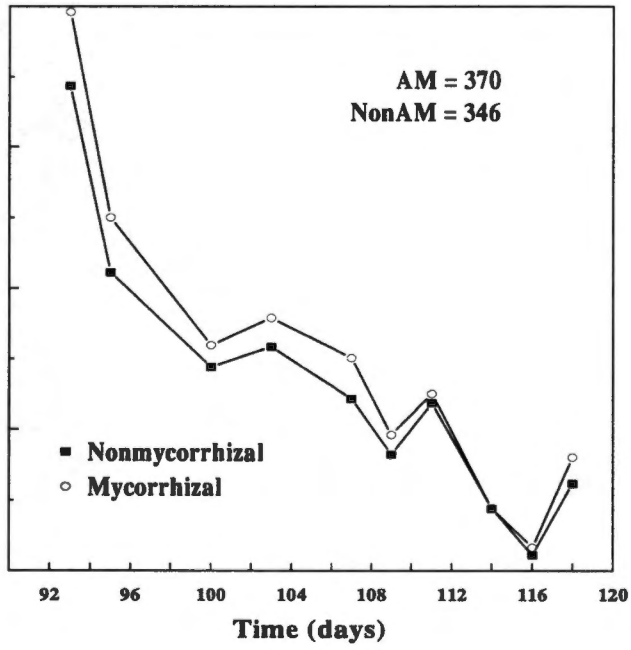

Figure 3 *In situ* leaf conductance of intact mycorrhizal and nonmycorrhizal leaves measured on 10 separate days. Numbers on the x axis refer to Julian days (the number of days into the year). Mean g_s given for each treatment.

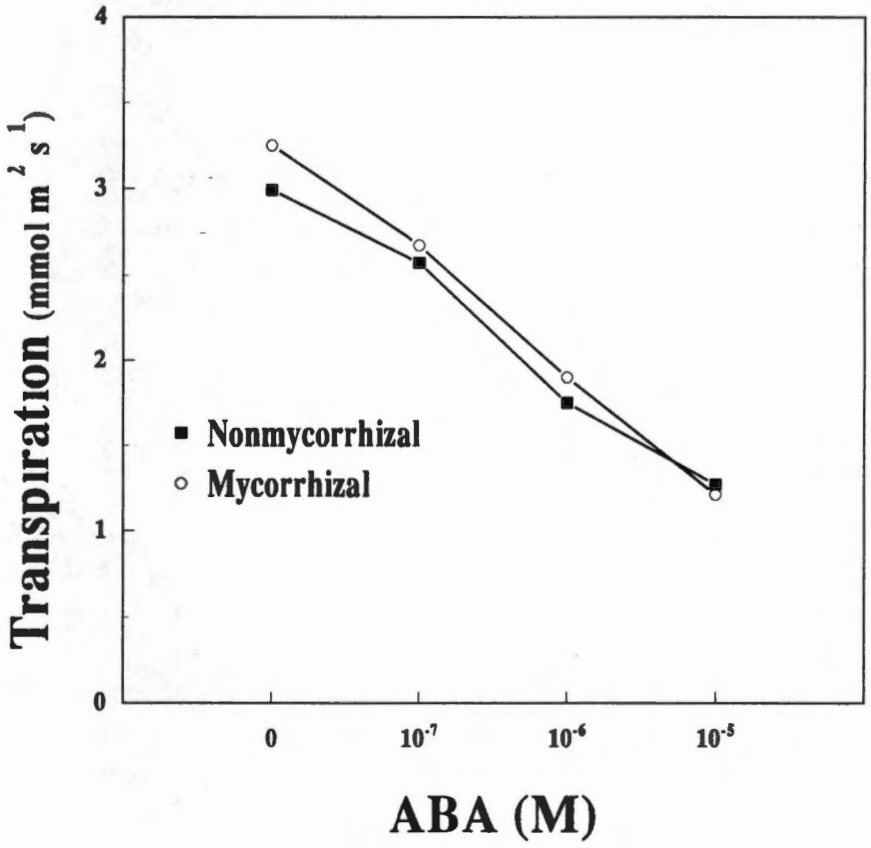

Figure 4. Absolute transpiration of detached mycorrhizal and nonmycorrhizal cowpea leaves at varying ABA.

significant effect on *E* overall, causing a decrease of 48 % with an increase in [ABA] from 10^{-7} to 10^{-5} µM. This proved that the assay procedure was satisfactory.

