Quantification of Water Uptake by Arbuscular Mycorrhizal Hyphae and its Significance for Leaf Growth, Water Relations, and Gas Exchange of Barley Subjected to Drought Stress

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Abstract: Arbuscular mycorrhizal fungi alleviate drought stress in their host plants via the direct uptake and transfer of water and nutrients through the fungal hyphae to the host plants. To quantify the contribution of the hyphae to plant water uptake, a new split-root hyphae system was designed and employed on barley grown in loamy soil inoculated with Glomus intraradices under well-watered and drought conditions in a growth chamber with a 14-h light period and a constant temperature (15°C; day/night). Drought conditions were initiated 21 days after sowing, with a total of eight 7-day drying cycles applied. Leaf water relations, net photosynthesis rates, and stomatal conductance were measured at the end of each drying cycle. Plants were harvested 90 days after sowing. Compared to the control treatment, the leaf elongation rate and the dry weight of the shoots and roots were reduced in all plants under drought conditions. However, drought resistance was comparatively increased in the mycorrhizal host plants, which suffered smaller decreases in leaf elongation, net photosynthetic rate, stomatal conductance, and turgor pressure compared to the non-mycorrhizal plants. Quantification of the contribution of the arbuscular mycorrhizal hyphae to root water uptake showed that, compared to the non-mycorrhizal treatment, 4% of water in the hyphal compartment was transferred to the root compartment through the arbuscular mycorrhizal hyphae under drought conditions. This indicates that there is indeed transport of water by the arbuscular mycorrhizal hyphae under drought conditions. Although only a small amount of water transport from the hyphal compartment was detected, the much higher hyphal density found in the root compartment than in the hyphal compartment suggests that a larger amount of water uptake by the arbuscular mycorrhizal hyphae may occur in the root compartment.

Key words: Barley, water uptake, drought, stress, Glomus intraradices, photosynthesis, water relations.

Introduction
Drought is one of the major problems limiting plant growth in the world. One mechanism to increase drought resistance in plants may be through the symbiotic effects of arbuscular mycorrhizal (AM) fungi, where 80% of studies on the effects of AM fungi on growth in plants that were subjected to drought stress have shown enhanced drought tolerance in the host plants (Auge, 2001). The increase in drought tolerance may be attributed, in part, to altered rates of water movement into, through, and out of the host plants, with its consequent effects on tissue hydration and plant physiology. Thus, direct uptake and transfer of water through the fungal hyphae (2–5 μm in diameter) that can penetrate soil pores inaccessible to root hairs (10–20 μm diameter) and thereby absorb water that is not available to non-AM plants, may be one important mechanism, even though improvement of P uptake by AM fungi has been considered a primary mechanism (Allen, 1982; Marulanda et al., 2003). Allen (1991) estimated that the rate of water transport from extraradical hyphae to the root was 100 nl H2O h−1 per hyphal infection point, which was considered sufficient to alter plant water relations. However, other authors have predicted rates of water uptake by hyphae based on the number of hyphal entry points per unit root length, hyphal cross-sectional areas, and water potential gradients, and suggested that hyphal water transport rates were negligible (Fitter, 1988; George et al., 1992; Koide, 1993). The conflicting reports may stem from a lack of reliable split-root-hyphae systems to separate the contributions of the fungal hyphae and the host plant roots to water uptake. Thus, a new two-compartment system was designed for this study, which, by inducing an air gap between the two compartments, minimizes the non-hyphal transport of water from one chamber to the other.

The increase in root water uptake in host plants under drought conditions has often been associated with changes in leaf growth rate, leaf water relations, stomatal conductance, and net photosynthetic rate (Dixon et al., 1994; Auge, 2001; Sanchez-Blanco et al., 2004; Porcel and Ruiz-Lozano, 2004). Leaf growth in grasses such as barley, maize, and wheat is highly sensitive to stresses such as drought and salinity (Schmidhalter et al., 1998; Hu et al., 2000). Therefore, to increase our understanding of the significance of and the mechanisms by which AM fungi increase water uptake of plants under drought stress, it is necessary to study leaf physiology during leaf growth and during drought periods. As such, the objectives of
this study were to quantify the contribution of the acquisition and transport of water through the hyphae into the AM plants and to determine the instantaneous leaf elongation rate, leaf water relations, and photosynthesis rate of AM barley plants during different drying cycles in an extended drought period.

