Arbuscular mycorrhizal fungi influence water relations, gas exchange, abscisic acid and growth of micropropagated chile ancho pepper (Capsicum annuum) plantlets during acclimatization and post-acclimatization

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Summary

Little is known about the role of arbuscular mycorrhiza fungi (AMF) on physiological changes of micropropagated plantlets during acclimatization and post-acclimatization. Using chile ancho pepper (Capsicum annuum L. cv. San Luis), measurements were made of water relations, gas exchange, abscisic acid (ABA), plantlet growth and AMF development. Plantlets had low photosynthetic rates (A) and poor initial growth during acclimatization. Relative water content (RWC) decreased during the first days after transfer from tissue culture containers to ex vitro conditions. Consequently, transpiration rates (E) and stomatal conductance (gs) declined, confirming that in vitro formed stomata were functional and able to respond ex vitro to partial desiccation – thus avoiding excessive leaf dehydration and plant death. Colonization by AMF occurred within 3 days after inoculation. Colonized plantlets had lower leaf ABA and higher RWC than noncolonized (NonAMF) plantlets during peak plant dehydration (6 days after plant transfer) – and a higher A and gs as early as days 5 and 7. During post-acclimatization [after day 8, when RWC increased and stabilized], A increased in all plantlets; however, more dramatic changes occurred with AMF plantlets. Within 48 days, 45% of the roots sampled of inoculated plantlets were colonized and had extensive arbuscule development. At this time, AMF plantlets also had greater E, A, leaf chlorophyll, leaf elemental N, P and K, leaf dry biomass and leaf area, fruit production and differences in carbon partitioning [lower root/shoot ratio and higher leaf area ratio] compared with NonAMF plantlets. Rapid AMF colonization enhanced physiological adjustments, which helped plantlets recover rapidly during acclimatization and obtain greater growth during post-acclimatization.

Key words: acclimatization – endomycorrhiza – gas exchange – micropropagation – pepper – photosynthesis

Abbreviations: ABA = abscisic acid. – AMF = arbuscular mycorrhizal fungi. – DM = dry mass. – FM = fresh mass. – LAR = leaf area ratio. – A = net photosynthesis. – NonAMF = noninoculated with AMF. – PPF = photosynthetic photon flux density. – RWC = relative water content. – SM = saturated mass. – gs = stomatal conductance. – E = transpiration

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Introduction

With many species, use of micropropagation is limited because of poor plantlet survival rates during acclimatization, which is the transition from in vitro to ex vitro conditions (Kozai 1991, Preece and Sutter 1991). In commercial micropropagation systems, plant losses of 10 to 40% and higher can occur (Varma and Schöpp 1995). Some micropropagated plantlets may develop functional roots during tissue culture (Apter et al. 1993 a, b) or may die due to the development of nonfunctional root systems and deficient vascular connections between the root and shoot system (Grout and Aston 1978). Micropropagated plantlets are photomixotrophic and typically have leaves with low chlorophyll content and low photosynthetic rates that impede growth (Grout and Millan 1985).

When micropropagated plantlets are exposed to a lower relative humidity (RH), which occurs during transferring from in vitro to ex vitro conditions, they usually undergo transplant stress. Transpiration rates are considerably higher in micropropagated plantlets than in vivo grown plants because of the poor stomatal control and the abnormally high cuticular water loss, which results in wilting, necrosis of leaves, and may include senescence and death of leaves and plantlets (Brauner and Fuchigami 1982, Diaz-Perez et al. 1995). During water deficits, stomatal regulation is mediated by signal metabolites produced from roots, which are translocated through the xylem to the shoots and leaves (Grantz 1990). These signal metabolites have the ability to decrease leaf conductance and may include the plant hormone abscisic acid (ABA), inhibitors, Ca^{2+}, or promoters such as cytokinins (Davies and Zhang 1991); however, evidence suggests that ABA is the primary root signal molecule (Zhang et al. 1997). Due to the important role that ABA has during soil water stress, ABA may also play a critical role during the acclimatization of micropropagated plantlets in controlling stomatal responses. Exogenous application of ABA can serve as an anti-transpirant to alleviate water stress during acclimatization (Pospisilova et al. 1997).

