Xylem Sap Abscisic Acid Concentration and Stomatal Conductance of Mycorrhizal Vigna unguiculata in Drying Soil

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**Summary**

This study was conducted to determine whether xylem abscisic acid (ABA) concentration is altered by mycorrhizal symbiosis of cowpea plants grown in drying soil, and to determine whether stomatal sensitivity to xylem ABA is altered by the symbiosis. We allowed the entire root zone to dry and found that at high soil water contents (θ), mycorrhizal plants had higher stomatal conductance (ĝs) and lower xylem [ABA] than did non-mycorrhizal plants, but the difference disappeared at low θ, probably because of stomatal closure. The altered ĝs and xylem [ABA] were apparently not related to plant water status since shoot water potential, xylem sap osmotic potential and shoot water content were similar for mycorrhizal and non-mycorrhizal plants across the range of soil moisture. These differences were also not related to P nutrition or plant size. The relationship of ĝs to xylem [ABA] was not affected by the symbiosis, indicating that either stomatal sensitivity to xylem ABA was not affected by other xylem constituents, or that more than one xylem constituent was altered by the symbiosis but was offsetting in its effect on ĝs. We conclude that the symbiosis altered ĝs non-hydraulically, and that the factor might be xylem ABA.

**Keywords:** Abscisic acid, stomatal conductance, cowpea, root signals, drought.

**Introduction**

Recent investigations have focused on abscisic acid (ABA) as a root-sourced signal which closes stomata of plants growing in drying soil (Davies & Zhang, 1991; Bano et al., 1993; Gowing, Jones & Davies, 1993; Khalil & Grace, 1993; Tardieu, Zhang & Gowing, 1993; Davies, Tardieu & Trejo, 1994; Tardieu, Lafarge & Simonneau, 1996). Drought-avoiding plants such as cowpea do not osmotically adjust but rather avoid damaging plant water deficits by closing stomata (Shackel & Hall, 1983; Fernandez & McKee, 1991). The extremely sensitivity of drought-avoiding plants such as cowpea should make them excellent model plants for studying the effects of signals moving through xylem on stomatal regulation of gas exchange.

In addition to soil moisture, mycorrhizal symbiosis is another edaphic factor that can strongly affect plant growth and development (Cooper, 1984; Smith & Gianinazzi-Pearson, 1988). Mycorrhizal symbiosis can alter plant nutrient uptake, especially P (Cooper, 1984; Smith & Gianinazzi-Pearson, 1988), and hormonal status of roots and shoots (Allen, Moore & Christensen, 1982; Danneberg et al., 1992; Drüge & Schönhbeck, 1992; Goicochea et al., 1995). It is not clear whether hormonal changes are directly related to or independent of nutritional factors, although some studies have shown altered ĝs or growth independent of nutrition (Augé, Schekel & Wample, 1986; Augé et al., 1994). It has also been
reported that $g_c$ can be altered non-hydraulically by mycorrhizal symbiosis (Augé & Duan, 1991; Augé et al., 1994). It is plausible that the nonhydraulic factor(s) might be phytohormones.

We recently published a report showing that xylem [ABA] was not consistently affected by mycorrhizal symbiosis in glasshouse-grown cowpea plants with half the root system dried; drought conditions which did not affect plant water status (Ebel et al., 1996). In this study, we applied a more severe stress to cowpea plants by allowing the soil to dry in the entire root zone in order to determine whether mycorrhizal symbiosis alters [ABA] in the xylem sap independent of shoot $P$, and whether [ABA] correlates with $g_c$.

MATERIALS AND METHODS

Plant culture and drought treatments

Mycorrhizal pot cultures were initiated by mixing separate sorghum pot cultures of six fungal isolates to a final ratio of 1:3 (v:v) sorghum pot culture:autoclaved medium. Six fungal isolates were used to ensure cowpea colonization and included *Glomus intraradices* UT143 (1:20), *Glomus mosseae* (1:20), *Gigaspora margarita* (1:20), *Glomus etunicatum* BR149-3 (1:30), *Glomus etunicatum* CLS19 (1:30) and *Glomus etunicatum* TN-101 (1:30). Non-mycorrhizal pot cultures consisted of sorghum non-mycorrhizal pot culture mixed with autoclaved medium to a final ratio of 1:3 sorghum pot culture:autoclaved medium. Soil media of both the pot cultures and new autoclaved media consisted of silica sand and calcined montmorillonite clay (Terrafine®, Industrial Materials Corp., Deerfield, Ill., USA) in a 2:1 ratio.