AM fungi

Statistically significant differences (Table 2) in *E* were observed between mycorrhizal and nonmycorrhizal rose leaves (Figure 5). Average values were 2.14 mmol m^{-2} s^{-1} for mycorrhizal and 1.98 mmol m^{-2} s^{-1} for nonmycorrhizal. Combining the data from the cowpea pH, calcium and phosphorus experiments gave no significant difference (P = 0.764) in *E* of mycorrhizal and nonmycorrhizal cowpea plants (Figure 6). However, colonization did alter the way ABA and pH interacted to affect stomatal opening in cowpea plants (Table 3). Detached mycorrhizal cowpea leaves had an *E* that was decreased to a lesser extent than nonmycorrhizal leaves fed the same concentration of ABA (Figure 7). At an [ABA] of 10^{-6} µM, AM leaves had an average *E* of 1.94 mmol m^{-2} s^{-1} whereas nonmycorrhizal leaves had an average *E* of 1.69 mmol m^{-2} s^{-1}. At a pH level of 6.0, mycorrhizal leaves' average *E* was 2.09 mmol m^{-2} s^{-1}, 0.28 mmol m^{-2} s^{-1} higher than the nonmycorrhizal average *E*. Changing the pH level to 5.5 caused mycorrhizal leaves to average 0.18 mmol m^{-2} s^{-1} lower than the nonmycorrhzial leaves. The rates were almost identical at pH 6.5. Colonization of geraniums did not alter either the overall *E* or the sensitivity

Table 2. ANOVA showing main effects and their interactions for rose pH experiment. Sigificant differences (P < .05) in bold.

SOURCE	PR>F
COLONIZATION	**0.0094**
pH LEVEL	**0.0001**
[ABA]	**0.0001**
COLONIZATION*pH	0.1144
COLONIZATION*[ABA]	0.0760
pH*[ABA]	0.3575

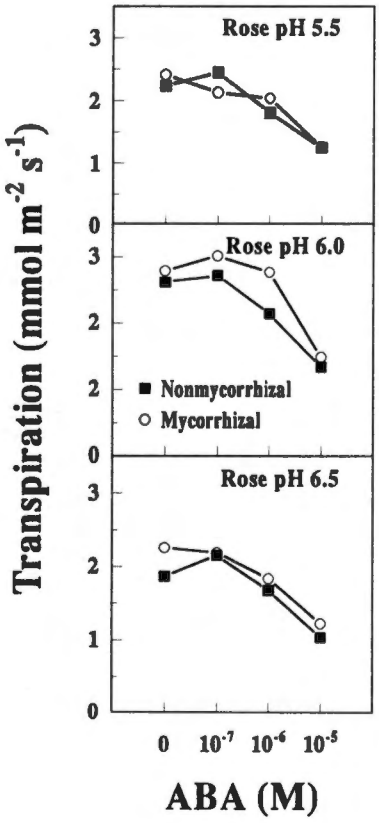

Figure 5. Absolute transpiration of detached mycorrhizal and nonmycorrhizal rose leaves at varying pH and ABA.

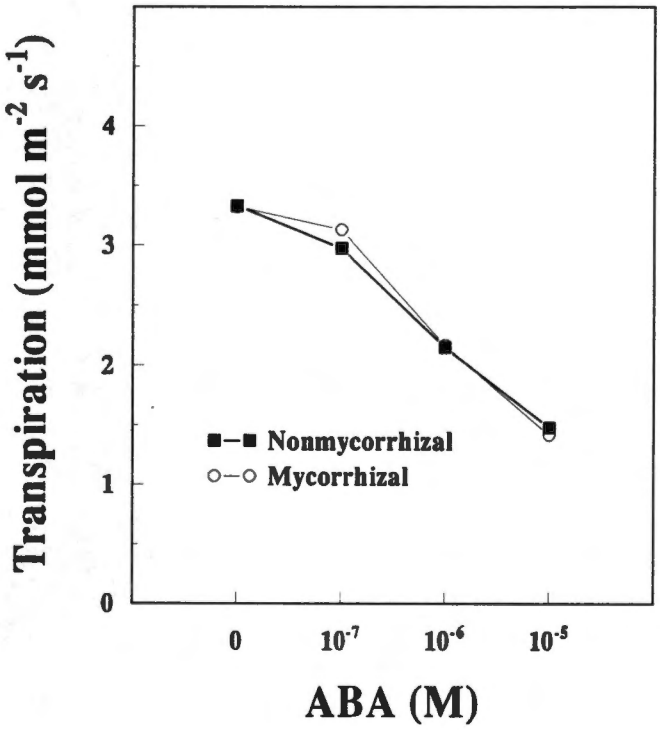

Figure 6. Absolute transpiration of detached mycorrhizal and nonmycorrhizal cowpea leaves combining data from pH, calcium and phosphorus experiments.