Acclimatization is critical because these abnormalities must be corrected to ensure survival and continued normal plant growth (Debergh and Zimmerman 1991, Preece and Sutter 1991). Post-acclimatization follows acclimatization, when plantlets become photoautotrophic, less variable and have more steady-state characteristics.

Treatments have been used to acclimatize plantlets for transplanting ex vitro (Van Huylenbroeck and Debergh 1996, Van Huylenbroeck et al. 1998). Environmental manipulation has included variations in light intensity, reduced RH, and increased CO_{2} concentrations of the culture containers (Vanderschaeghe and Debergh 1987, Pospisilova et al. 1997). Colonization of micropropagated plantlets with arbuscular mycorrhiza fungi (AMF) after ex vitro transplantation can also enhance acclimatization, survival, and plantlet performance (Sbrana et al. 1994, Azcón-Aguilar and Barea 1997, Naqvi and Muerj 1998). Mycorrhiza can stimulate earlier resumption of plantlet growth (Azcón-Aguilar et al. 1994, Nouaim and Chauvad 1994, Fortuna et al. 1996), enhance nutrient uptake (DeClerck et al. 1994, Nouaim et al. 1994, Estrada-Luna et al. 2000), increase pathogen resistance (Guillemin et al. 1994), and enhance tolerance to environmental stress (Varma and Schöpp 1995). The establishment of mycorrhiza under in vitro conditions in tissue-cultured plantlets increased RWC under well-watered conditions (Hernández-Sebastiá et al. 1999). However, the role of AMF on physiological changes of plantlets during acclimatization is not well understood.

Chile pepper is an important vegetable crop that is cultivated on 1.2 million ha worldwide, with a production estimated at over 10 million tons in 1993 (FAO 1994). Chile peppers add flavor and aroma, have medicinal value, are an excellent source of vitamin A and C, and are low in caloric value (Villalon 1981, Andrews 1995). This crop is usually propagated by seed; however, micropropagation has become an important tool in clonal regeneration and breeding programs, particularly during the processes of selecting somaclonal variants, production of transgenic plants, and in the development of haploids and interspecific hybrids (Harini and Sita 1993, Mityko et al. 1995, Ezura 1997). Micropropagation is also of great importance in maintaining and propagating breeding lines of chile ancho, particularly with sterile individuals where it is the only method of multiplication (Valera-Montoro and Ochoa-Alejo 1992, Ezura 1997). Following tissue culture, these valuable regenerated materials may fail to survive during acclimatization.

Chile Ancho ‘San Luis’ is highly mycorrhiza dependent and thus a good potential model system for studying AMF-induced physiological effects during acclimatization. This research was conducted with a mixed isolate of Glomus spp. from Mexico (ZAC-19) that enhances drought resistance and nutrient uptake of chile ancho seedling peppers (Davies et al. 2000, 2002). This study focused on mycorrhizal influence on water relations, gas exchange, ABA, and growth of the micropropagated plantlets during acclimatization and post-acclimatization.

Materials and Methods

Plant material and micropropagation procedures

Chile ancho (Capsicum annuum L. cv. San Luis) plantlets were multiplied through micropropagation. Cultures were started from aseptic germination of seeds, which were initially treated for cleaning and surface disinfection. The cleaning process started by packing a set of seeds in cheesecloth. The seed pack was placed in a plastic container and soaked in distilled water containing commercial detergent (Liqui-Nox, Alconox, Inc. New York) and mechanically agitated. The cleaning solution was changed every 15 min for 2 h. Under aseptic conditions, seeds were then immersed in ethanol (70% [v/v]) for 5 min. The seeds were subjected to a surface disinfecting treatment for 25 min with commercial bleach (Clorox (6% NaOCl solution [20% (v/v)] plus tween-20 (0.1%))). Subsequently, the package was rinsed
5 times with distilled sterilized water and soaked for 5 min in an antibiotic solution containing carbenicillin disodium salt (Sigma Chemical Co.) and cefotaxime disodium salt (Sigma Chemical Co.), both at a concentration of 100 mg L^{-1}.