Three seeds of *Vigna unguiculata* (L.) Walp. cv. White Acre were planted in the pot culture near the rims and allowed to grow for 7 wk before being excised near the soil line. One week before the plants were excised, two additional seeds were planted in the centre of the pots, and 10 d later the smaller of the two plants was removed. The process of planting the experimental plants directly in pot culture media with the same species growing in it allowed mycorrhizal colonization of roots without soil disturbance.

All plants were fertilized daily with 11 mM N as Peter’s® 15–0–15 and soluble trace elements at 15 μM Mn as Peter’s S.T.E.M. (Grace-Sierra, Milpitas, CA, USA). Phosphorus was applied weekly as 3 mM KH$_2$PO$_4$ for non-mycorrhizal plants and 1, 2, 3 and 4 mM KH$_2$PO$_4$ for mycorrhizal plants. The range of P fertilization to mycorrhizal plants assured overlap in plant size between the two mycorrhizal treatments.

Plants were grown in a clear glasshouse. Daytime temperatures ranged from 20 to 35°C and night temperature was maintained near 20°C. Mycorrhizal and non-mycorrhizal plants with equal leaf numbers and size were paired and allowed to dry by withholding water for 2 h to 7 d before plant measurements. Pairing removed the effect of plant size on plant response to drying.

Data collection

Pairs of mycorrhizal and non-mycorrhizal plants were randomly selected by drying duration for sampling. Abaxial $g_c$ was measured with a diffusion porometer (AP4, Delta-T Devices, Cambridge, England) calibrated each day immediately before sampling. Stomatal conductance was measured on the terminal leaflet of the five most recently expanded leaves of each plant. Measurements were made between 1000 and 1600 hours, a time for which previous tests showed no consistent, significant diurnal changes. Photosynthetic photon flux density (PPFD) was measured with a quantum sensor (LiCor, Lincoln, NE) with each $g_c$ measurement. PPFD ranged from 200 to 1100 μmol m$^{-2}$ s$^{-1}$, with the high variability principally due to leaf orientation to the sun. Relative humidity ranged from 20 to 55 %, and air temperatures from 17 to 23°C, during measurements.

Immediately after $g_c$ was measured, the entire shoot was excised 1–2 cm above the soil line and its $P$ measured in a pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA, USA). A 2 cm piece of Tygon® tubing was then fitted over the cut end of the stem to form a well. Pressure was raised about 0.3 MPa above the balance pressure and the first 10 μl of xylem sap expressed was discarded. An additional 10 μl of xylem sap was expressed onto a filter paper disc and osmotic potential measured with a vapour pressure osmometer (Model 5500, Wescor, Logan, UT, USA) to determine whether osmotic potential of xylem sap was affected by the symbiosis. Pressure was raised 0.3 MPa to 0.6 MPa above the balance pressure and c. 400 μl of xylem sap was collected from the well with a syringe. The xylem sap was quickly injected into an opaque microcentrifuge tube, capped and frozen in liquid N$_2$ for later ABA analysis.

A soil sample was removed with a cork borer and water content analysed gravimetrically.

Shoots were removed from the pressure bomb, the leaves were removed and total plant leaf area was measured (LI-3000A, LiCor). Shoots were dried at 40°C for 4 d to constant weight and weighed.

The fifth oldest leaf was segregated from the rest of the leaves and P concentration determined spectrophotometrically using the vanadate-molybdate-yellow method on samples dry-ashed with magnesium nitrate at 750°C for 2 h then digested in nitric acid (Chapman & Pratt, 1961). Hyphal, arbuscular and vesicular colonization of roots was determined on 100 1-cm root pieces from
Figure 1. Effect of \( \theta \) on \( g_s \) of mycorrhizal (○) and non-mycorrhizal (●) cowpeas. Since there was a significant interaction between mycorrhizal treatment and \( \theta \) (Table 1), correlation coefficients were determined for mycorrhizal and non-mycorrhizal relationships. Significance: ** \( P \leq 0.01 \); *** \( P \leq 0.001 \).