Table 3. ANOVA showing main effects and their interactions for cowpea pH experiment. Significant differences (P< .05) in bold.

SOURCE	PR>F
COLONIZATION	0.6020
pH LEVEL	0.2587
[ABA]	**0.0001**
COLONIZATION*pH	**0.0001**
COLONIZATION*[ABA]	**0.0056**
pH*[ABA]	0.7866

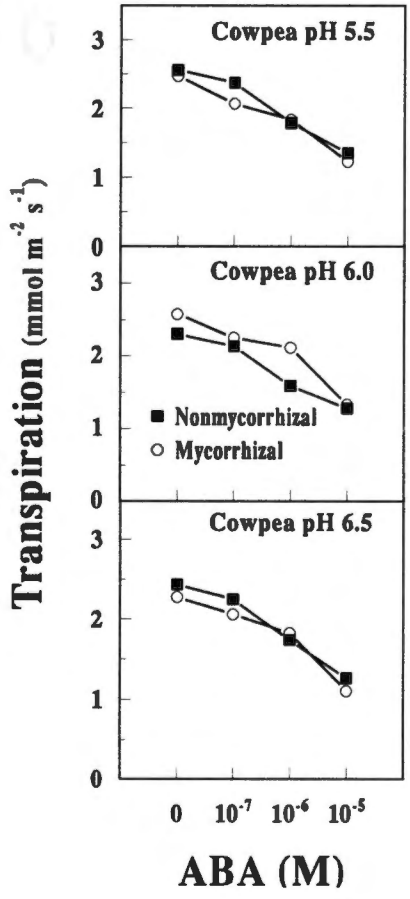

Figure 7. Absolute transpiration of detached mycorrhizal and nonmycorrhizal cowpea leaves at varying pH and ABA

of their stomates to changes in [ABA] or pH (Table 4, Figure 8).

ABA

Transpiration declined similarly in each host species as the concentration of ABA in the feeding solution increased from 10^{-7} to 10^{-5} µM in a near linear manner (Figures 3-10). The *E* for each level of ABA was significantly different ($Pr \leq 0.05$) for each species in all the transpiration experiments (Tables 2-6). The average decrease in *E* accompanying an increase of 10x in the [ABA] was 18.9% for cowpea, 26.4% for geranium and 15.5% for rose. In rose (Figure 5), adding 10^{-7} µM was not sufficient to decrease *E*.

Level of pH

Cowpea and rose *E* were also partially controlled by pH. For rose (Figure 5), solutions with a pH of 6.0 were significantly separated from the other pH levels (Table 2) and resulted in the greatest *E* of 2.36 mmol m^{-2} s^{-1}. Increasing or decreasing the pH by 0.5 caused an 83 % and 73 % decrease, respectively. In cowpea leaves (Figure 7) pH alone did not alter *E*, but as mentioned in the AM fungi section, there was a significant interaction between pH and colonization (Table 3). Geranium *E* was not affected by changes in pH (Table 4, Figure 8).

Table 4. ANOVA showing main effects and their interactions for geranium pH experiment. Significant differences (P < .05) in bold.