**Materials and Methods**

**Split-root-hyphae system**

The split-root-hyphae system was constructed from grey Plexiglas (3 mm in thickness) and consisted of two soil compartments, i.e., hyphal compartment (H x L x W: 30 x 19 x 3 cm) and root compartment (30 x 19 x 5 cm) separated by two layers of nylon net with a pore size of 30 μm. There was a 5-mm air gap between the two nylon nets, which was sufficient to prevent water diffusion and mass flow from the root compartment to the hyphal compartment, but did allow hyphal growth from the root to the hyphal compartment. To avoid water loss by evaporation in both the hyphal and root compartments, the soil surface at the top of the hyphal compartment was completely sealed with a plastic film during the experiment, and the soil surface at the top of the root compartment was covered by 100 g sand (Ø 2.5 mm) and with a perforated plastic film, where plants could grow through the small holes.

**Growth conditions**

Loamy soil was collected from the soil surface (0 – 15 cm) and consisted of 23 % clay, 48 % silt, and 29 % sand, with the organic matter content being 1.7 %. The soil was air-dried, ground, passed through a 5-mm mesh screen, and sterilized using an autoclave before being filled into both compartments. The initial soil water content (23 % on a dry soil basis) was achieved by adding nutrient solution of 0.2 g l⁻¹ NH₄NO₃, and then the soil was thoroughly mixed. Thereafter, the soil was sieved through a 5-mm sieve and placed into pots. The initial soil bulk density was 1.4 g cm⁻³.

Ten seeds of barley (*Hordeum vulgare* L. cv. Scarlett) were sown in the root compartment and were thinned to six seedlings per pot one week after emergence. The plants were grown in a growth chamber with an air temperature of 15 °C, a relative humidity of 70 %, and a light period of 14 h at a photosynthetic photon flux density (PPFD) of 550 μmol m⁻² s⁻¹.

There were four treatments in this study: well-watered (W) and droughted (D) plants, each with and without AM. All treatments were replicated three times. For the AM treatments, 25 g of pre-cultivated inoculum (*Glomus intraradices*) (Inst. of Pathology, Univ. Hannover, Germany) was placed below the barley seeds in each root compartment. This amount of inoculum was determined in preliminary tests as being the optimal amount to produce good colonization for the total amount of soil in the pot.

To measure gravimetric soil water content and maintain the well-watered and droughted treatments in the root compartment, the pots were weighed daily. Since the daily water status change in the hyphal compartment was very small compared with the root compartment, the water loss for the well-watered treatments was replaced by adding tap water daily during the experiment, presumably to the root compartment. The drought conditions were initiated at day 21 after sowing and were represented by eight drying cycles, each of about 7 days duration without watering, between day 21 and 77 after sowing (see also Fig. 1). At the end of each drying cycle, the root compartments were watered to the initial water content again. Furthermore, the same concentration of N solution as that initially used was applied another three times at 20, 40, and 60 days after sowing.

At 90 days after sowing, the soil was carefully removed from the hyphal compartment and cut horizontally into five 5-cm sections. To quantify the water uptake by the hyphae, the gravimetric soil water content of each section in the hyphal compartment (GSWC) for all treatments was determined by weighing before and after drying at 105 °C for 36 h. The gravimetric soil water content (GSWC) (expressed in %) was calculated from the equations:

\[
\text{GSWC} = 100 \cdot \frac{(\text{Soil}_{\text{wet}} - \text{Soil}_{\text{dry}})}{\text{Soil}_{\text{dry}}}
\]

where the \( \text{Soil}_{\text{wet}} \) and \( \text{Soil}_{\text{dry}} \) are the weight of soil before and after drying, respectively.