Each plant (McGonigle et al., 1990). Roots were cleared with 10% KOH in an autoclave at 121 °C for 20 min, stained with trypan blue for 1 h and held in destaining solution until measurement.

Concentrations of sucrose, fructose and glucose in xylem sap were measured by HPLC (Model DX-300, Dionex Corp., Sunnyvale, CA) equipped with a pulsed amperometric detector, using a CarboPac® PA1 column. Sugar concentrations have been used previously (Zhang & Davies, 1990) and here to estimate extent of contamination by phloem sap.

ABA in xylem sap was measured using indirect ELISA (Walker-Simmons, 1987), with ABA conjugate made according to Quarrie & Galfre (1985) except that the conjugate was dialysed three times in 51 of buffer. Serial dilutions of xylem sap were assayed to ensure that ABA concentrations of samples would fall within the range of the standard curve. Three separate aliquots of xylem sap were measured from each plant. Plates were incubated at 25 °C in the dark for c. 1 h until the wells with no ABA gave an absorbance of c. 1.0 at 405 nm. Triplicate ABA standards were assayed for each plate. Validation of the ELISA assay for use with unpurified xylem exudate was confirmed by a dilution/spike recovery test for nonspecific interference. Either 22.5 or 25.0 μl of 10% xylem sap diluted with distilled water were added to four concentrations of (±) ABA standard and assayed. Plots of ABA added vs. ABA detected produced lines parallel to the ABA standards, demonstrating the absence of nonspecific interference (Jones, 1987).

### Experimental design and statistical analysis

Data were analysed using the General Linear Model procedure of the Statistical Analysis System (SAS/STAT User’s Guide, 1988). PPFD was used as a covariate in models with \( g_s \) as the dependent variable to remove effects of leaf orientation to the sun. The day plants were sampled (three consecutive days) and the time of day they were sampled (linear and quadratic) were initially included in all analyses but removed since they were not significant. The lack of a difference in the time of day plants were sampled indicates that there was no diurnal effect across our 6 h sampling period at midday.

| Table 1. Results of ANOVAs conducted for data presented in Figures 1–4 |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| | \( g_s \) (Fig. 1) | \( [ABA]^{-1} \) (Fig. 2) | \( g_s \) (Fig. 3) | Shoot \( \Psi \) (Fig. 4a) | \( g_s \) (Fig. 4b) |
| Independent variable | | | | | |
| VAM | n.s.† | n.s. | n.s. | n.s. | n.s. |
| Soil \( \theta \) | **** | *** | *** | *** | — |
| VAM × soil \( \theta \) | — | — | — | — | — |
| [ABA]^{-1} | § | — | — | — | — |
| VAM × [ABA]^{-1} | — | — | n.s. | — | — |
| Shoot \( \Psi \) | — | — | — | — | n.s. |
| VAM × shoot \( \Psi \) | — | — | — | — | n.s. |
| Model \( r^2 \) | 0.36*** | 0.38* | 0.71* | 0.60*** | 0.54*** |

† n.s. indicates not significantly different.

‡ *, **, *** indicate differences at the 0.05, 0.01 and 0.001 levels of significance, respectively.

§ — indicates the independent variable was not included in the ANOVA model.

Water was withheld from potted mycorrhizal and non-mycorrhizal cowpea plants for 2 h to 7 d before making measurements. Five ANOVAs were conducted with independent variables showing results included in the models. The model \( r^2 \) is included to show overall variability explained by the independent variables. Xylem [ABA] was arbitrarily transformed using an inverse function (indicated by [ABA]^{-1}) to linearise the data for ANOVA.
RESULTS

The slopes of the \( g_s \) vs. \( \theta \) linear regressions differed in mycorrhizal and non-mycorrhizal plants (Fig. 1) as indicated by the significant VAM \( \times \) \( \theta \) interaction (Table 1). At low \( \theta \), \( g_s \) was similar for mycorrhizal and non-mycorrhizal plants but at high \( \theta \), mycorrhizal plants had higher \( g_s \). There was considerable variation in the data which could be partly explained by PPFD which was significant in the statistical analyses (analysis not shown). Despite the variability, \( \theta \) and \( g_s \) were significantly correlated in both mycorrhizal and non-mycorrhizal plants.