After disinfection, individual seeds were placed in 50 mL test tubes containing 10 mL of MS basal salt formulation medium (Murashige and Skoog 1962) supplemented with sucrose (Sigma Chemical Co.) [2% (w/v)], Bacto-agar (Difco Laboratories, Detroit, Mich) [0.6% (w/v)] and an adjusted pH of 5.8.

For the shoot system of microcuttings, seedlings with the first pair of expanded true leaves (13 to 15-day-old) were selected, and their radicles dissected and discarded. Microcuttings were transferred to glass containers (200 mL) with 30 mL of MS basal salt formulation medium supplemented with 0.4 mg L^{-1} K-IBA (Sigma Chemical Co.), 3% sucrose (Sigma Chemical Co.), 0.6% Bacto-agar, and an adjusted pH of 5.7 to produce adventitious roots. All cultures were grown in a culture room at 25 ± 2°C with light at 100 μmol m^{-2} s^{-1} PPF (GTE, Sylvania, USA cool white bulbs, 75 W) at plant level and a photoperiod of 16 h.

**Plant transfer, transplanting, ex vitro inoculation, acclimatization and culture conditions**

Uniform rooted micropropagated plants about 6-cm tall and with eight true leaves were selected from the tissue culture containers and transferred to a growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) set at 25 ± 2°C with 300 μmol m^{-2} s^{-1} PPF (Philips USA cool white bulbs, 210 W) and a photoperiod of 16 h. Inside the growth chamber, each plantlet was transplanted into individual commercial plastic pots (7.5 × 8.5 × 5.0 cm) containing a steam-sterilized mixture of peat moss:perlite (1:1, v/v). Remnants of agar were washed from the roots systems with sterilized distilled water.

At this step, half of the plantlets were not inoculated (NonAMF) and the remainder inoculated (AMF) with a mixed isolate, ZAC-19, collected in the State of Zacatecas, Mexico that contained Glomus abilium (Walker and Rhodes), G. claroideum (Schenk and Smith), and G. diaphanum (Morton and Walker) (Chamizo et al. 1998). ZAC-19 was collected from a sandy-loam soil with pH of 5.4, low P level (16 μg g^{-1}), and 1.1% organic matter in a semi-arid area dedicated to commercial bean production in the State of Zacatecas, Mexico (Contreras and Ferrera-Cerrafo 1989). The 100 g of inoculum was applied in a band (after the pot was 75% full of media), and consisted of a dried pasteurized sand: soil carrier containing sections of mycorrhizal roots (onion and rye grass), extraradical hyphae and approximately 4,000 fungal spores. In addition to the banded inoculum, 2 mL of an aqueous spore suspension containing 180 to 200 spores of the mycorrhizal isolate were also applied to the root system of each plantlet. The ZAC-19 isolate was selected because of its effectiveness (29%, 7% and 3% for total colonization, arbuscule and vesicle development, respectively) and rapid colonization (within 3 days) of chile ancho pepper plantlets during a 10 day study (Estrada-Luna 1999).

After transplanting, plantlets were subjected to an acclimatization treatment of 3 days of gradual reductions in relative humidity (80, 70 and 60% per day, respectively), which were controlled by adjusting the growth chamber environment. A multi-channel HOBO 8K recorder for temperature and relative humidity (Onset Computer Corp., USA) was used to monitor the growth chamber environment. Six days after plant transfer and inoculation, the plantlets were transferred to the glasshouse and grown for 42 more days. The average minimum/maximum glasshouse RH was 62/82%, with average minimum/maximum temperature 25/30°C, and a maximum PPF of 1000 μmol m^{-2} s^{-1} at plant height. All pots were fertilized weekly with 100 mL of Long Ashton nutrient solution (Hewitt 1966) modified to supply 11 μg mL^{-1} of P at each irrigation.