**Instantaneous measurements of leaf elongation rate and leaf length**

Following Hu et al. (2000), instantaneous measurements of leaf growth in all treatments were conducted using linear variable differential transducers (LVDT) when the visible part of leaf 5 of the main stem was 1 – 2 cm long (about one day after leaf emergence). The tip of the leaf was connected to the LVDT by a fishing line (0.22 mm diameter) that was attached to the leaf tip using a small clamp cushioned with rubber to avoid damaging the leaf. The force on the fishing line was 10 g to eliminate oscillations in LVDT output resulting from slipping and friction in the measurement system. According to our pre-experiment, this force did not affect the leaf elongation rate (LER) during measurements. One reading was taken from each transducer every 30 min. During this 30-min period, the average of six values was stored by a data logger (Delta-T Device, Cambridge, UK). The measurements of LER were completed when the rate (mm h⁻¹) was approximately zero. LER was calculated by dividing the increase in length by the time interval.

Leaf length can be viewed as the integral of LER. Once the measurement of each leaf was finished, the final leaf length was also determined with a ruler to compare to the results of the LVDT method, which confirmed that there was no influence of the 10 g weight on leaf elongation.

**Photosynthetic parameters**

Photosynthesis rate (A) and stomatal conductance (gs) were non-destructively measured on the second youngest leaf that was fully expanded (at least three leaves per pot) at the end of each drying cycle for all treatments before re-watering. Measurements were made with a LI-COR 6400 portable gas exchange system (Analytical Development Company, England).
as described by El-Hendawy et al. (2005). Measurements were performed 2 h after the start of the light period in the growth chamber.

Measurements of leaf water relations

Leaf water potential and osmotic potential from the middle of the second youngest fully developed leaf blades were measured at the end of each drying cycle for all treatments. Leaf water potential was measured with a pressure bomb (PMS Instrument Co., model 1002, Corvalis Co., Oregon, USA) following Scholander et al. (1965). Immediately after leaf water potential was determined, the same leaf material was frozen in dry ice. The leaf samples were then thawed at room temperature and placed in a syringe, with the leaf sap expressed under pressure. Osmotic potential was then determined with a vapor pressure osmometer (Wescor 5100C, Wescor Inc., Logan, USA). Turgor pressure was estimated as the difference between osmotic potential and water potential.

Quantification of AM hyphae

Three halves of cellulose acetate/cellulose nitrate membrane filters (MF-Millipore, Millipore Corporation, 47 mm diameter, 0.45 μm pore size), wetted with deionized water, were inserted vertically into the soil of both the hyphal and root compartments at the beginning of the experiment (Balaz and Vosatka, 2001). The membrane filters were placed into those zones where either the mycorrhizosphere or the hyphosphere were predicted to develop. After plants were harvested at 90 days after sowing, the membranes with adhering AM hyphae were removed carefully and washed gently with deionized water. All membranes were then placed into small Petri dishes (50 mm diameter), flooded with 10 ml of a 0.5 % solution of trypan blue, and then mounted on microscope slides for examination and quantification of the stained AM hyphae.

Statistical analysis of data

A randomized complete block design was used. Data were analyzed by an analysis of variance (ANOVA) using JMP 4.02 (SAS, 2000) to test the significance of the main effects. Means separation on data was conducted using LSD multiple range tests. Terms were considered significant at \( p < 0.05 \).

Results

Plant biomass production

Drought significantly reduced \( p < 0.05 \) the dry weight of the shoots and roots of both AM and non-AM barley day 90 after sowing, compared to plants in the well-watered treatments (Table 1). For AM plants, drought reduced the dry weight by 38% for the shoots and by 44% for the roots compared with the control treatments; the respective values for the non-AM plants were 70% and 53%. Under drought conditions, therefore, growth of the shoots and roots in barley was significantly improved \( p < 0.05 \) by AM, with the shoot and root dry weights of the AM plants being 50% and 17% higher, respectively, than those of the non-AM plants.