SOURCE	PR>F
COLONIZATION	0.4069
pH LEVEL	0.09791
[ABA]	**0.0001**
COLONIZATION*pH	0.5469
COLONIZATION*[ABA]	0.7117
pH*[ABA]	0.6949

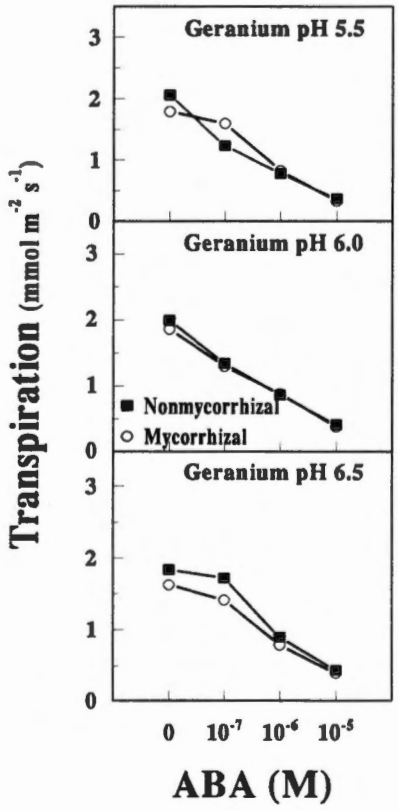

Figure 8. Absolute transpiration of detached mycorrhizal and nonmycorrhizal geranium leaves at varying pH and ABA.

Table 5. ANOVA showing main effects and their interactions for cowpea calcium experiment. Significant differences (P < .05) in bold.

SOURCE	PR>F
COLONIZATION	0.5195
CALCIUM LEVEL	**0.0074**
[ABA]	**0.0001**
COLONIZATION*CALCIUM	0.4927
COLONIZATION*[ABA]	0.9871
CALCIUM*[ABA]	**0.0272**

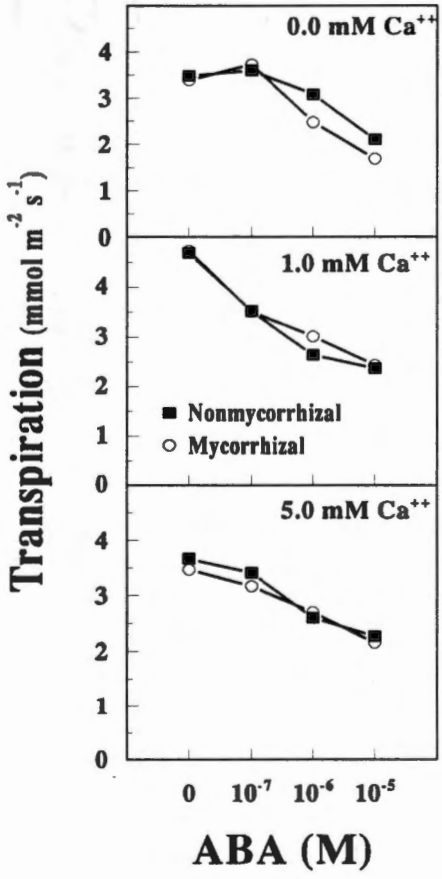

Figure 9. Absolute transpriation of detached mycorrhizal and nonmycorrhizal cowpea leaves at varying calcium and ABA.

Table 6. ANOVA showing main effects and their interactions for cowpea phosphorus experiment. Significant differences (P < .05) in bold.

SOURCE	PR>F
COLONIZATION	0.1665
Phosphorus LEVEL	0.7123
[ABA]	**0.0001**
COLONIZATION*Phosphorus	0.2321
COLONIZATION*[ABA]	0.5954
Phosphorus*[ABA]	**0.0222**

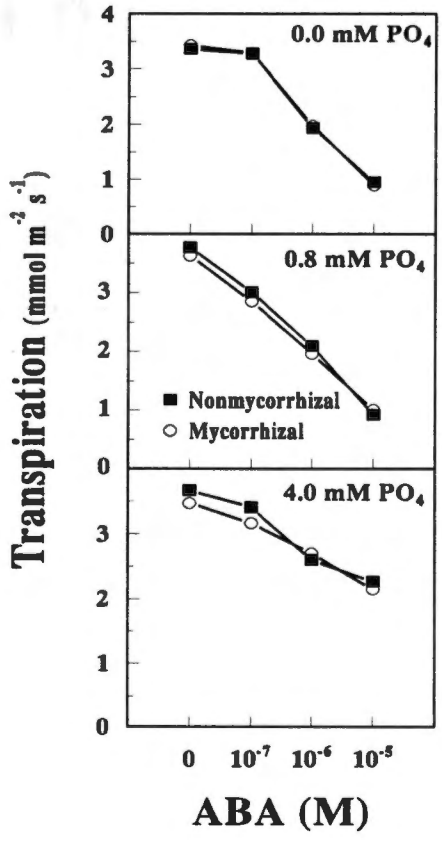

Figure 10. Absolute transpiration of detached mycorrhizal and nonmycorrhizal cowpea leaves at varying phosphorus and ABA.