Since xylem [ABA] values were not linearly related to \( \theta \) (Fig. 2a), the data were linearised by an arbitrary transformation using the inverse of the xylem [ABA] values before conducting an ANOVA (Fig. 2b). The inverse of xylem [ABA] gave a better linear relationship than did the log form. There was a significant interaction of VAM \( \times \) \( \theta \) indicating that the slopes of the lines differed for mycorrhizal and non-mycorrhizal plants. Mycorrhizal plants had lower xylem [ABA] at higher \( \theta \) than did non-mycorrhizal plants but similar xylem [ABA] concentrations at low \( \theta_s \). The lower xylem [ABA] at high \( \theta_s \) of mycorrhizal plants compared with non-mycorrhizal plants corresponded to higher \( g_s \).

Mycorrhizal symbiosis did not change the relationship between xylem [ABA] and \( g_s \) (Fig. 3a, b).

Shoot water relations were not affected by the mycorrhizal symbiosis. Shoot \( \Psi \) did not vary between mycorrhizal and non-mycorrhizal plants across the range of \( \theta_s \) (Fig. 4a) nor was there a difference between mycorrhizal treatments in the shoot \( \Psi \) and \( g_s \) relationship (Fig. 4b). Xylem sap osmotic potential was \( 0.12 \pm 0.013 \) MPa for mycorrhizal plants and \( 0.14 \pm 0.014 \) MPa for non-mycorrhizal plants. Shoot water contents of mycorrhizal plants and non-mycorrhizal plants were \( 89.1 \pm 0.17 \% \) and \( 89.1 \pm 0.14 \% \) respectively, and did not vary across \( \theta \) (data not shown).

We conducted several tests on xylem sap of plants other than those in the present experiment to determine whether artefacts which might have

Figure 2. Relationship of \( \theta \) to cowpea xylem [ABA] expressed on a log scale, which was the best graphical presentation of the data (a), and after being transformed by an inverse function (b) which was performed in order to linearise the data for ANOVA. Since there was a significant interaction between mycorrhizal treatment and \( \theta \) (Table 1), correlation coefficients were determined separately for mycorrhizal (○) and non-mycorrhizal (●). Significance: * \( P \leq 0.05 \); ** \( P \leq 0.01 \); *** \( P \leq 0.001 \).

Figure 3. Relationship of cowpea \( g_s \) to xylem [ABA] expressed on a log scale, which was the best graphical presentation of the data (a), and after being transformed by an inverse function (b) which was performed in order to linearise the data for ANOVA. Mycorrhizal (○) and non-mycorrhizal (●) plants were statistically similar (\( P \leq 0.05 \)).
reduced accuracy in quantification of xylem [ABA] were introduced in the procedure. First, we determined whether solute concentration changed as more sap was expressed by comparing osmolality of the first 200 µl to the second 200 µl and found no difference. Second, we determined whether there was contamination by phloem sap by measuring soluble carbohydrates which are in very low concentrations in xylem sap but high in phloem. Carbohydrate concentrations in the sap collected were 0.69 mM glucose, 0.59 mM fructose, and 0.94 mM sucrose. Total carbohydrate concentrations (3.2 mM glucose equivalents) were lower than those of xylem sap expressed from maize (Zhang & Davies, 1990). We conclude that there was very little contamination of sap from the phloem. Third, we tested whether the amount of over-pressure to express sap affected its concentration of ABA. Plants subjected to 0.3 and 0.6 MPa over-pressure had xylem sap [ABA] of 60 ± 11 and 42 ± 10 nM respectively, for the first 200 µl sample collected and 50 ± 4 and 46 ± 9 nM for the subsequent 200 µl sample collected. It appears that pressurization had little effect on dilution of xylem ABA, which is supported by others who conducted a more thorough sequential sampling of xylem sap and also found little effect (Zhang & Davies, 1990). We conclude that our protocol for sampling xylem sap ABA probably introduced little error in measuring its concentration.