Daily soil water status

The gravimetric soil water content in the root compartments in the well-watered treatments was always maintained at the original water content of 23%. For the drought treatments, the gravimetric water content rapidly decreased during the first 3 – 4 days of each drying cycle (Fig. 1). At the end of each drying cycle, the gravimetric soil water content in the root compartments, either with or without AM, was around 8 – 11% (Fig. 1). A significantly lower gravimetric soil water content \( p < 0.05 \) in AM treatments compared to the non-AM treatments, was observed for the last 2 – 3 days for the second, third, fifth, seventh, and eighth drying cycles. From the second to eighth drying cycle, the plants in the root compartment took up about 7% (w/w dry soil) more water in AM treatments than that in non-AM treatments.

Table 1  Dry weight of shoots and roots of barley plants with and without vesicular-arbuscular mycorrhizae (AM and non-AM) that were subjected to well-watered (W) and drought stress (D) conditions on day 90 after sowing

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dry weight (g plant(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoots</td>
</tr>
<tr>
<td>W-AM</td>
<td>3.6 a</td>
</tr>
<tr>
<td>W-non-AM</td>
<td>3.7 a</td>
</tr>
<tr>
<td>D-AM</td>
<td>2.2 b</td>
</tr>
<tr>
<td>D-non-AM</td>
<td>1.1 c</td>
</tr>
</tbody>
</table>

Means followed by the same letters in columns for shoots or roots are not statistically different at \( p < 0.05 \).
Contribution of AM hyphae to root water uptake and length of AM hyphae

The results of the quantification of water transport through the hyphae, as determined from the split-root-hyphae system (Table 2), show that the gravimetric soil water content in the hyphal compartment remained unchanged over the course of 90 days for the AM and non-AM treatments under well-watered conditions and for only the non-AM treatments under drought conditions. By contrast, the soil water content was decreased by about 4% for the AM treatment under drought stress, which translates to about 80 ml H$_2$O that was transferred from the hyphal compartment through the hyphae. Hyphal density data in the AM treatments (Table 2) demonstrate that hyphae were only detected for AM treatments in the hyphal compartment. The hyphal density was higher in the root compartment than that in the hyphal compartment for both the well-watered and drought conditions. For example, compared to the hyphal compartment, hyphal density in the root compartment was six times higher in well-watered conditions and two times higher in drought conditions. A significant difference ($p < 0.05$) in hyphal density between the well-watered and drought conditions was also observed (Table 2).

Kinetics of leaf elongation

The results of analysis of the elongation rate of leaf 5 on the main stem show a similar pattern of kinetics of leaf elongation in all treatments and a distinct diurnal variation during the entire period of leaf elongation (Fig. 2). The LER generally decreased with time and ceased at about 7 days after emergence of the leaf, regardless of treatments. On a diurnal scale, LER was higher during the light period than during the dark period. LER also decreased with time during both the light and dark periods. Drought significantly reduced LER in both AM and non-AM plants compared to the well-watered treatment. The average LER over the first 2 days of drought conditions was reduced by 45–55% and 55–65% in the light and dark periods, respectively. In well-watered conditions, there was no difference in LER between the AM and non-AM treatments, whereas there was a higher LER in the AM plants under drought conditions. The difference in LER between the AM and non-AM treatments under drought conditions increased with time in both the light and dark periods. For instance, compared to the non-AM treatment, LER of leaf 5 in the AM treatment was about 20% higher on the first day of measurement during the light period and 40% higher on the fourth day. However, the increase in LER by the AM was greater during the light period than during the dark period. Again, compared to the non-AM treatment, LER in the AM treatment was almost the same on the first day of measurement during the dark period and about 20% higher on the fourth day.

The final length of leaf 5 was about 20 cm for both the AM and non-AM plants in well-watered conditions and about 12.7 cm and 9 cm for the AM and non-AM plants, respectively, in drought conditions.