Level of Calcium

E of detached cowpea leaves was responsive to varying levels of Ca^{++} supplied in the assay solution (Figure 11). When the concentration was 1.0 mM, the average *E* was 3.63 mmol m^{-2} s^{-1}. Increasing the concentration to 5.0 mM or decreasing the concentration to 0.0 mM caused a decrease in *E* of 87%. A significant interaction with ABA also appeared (Table 5). Stomata of cowpea have an apparently different sensitivity to a 1.0 mM concentration of Ca^{++} at 0.0 µM ABA than to either 5.0 mM or no Ca^{++} at the same ABA concentration. The average difference in *E* between 1.0 mM Ca^{++} at 0.0 µM ABA and 0.0 mM Ca^{++} was 1.27 mmol m^{-2} s^{-1}. For 1.0 mM Ca^{++} and 5.0 mM Ca^{++} the difference is 1.14 mmol m^{-2} s^{-1} at 0.0 µM ABA.

Level of phosphorus

Cowpea stomata did not differ in their sensitivity to phosphorus supplied in the assay solution (Table 6, Figure 10). Mean *E* for both 0.8 mM and 4.0 mM PO_4 only differed from controls by 0.03 mmol m^{-2} s^{-1}. The two concentrations differed from each other by an average of 0.06 mmol m^{-2} s^{-1}. There were no significant interactions between the level of colonization and level of phosphorus supplied (Table 6). Phosphorus did alter stomatal sensitivity to ABA but only the 0.8 mM at a 10^{-7} concentration (Table 6).

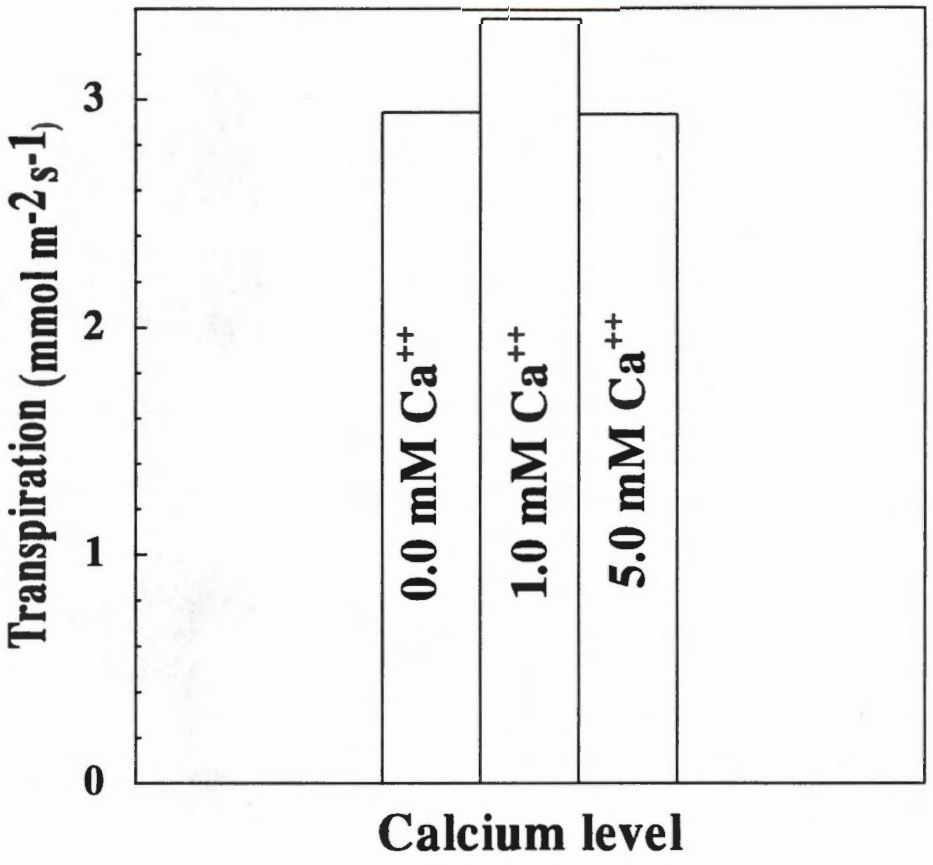

Figure 11. Absolute transpiration of detached mycorrhizal and nonmycorrhizal cowpea leaves at varying calcium.