**Assessment of mycorrhizal colonization**

Roots from three plantlets of both AMF and NonAMF treatments were harvested at 0, 3, 4, 5, 6, 7, 8, 10, 12, 21, and 48 days after transplanting in order to assess colonization. Root systems were fixed in FAA, cleared with 2.5% KOH and stained with 0.05% trypan blue in acidic glycerol (Phillips and Hayman 1970). Subsequently, 15 slides per treatment containing 10 root pieces (1 cm) per slide were examined under a compound microscope (Nikon, Alphaphot YS) at 400X by making 3 passes per slide (top, middle, and bottom). Arbuscules, vesicles, internal hyphae, and total colonization in root cortical cells were determined (n = 450).

**Chlorophyll determination**

Leaf chlorophyll was determined with a SPAD-502 portable chlorophyll meter (Minolta Camera Co. LTD, Japan). The SPAD-502 meter readings were correlated with a chlorophyll content prediction equation: y = −25.37 + 6.85x, where y = chlorophyll content (mol·m⁻²), x = meter reading (r² = 0.96). This equation was obtained by running a linear regression analysis between the SPAD-502 readings obtained from the first pair of fully expanded leaves of 10 different chile ancho plantlets and the total chlorophyll content of the same pair of leaves. Leaf chlorophyll was extracted with aqueous acetone (80%) and the total content was determined by optical density of filtered aqueous acetone supernatant, which was measured at 645 nm and 663 nm with a spectrophotometer (Yadava 1986). Three readings were performed on different locations from each of the first pair of fully expanded leaves obtained from each plant and the average per leaf was calculated. Each leaf was a single replication, and there were 10 replications per treatment (n = 10). Sample dates were 0, 3, 4, 5, 6, 7, 8, 10, 12, 21, and 48 days after transplanting.

**Relative water content**

Relative water content (RWC) of leaves was determined at 0, 3, 4, 5, 6, 7, 8, 10, 12, 21, and 48 days after transplanting by the formula: RWC (%) = [(FW-DM)/(SM-DM)]×100, where FM = fresh mass, DM = dry mass, and SM = saturated mass (Kramer and Boyer 1995). The FM was determined by immediately weighing a 4.1 cm² leaf disc cut from the first pair of fully expanded leaves. The SM was determined by weighing the same leaf disc after flotation in distilled water and rehydration for 4 h in a near 100% relative humidity environment. The DM was obtained by weighing the disc after it had been dried in an oven at 60°C for 72 h. One disc per leaf of each of three plantlets was used in the determination (n = 3).

**Gas exchange measurements**

Gas exchange measurements included net photosynthesis (A), stomatal conductance (gₛ), and transpiration rate (E). Before gas exchange measurements were made, plantlets were acclimated for 20
minutes on light produced by a 1,000 W metal halide lamp (PPF was 1,000 μmol m⁻² s⁻¹ at plant height), which was filtered through a 5-cm non-circulating water bath enclosed in a Plexiglas box. Measurements were performed between 8:00 and 11:00 on each leaf of the first pair of fully expanded leaves from the shoot apex using a Li–6200 Portable Photosynthesis System (LI-COR Inc., Lincoln, NE). Each leaf was a single replication, and there were 10 replications per treatment (n = 10), which represented 5 plants per treatment. Sample dates were 0, 3, 4, 5, 6, 7, 8, 10, 12, 21, and 48 days after transplanting.

Plant growth measurements and leaf nutrient analysis

Total shoot length, leaf number, leaf area and DM of leaves, roots, and stems were measured at 0, 3, 4, 5, 6, 7, 8, 10, 12, 21, and 48 days after transplanting to characterize plant growth. From these growth variables, the leaf area ratio [LAR, (total plant leaf area) / (total plant DM)] and root to shoot ratio were calculated. Only data from day 48 (experiment termination) is presented. Five plantlets per treatment were sampled on each date (n = 5). Fruit number was included in the last sample.