Leaf water relations

The leaf water potential, osmotic potential, and turgor pressure remained almost unchanged or decreased slightly with time in all treatments (Fig. 3). The water and osmotic potentials were significantly lower ($p < 0.05$) in the drought treatments than those in the well-watered treatments. In the well-watered treatment, a difference in water potential between the AM and non-AM treatments was observed only from day 42 to 63 after sowing; in the droughted treatments, a significantly
higher water potential ($p < 0.05$) in the AM treatments was found from day 49 to 77. Similarly, a difference in osmotic potential between the AM and non-AM treatments in well-watered conditions was observed on days 56 and 63 after sowing, whereas a difference was only observed on days 42, 56, 70, and 77 after sowing in the drought treatments. Interestingly, the turgor pressure was usually highest in the AM plants subjected to drought stress and usually lowest in the non-AM plants subjected to drought stress (Fig. 3). There was no difference in turgor pressure between the AM and non-AM plants in well-watered conditions. The negative turgor pressure under drought conditions may be due to an artificial effect during the measurement rather than a real effect; thus the negative values should be set to zero (Fig. 3).

Leaf net photosynthetic rate and stomatal conductance

$A$ of both the AM and non-AM plants in well-watered conditions and of only the non-AM plants in drought conditions decreased with time; it was increased in AM plants subjected to drought conditions from day 35 to 63 and then decreased with time thereafter (Fig. 4). Drought reduced $A$ regardless of the mycorrhization from day 35 to 63 after sowing. The $A$ of AM plants was not affected by drought after this time. Under drought conditions, $A$ in AM plants was always higher than in non-AM plants.

Similar to the results for $A$, $g_s$ in well-watered conditions decreased with time and no difference between the AM and non-AM plants was observed (Fig. 4). However, $g_s$ of AM plants in drought conditions increased from day 35 to 49 and then decreased with time thereafter. Stomatal conductance of the AM plants subjected to drought stress remained unchanged up to day 56 and then decreased with time. In contrast to well-watered conditions, mycorrhization enhanced $g_s$ under drought conditions.

Fig. 3 Leaf water potential, osmotic potential and turgor pressure of barley plants with and without vesicular-arbuscular mycorrhizae (AM and non-AM) that were subjected to well-watered (W) or drought stress (D) conditions. All measurements were made at the end of each drying cycle. Within measuring dates, means accompanied by different letters are significantly different ($p < 0.05$); ns, not significantly different.

Fig. 4 Leaf net photosynthetic rates ($A$) and stomatal conductance ($g_s$) of barley plants with and without vesicular-arbuscular mycorrhizal (AM and non-AM) fungi that were subjected to well-watered (W) or drought stress (D) conditions. All measurements were made at the end of each drying cycle. Within measuring dates, means accompanied by different letters are significantly different ($p < 0.05$); ns, not significantly different.
Discussion

Auge (2001) reported that AM plants were found to be larger than non-AM plants in about 80% of the AM studies on plant growth during drought. In this study, the observations that both shoot and root biomass were enhanced by AM under drought conditions (Table 1) agree with findings in the literature. Importantly, mycorrhization enhanced the elongation rate of leaf 5 in barley subjected to drought stress in the early growth stages. This suggests an important role for AM fungi in promoting drought resistance of their host plants, which is associated with changes in the leaf physiological functions.

At the end of the drying cycles between days 35 and 77 after sowing, A was consistently higher in AM plants than in non-AM plants (Fig. 4). Higher photosynthetic rates have been found in other studies of AM Bouteloua gracilis (Allen et al., 1981), Medicago (Sánchez-Díaz et al., 1990), Zea mays (Subramanian and Charest, 1995), and Rosmarinus officinalis (Sánchez-Blanco et al., 2004) subjected to drought stress. The higher photosynthetic rates associated with mycorrhization can result in higher concentrations of soluble sugars and other photosynthetic byproducts in the leaf symplasm, which can manifest itself as an increased cytoplasm osmolality in AM plants as against non-AM plants (Auge, 2001; Porcel and Ruiz-Lozano, 2004). In contrast, the similar osmotic potential for AM and non-AM plants in this study (Fig. 3) may suggest that greater sink strength of AM root systems may result in greater rates of sugar movement out of leaves. Compared with non-AM plants, a maintained turgor pressure and a higher stomatal conductance stress were also found for AM plants subjected to drought in this study (Figs. 3, 4), indicating higher osmotic adjustment in the AM plants. Adjustments in the leaf osmotic potential and stomatal conductance are often related (Ludlow, 1989). The lowered leaf osmotic potential in AM plants may explain the AM-induced promotion of stomatal conductance (Auge et al., 1986), with a higher stomatal conductance often observed in AM plants under drought conditions compared to that in non-mycorrhizal plants (Duan et al., 1996; Auge, 2001; Sanchez-Blanco et al., 2004). In the same way, the reports in the literature have shown that AM plants postpone decreases in leaf water potential during drought stress (Davies et al., 1992; Subramanian et al., 1997; El-Tohamy et al., 1999; Porcel and Ruiz-Lozano, 2004). Our study also showed that leaf water potential for non-AM plants subjected to drought stress was lower between day 49 and 77 after sowing (Fig. 3). In the same period, however, the gravimetric soil water content during a given drying cycle was higher in the non-AM treatment compared to that in the AM treatment (Fig. 1). Thus, the higher water potential in AM plants may be due to increased water uptake by hyphae, improved root conductance to water flow (Safir et al., 1972; Cui and Nobel, 1992; Koide, 1993; Morte et al., 2000), and the effects on the soil moisture characteristics and soil structure, even though root mass and root length were similar in the AM and non-AM soils (Auge et al., 2001; Auge, 2004).