Shoot dry weight, leaf phosphorus concentration, total leaf area and root colonization

Data for total leaf area and percent root colonization by AM fungi for all experiments are given in Table 7. Shoot dry weight, and leaf phosphorus data for all experiments are give in Table 8. Only in the cowpea experiment with added phosphorus did leaf phosphorus concentration differ between mycorrhizal and nonmycorrhizal leaves. Since mycorrhizal colonization did not alter stomatal sensitivity to phosphorus, I do not consider such a difference relevant. Shoot dry weight of mycorrhizal plants was significantly higher than nonmycorrhizal plants ($P = 0.016$) in the cowpea pH experiment, but by only 11.7%. Such a difference is not usually enough to account for the difference associated with mycorrhizal colonization in that experiment. For all other experiments, no differences were found in these variables, suggesting that nutrition was not the controlling influence here.

Table 7. Percent colonization and leaf area for each experiment.

Experiment	% Colonization: VAM Only			Leaf Area	
	Vesicles	Arbuscules	Hyphae	AM	NonAM
AM vs NonAM	66.7	68.3	88.3	-	-
Cowpea pH	59.5	85	94.5	3071.5	3286.8
Cowpea Phos	55.3	77.8	95.5	954.5	1246.3
Cowpea Ca	57	83.5	98	182.5	223.8
Geranium pH	19	23.3	60.8	1608.0	1704.9
Rose	78.7	6.7	67.7		

Table 8. Shoot dryweight and leaf phosphorus for each experiment. Significant differences in bold.

Experiment	Shoot Dry weight		Leaf Phos	
	AM	NonAM	AM	NonAM
AM vs NonAM	-	-	1.7	2.3
Cowpea pH	27.1	**30.7**	3.5	4.3
Cowpea Phos	6.36	9.25	2.3	**4.3**
Cowpea Ca	2.08	1.96	0.8	1.1
Geranium pH	17.3	17.3	3.7	4.2
Rose	-	-	1.4	1.2

IV. DISCUSSION

Transpiration assays were performed using three host species to test for any residual mycorrhizal effects on stomatal sensitivity to the potential chemical signals ABA, pH, calcium, and phosphorus, as well as the ability of these factors to independently affect stomatal behavior. Finding a difference in g_s, between mycorrhizal and nonmycorrhizal plants with intact leaves (leaves still attached to plants) was necessary in order to continue this study. If no effect was present in intact leaves, one would not be expected in detached leaves. Previously, colonization by mycorrhizal fungi has led to increased g_s in intact leaves of both rose and cowpea (Augé *et al.* 1986a, Ebel *et al.* 1996). Similar results were found in the preliminary g_s experiment performed at the beginning of this study. g_s was consistently higher in AM colonized plants for the 10 days of the test. Several possibilities exist as to the nature of the mycorrhizal influence on stomatal activity. Most authors relate such changes to root functions often altered by mycorrhizal colonization: for example, altered radial or axial hydraulic conductivity (Hardie and Leyton 1981), altered root system architecture (Kotari *et al.* 1990), increased water uptake via soil hyphae (Faber *et al.* 1991), and altered root-to-shoot hormonal relations (Hardie and Leyton 1981). This study investigated whether AM fungi imposes a persistent influence on stomata that remains after the fungi (i.e. the roots) are removed

and, if this influence would change the sensitivity of the stomata to the potential chemical signals in question.

One change in leaf water relations that could possibly be brought about by AM fungi colonization is altered leaf osmotic potential. In rose leaves, colonization by either the AM fungi *Glomus deserticola* Trappe, Boss, and Menge or *G. intraradices* Schenck and Smith led to lower ψ_π at full turgor and at the turgor loss point (Augé *et al.* 1986b). Consequently, the plant was able to maintain leaf turgor and g_s at greater tissue water deficits and lower leaf and soil water potentials, compared to nonmycorrhizal plants In my study, the only host species to have detached leaves with altered transpiration due directly to AM colonization was rose plants. Although leaf ψ_π was not monitored in this study, it may be that these differences were due to altered ψ_π similar to that found in Augé's 1986 study.