Leaf tissue was harvested at the termination of the experiment (day 48) and the mineral status of plants was determined on a dry mass basis. Leaf tissue elemental analysis was conducted on an inductively coupled plasma atomic emission spectrophotometer (J. R. Peters/Scott's Testing Laboratory, Allentown, Penn) using 4 replicates of composite leaf mass samples per treatment (n = 4). Each composite sample was a pool of leaves from 3 plantlets.

Abscisic acid (ABA) determination

Endogenous concentrations of ABA were determined in roots, stems, and leaves of three plantlets per treatment (n = 3) at 0, 3, 6 and 12 days after transplanting. The last two sample dates corresponded to 0 and 6 days after transferring the plantlets to a glasshouse. ABA was determined by using methods described by Creelman and Zeevaart (1987), Zeevaart and Creelman (1988), with some modifications (Estrada-Luna 1999). After harvest, the plantlets were split into roots, stems, and leaves, frozen in liquid nitrogen and stored at −80 °C. For sample preparation, the tissues were lyophilized in a LABCONCO freeze dryer for 48 h and ground in a porcelain mortar for homogenization. Subsamples of 20 mg were weighed for analysis. ABA was extracted by performing two warm extractions (90 and 30 min each) at 60 °C in acetone (100%). Before quantifying the ABA concentration, the extracted samples were purified with high performance liquid chromatography (HPLC) using a Waters 600E System that was connected to an autosampler (Waters 717 Autosampler) (Milford, Mass). The column (Waters µBondapak C18) had a 3.9 mm internal diameter and was 250 mm long. The retention time was 10.35 to 11.5 min. Fractions collected were methylated with ‘diazomethane’ (25 μL). A gas chromatograph (GC) [5890 GC, Hewlett Packard, Palo Alto, Ca, USA, column type DB-5 (0.32 mm internal diameter X 15 m long at 1 μm, J&W Scientific)] was used to measure the samples. The GC was coupled with a transfer line 280 to a mass selective detector (5970 B, Hewlett Packard, Palo Alto, CA, USA) to perform the determination. Linear flow in the GC was 30 cm s⁻¹. The monitored ions were 125, 162 and 190 for endogenous compounds and the corresponding ions for the standard were 128, 165 and 193, using a dwell of 100 μsec. The retention time was 4.8 min.

Experimental design and statistical data analysis

Each sample date was treated as a single experiment arranged in a completely randomized design. Treatments included the presence and absence of mycorrhiza (AMF and NonAMF plantlets). Data from all variables were analyzed by using analysis of variance (ANOVA) (SAS Institute Inc., 1996), and Tukey (α = 0.05 %) was used for the means separation.

![Figure 1. Development of mycorrhizal structures in root cortical cells of arbuscular mycorrhiza colonized (AMF) micropropagated chile ancho pepper (Capsicum annuum L. cv. San Luis) plantlets during acclimatization and post-acclimatization. Means with ± SE; n = 450.](image-url)
Results

Mycorrhizal colonization

Extensive AMF colonization was observed in root systems of inoculated plantlets (Fig. 1), while no colonization occurred with NonAMF plantlets. Mycorrhizal colonization occurred rapidly, with intraradical hyphae observed 3 days after inoculation. By the end of the experiment (48 days after transplanting), 45% of the roots sampled were colonized. Arbuscule and vesicle formation were observed 10 days after inoculation and increased in each successive sampling date, reaching 37% by day 48. Internal spores were not observed until day 12, and reached 6% at the experiment termination (Fig. 1).

Relative water content

Leaf relative water content (RWC) was reduced ($P \leq 0.05$) from about 97% to 83% from days 4 to 6 when the plantlets were transferred from the growth chamber to glasshouse (Fig. 2a). However, by day 8, plantlets were acclimatized and dehydration was no longer observed; RWC increased from 83% to 93% and remained constant through the duration of the experiment.
the experiment. AMF significantly enhanced RWC at day 6 during peak leaf dehydration.