The studies of Bryla and Duniway (1997), Faber et al. (1991), and Ruiz-Lozano and Azcon (1995) have suggested that, under drought conditions, any increase in water uptake by fungal hyphae would play a vital role in increasing plant drought resistance through improving leaf water potential, maintaining turgor pressure, and increasing the net photosynthetic rate and stomatal conductance. However, our results in Table 2 showed that about 4% of the water (about 80 ml) in the hyphal compartment was apparently transferred to the root compartment through the AM hyphae over the course of 90 days under the drought conditions. The daily contribution to root water uptake from the hyphal compartment was only approximately 0.15 ml plant⁻¹ day⁻¹, which was too small when compared to transpiration of about 10 ml H₂O plant⁻¹ day⁻¹ that was averaged from each drying cycle (Fig. 1). Therefore, such a small amount of water from hyphae may not explain why leaf water potential and turgor pressure were improved, and net photosynthetic rate and stomatal conductance increased, which is in agreement with reports from Auge (2001) and Marulanda et al. (2003). However, there may be a possibility of higher uptake of water by AM roots in the root compartment. For example, the hyphal density in the root compartment of AM plants subjected to drought conditions was twice as high as that in the hyphal compartment (Table 2), indicating that the larger amount of water taken up by the hyphae may occur in the root compartment. This was supported by the higher water uptake of AM plants in the root compartment compared to non-AM plants subjected to drought conditions (Fig. 1). Furthermore, the air gap might have caused hyphae to desiccate somewhat, resulting in them being less effective for transport of water and suggesting that higher hyphal transport potential may exist in drought-AM plants. As mentioned above, there are conflicting reports in the literature regarding hyphal water transport. Bryla and Duniway (1997) investigated the role of fungal hyphae in water uptake in conditions ranging from well-watered to severe drought and concluded that mycorrhization did not affect the rates at which roots extracted water from the soil. By contrast, Bethlenfalvay et al. (1987) found that AM soybean plants depleted soil water to a greater extent than non-AM plants, a finding that is supported by other studies (Faber et al., 1991; Ruiz-Lozano and Azcon, 1995). In addition, gravimetric soil water content in the hyphal compartment for well-watered-AM, well-watered-non-AM, and drought-non-AM treatments was about 1 – 2% higher than that for their initial values (Table 2), resulting from each watering that caused a small water transfer at the bottom of the pots from the root to the hyphal compartment. This was detected by a kinetic study on the water status in both compartments using a capacitance sensor (data not shown).

In conclusion, this study shows that AM increases the drought resistance of barley in that leaf elongation, net photosynthetic rates, stomatal conductance and turgor pressure are less reduced in AM plants as compared to non-AM plants under drought stress. Quantifying the contribution of the hyphae to root water uptake confirms that there is transport of water by the hyphae under drought conditions. Although only a small amount of water transport in the hyphal compartment of our split-root-hyphae system was detected, the much higher hyphal density in the root compartment indicates that a larger amount of water uptake by the hyphae may occur in the root compartment.

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