Hyphal colonization was 18% and 2% for mycorrhizal and non-mycorrhizal plants respectively, and arbuscular colonization was 7% and 1% respectively. No vesicles were observed. Leaf P concentrations were not significantly different (P ≤ 0.05) in mycorrhizal (+8 ± 0.24 mg g⁻¹ d. wt, n = 34) and non-mycorrhizal (+6 ± 0.21 mg g⁻¹ d. wt, n = 30) plants. Shoot d. wt and leaf areas were also not affected by the mycorrhizal symbiosis (P ≤ 0.05). Shoot d. wt and leaf areas were 3.5 ± 0.12 g and 831 ± 29 cm² respectively in mycorrhizal plants (n = 30) and 3.4 ± 0.13 g and 822 ± 31 cm² respectively in non-mycorrhizal plants (n = 29).

**Discussion**

The symbiosis of mycorrhizal fungi and their host plants is complex, with the physiology of the host plant often altered. Some reports have shown altered gs by mycorrhizal symbiosis independent of plant size and nutrition (Augé et al. 1986; 1991; 1995). It seems plausible that the fungal symbiont alters xylem [ABA] in the host either directly, by producing ABA itself and transporting it into the host, or indirectly, by altering plant metabolism which results in altered production or redistribution of ABA. Considering the complex physiology of the symbiosis, it is possible that more than one mechanism might be involved, and the mechanism probably varies with extent of colonization, the particular host and symbiont species involved, stage of plant development, and the environmental conditions during growth. Indeed, it seems that even in non-mycorrhizal plants several xylem constituents can influence shoot behaviour, and the influence of these constituents probably differs between species (Davies et al., 1994).

In the present experiment, mycorrhizal plants had higher gs and lower xylem[ABA] than did non-mycorrhizal plants at high θb, despite the considerable variability in the data. It is possible that Type II statistical errors were committed in the ANOVAs, that is, concluding that the interactions between VAM × θ and VAM × xylem[ABA] were different when in reality they were not. However, it is highly unlikely that Type II statistical errors were committed for both interactions. Although we found a significant correlation for xylem[ABA] and gs, we cannot conclude that xylem[ABA] caused stomatal closure since it is possible that xylem[ABA] was diluted by a higher transpiration rate. The mycorrhizal plants had more open stomata and therefore probably had higher transpiration rates which would have increased the rate of water movement into the xylem from the soil, thus diluting ABA that was also
being loaded into the xylem. Nevertheless, some factor caused stomata in the mycorrhizal plants to be more open and that factor was not related to plant water status, P nutrition, plant size or differences in soil moisture since these were not affected by the symbiosis. It is possible that the mycorrhizal fungi caused a small decrease in xylem[ABA] which caused stomata to open more and then ABA would have been further diluted by increased transpiration. The suggestion that stomata are very sensitive to relatively small changes in xylem[ABA] concentrations against a background of high concentrations of xylem [ABA] has been made before (Trejo & Davies, 1991). On the other hand, it is also possible that some other factor closed stomata. Other factors influencing stomatal closure have been reported, and not all species respond to xylem ABA (Davies et al., 1994).

The lack of a difference between mycorrhizal and non-mycorrhizal plants in the relationship of xylem[ABA] to gs is important because it demonstrates that other factors either did not alter stomatal sensitivity to xylem[ABA], assuming that stomata were responding to xylem[ABA], or that other factors affecting gs were offsetting. Calcium, nitrate, phosphate, amino acids and pH in the xylem (Atkinson et al., 1989; Atkinson, Mansfield & Davies, 1990; Gollan, Schurr & Schulze, 1992; Schurr, Gollan & Schulze, 1992) and leaf Ψ (Trejo & Davies, 1991; Tardieu & Davies, 1992; Tardieu et al., 1993) might alter sensitivity of stomata to xylem hormones.

We conclude that under the growth conditions used in this study, mycorrhizal symbiosis altered stomatal conductance of cowpea non-hydraulically, which might be related to xylem [ABA]. We suggest that these be conducted to calculate flux rates for xylem [ABA] and relate that to gs in order to determine influence by mycorrhizal symbiosis.

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