**Gas exchange**

Rates of $E$, $g_s$, and $A$ varied according to plantlet water status following transplanting (Fig. 2b, c, d). The rates of $E$ and $g_s$ quickly declined as plantlets experienced water stress during acclimatization (day 3 to 8 after transplanting) and increased during post-acclimatization (after day 8) (Fig. 2b and 2c). On day 3 after inoculation, AMF plantlets had significantly higher $E$ and $g_s$ as compared to NonAMF plantlets. On days 10 and 12, AMF plantlets had lower $E$ and $g_s$ than NonAMF plantlets but higher $E$ at day 48. Plantlets had very low $A$ on day zero ($4.3 \mu mol m^{-2} s^{-1}$), which remained low during acclimatization. The rate of $A$ did not show significant increases until 12 days after plantlets had been moved to the glasshouse. AMF plantlets had higher $A$ on day 7, 8, 21, and 48 compared to NonAMF. At the termination of the experiment, AMF had higher $A$, $E$ and a trend of higher $g_s$ than NonAMF plantlets.

Both $E$ and $g_s$ were highly correlated with leaf RWC. Correlation coefficients were high in both AMF ($r = 0.90$ and 0.88, respectively) and NonAMF ($r = 0.77$ and 0.76, respectively) plantlets. $E$ was highly correlated to $g_s$ and $A$ between AMF ($r = 0.95$ and 0.62, respectively) and NonAMF ($r = 0.92$ and 0.64, respectively) plantlets. There was a high correlation of $g_s$ to $A$ between AMF and NonAMF plantlets ($r = 0.72$ and 0.67, respectively).

**Evolution of ABA during plant acclimatization**

Levels of ABA were low in leaves, stems, and roots (Fig. 3), when plantlets were not experiencing water stress – i.e. high RWC during days 0, 3 and 12 days after *ex vitro* transplanting (Fig. 2a). However, on day 6 when plantlets were experiencing water stress and had the lowest tissue RWC, ABA levels increased in all plant organs (Fig. 3). Dramatic increases in ABA occurred in leaf tissues, which were 2-fold and 5-fold greater than levels in stem and roots, respectively. ABA concentrations were lower in roots and stems of AMF than
Table 1. Effects of mycorrhiza on leaf area, leaf, shoot, root and total plant dry mass (DM), fruit number, leaf area ratio, and root: shoot ratio of micropropagated chile ancho pepper (Capsicum annuum) plantlets after acclimatization and post-acclimatization.

<table>
<thead>
<tr>
<th>Mycorrhiza</th>
<th>Leaf area (cm²)</th>
<th>Leaf DM (g)</th>
<th>Shoot DM (g)</th>
<th>Root DM (g)</th>
<th>Total Plant DM (g)</th>
<th>Fruit number</th>
<th>LAR (cm² g⁻¹)</th>
<th>Root: Shoot ratio (g g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>985 a *</td>
<td>4.2 a</td>
<td>2.4 a</td>
<td>2.4 a</td>
<td>9.0 a</td>
<td>9 a</td>
<td>110 a</td>
<td>0.36 b</td>
</tr>
<tr>
<td>Absent</td>
<td>842 b</td>
<td>3.5 b</td>
<td>2.5 a</td>
<td>2.7 a</td>
<td>8.7 a</td>
<td>6 b</td>
<td>96 b</td>
<td>0.42 a</td>
</tr>
</tbody>
</table>

* Means in each column followed by different letters are significantly different (P ≤ 0.05) according to Tukey's multiple range test; n = 5.

NonAMF plantlets 3 days after inoculation but similar at 6 and 12 days. ABA concentrations were higher in leaves of NonAMF than AMF plantlets 6 days after inoculation, and similar at 3 and 12 days. Leaf, stem and root ABA concentration was negatively correlated to leaf RWC of both AMF (r = -0.97, -0.94, -0.84, respectively) and NonAMF plantlets (r² = -0.99, -0.99, -0.98, respectively).