Previously, AM colonization did not alter *E* of unstressed cowpea leaves (Duan *et al.* 1996). Similar results were found in my study when *E* and colonization were the only variables. When the [ABA] and pH level are also considered, AM fungi colonizing plant roots do influence cowpea stomata of detached leaves. Here again, no tests were performed to determine if the biochemistry or the metabolism of the leaves had been altered, thus no conclusions as to the specific nature of the mycorrhizal effect can be made. It is possible that cowpea undergoes changes in leaf

ψ_π similar to those in rose, except that the reception of ABA and/or pH changes at the stomata is the specific parameter changed and not just overall *E*. It appears that these changes in stomatal sensitivity were not due to phosphorus nutrition, as the leaf concentration of P did not differ between AM and nonmycorrhizal cowpeas.

The *E* of geranium leaves was not altered by AM colonization, nor did mycorrhizae change the apparent sensitivity of geranium stomata to ABA or pH. Similarly, colonization did not alter the sensitivity of stomata in cowpea leaves to Ca^{++} or P , either independently or in conjunction with ABA.

ABA is a potent inhibitor of g_s (Davies and Zhang 1991). Root-sourced ABA is most likely the molecule acting as the chemical signal of soil drying in plants whose leaves have not yet suffered drought-induced dehydration. Roots produce ABA in sufficient quantities to decrease stomatal activity in the leaf (Zhang *et al.*1990). ABA in the assay solution simulates root-sourced ABA by traveling through the xylem tissue and does decrease *E* of the three test species dramatically in proportion to the amount supplied. This would seem to support the idea of root-sourced ABA being the primary signal but whether or not this depicts actual leaf physiology accurately is unknown.

Leaves also produce ABA, which may be the molecule responsible for inhibiting stomatal opening before any dehydration stress is suffered. If so, some other signal must alert the leaf of drying conditions which would

then stimulates the release of leaf ABA from symplastic compartments into the apoplast, and thence to guard cells. A possible candidate is pH. In the leaf, ABA is in two forms - membrane impermeable ABA and permeable ABAH. The impermeable form is the active stomatal closing form and the permeable form is stored in alkaline compartments (Hartung *et al.* 1990). With increasing stress, pH values change causing ABAH to be deprotonated and allowing the active form to function in closing the stomata. The pH of the assay solution in my study was altered in rose, cowpea and geranium to find if such changes can function as a signal or alter stomatal sensitivity to supplied ABA. If pH increases acted as a signal, a near linear change in *E* should be associated with sequential changes in pH. This did not happen, thus the leaf-sourced ABA is most likely not the active form of ABA controlling stomata. Rose leaves were sensitive to pH changes, but not in a correlated pattern as expected. Instead the pattern shows that, for rose, a pH of 6.0 gives the optimal *E* of the 3 pH's examined. Cowpea and geranium stomata were not sensitive to pH changes, nor to interactions between pH and ABA.

Calcium has also been shown to inhibit stomatal conductance (Atkinson *et al.* 1990) and was examined as a possible chemical signal affecting stomata. Although Ca^{++} applied directly to or fed to transpiring leaves can cause decreased g_s and transpiration (Atkinson 1990), its function as a chemical signal is uncertain. Plants grown in conditions of

high rhizospheric Ca^{++} can show higher g_s (Atkinson 1991). This type of incongruity limits the hypothesis that Ca^{++} is a chemical signal alerting the plant of soil conditions. Similar results were found in this study. Calcium did inhibit *E* at the highest concentration, but the lowest concentration actually increased *E* over the control [Ca^{++}]. Again, if Ca^{++} was a chemical signal, a near linear decrease in *E* would be expected with increasing concentration.

Finally, phosphorus has been shown to influence the sensitivity of stomata to ABA. Plants grown in low P conditions had stomata with increased sensitivity to ABA (Radin 1984). Phosphorus was examined as a potential chemical signal in my study primarily because AM colonization often alters leaf [P]. Colonization did not alter the reception of P and P did not alter *E*. Phosphorus did interact significantly with ABA, but not in a signal manner. Increasing the [P] did not cause a linear change in *E* as expected by a chemical signal.

With the exception of rose plants, there appears to be no consistent mycorrhizal effect on g_s that remains in leaves once they are detached form the plant. Duan *et al.* (1996) came to a similar conclusion and suggested that in their study, AM effects on host g_s had to be mediated by the root system.

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