Plant growth

Overall plant growth was initially low. However, growth increased as plantlets became acclimated. Root, shoot and total plant biomass (Table 1), plant height and leaf number (data not presented) were comparable between AMF and NonAMF plantlets through post-acclimatization. However, by the time the experiment was terminated (day 48), AMF plantlets had significantly greater leaf area [985 vs. 842 cm²] leaf DM [4.2 vs. 3.5 g], fruit number [9 vs. 6], LAR [110 vs. 96 cm² g⁻¹], and lower root/shoot ratio [0.36 vs. 0.42] than NonAMF plantlets (Table 1).

Leaf chlorophyll

At the beginning of the experiment, when the plantlets were transferred from in vitro conditions, leaves had very low chlorophyll (170 μg m⁻²), which was correlated with low A (r = 0.67 and 0.41, respectively for AMF and NonAMF plantlets). During the first 5 days of acclimatization, when the plantlets were grown in the growth chamber, chlorophyll levels increased to 220 μg m⁻². After plantlets were transferred to the glasshouse, chlorophyll levels dropped 36% (140 μg m⁻²) by day 10 (Fig. 4). However, during post-acclimatization, chlorophyll levels increased 2.3-fold from day 12 through the duration of the experiment; photosynthesis increased 2-fold during the same period. In general, AMF and NonAMF plantlets had comparable leaf chlorophyll during acclimatization, however, during post-acclimatization, AMF had higher chlorophyll than NonAMF plantlets (P ≤ 0.05).

Leaf elemental content

AMF significantly enhanced leaf elemental N, P and K compared to NonAMF plantlets (Table 2). There were no differences in Ca, Fe, B, Ca or Cu between treatments. NonAMF had greater Mg, Zn and Mn levels than AMF plantlets.

Discussion

This work provides new information on physiological adjustments of AMF colonized chile ancho plantlets during acclimatization and post-acclimatization. AMF colonization occurred
very rapidly (within 3 days), and influenced ABA levels, RWC [during peak dehydration – day 6], and gas exchange of the plantlets during acclimatization.

Upon initial transfer from tissue culture to ex vitro conditions, the plantlets had very low chlorophyll content and reduced A, and high E and gs. These photomixotrophic characteristics have been observed in nonmycorrhizal studies of micropropagated species (Preece and Sutter 1991).

Physiological adjustments were observed in the plantlets after transplantation, which may help correct abnormalities developed during tissue culture and enhance better plant survival and function. All plantlets experienced an abrupt decrease in RWC during the first seven days after transplanting. In response, plantlets reduced gs and E through stomatal control, thus avoiding excessive plant dehydration. This was contrary to some reports that micropropagated plantlets develop non-functional stomata in vitro (Fuchigami et al. 1981, Brainerd and Fuchigami 1982). Brainerd and Fuchigami (1982) reported that slow stomatal response to water losses after plant transfer to a relatively low humidity environment led to significant water stress of micropropagated apple plantlets.

Plantlet dehydration and consequently low RWC values during acclimatization are the result of changes in stomata response or cuticle development. By following the procedures of Holloway and Baker (1968), we found (data not shown) that cuticle was present on the leaves of the tissue cultured pepper plantlets. Hence, any cuticular transpirational loses were negligible in the pepper plantlets, and not a contributing factor to plant dehydration.

Due to the important role of ABA in stomatal control, we followed the changes of ABA in leaves, roots and stems during acclimatization and early post-acclimatization. At day 0, 3 and 12 after ex vitro transplantation, low levels of ABA were detected regardless of AMF. This corresponded with high E, gs and RWC, indicating the plantlets were not under water stress. At day six, plantlets were exposed to a high desiccation environment for the first time when moved to the greenhouse [lower RH, higher temperature and radiant energy]. This resulted in a 14% loss of RWC from 97 to 83% and correlated with substantial decreases in E and gs, and increased ABA production.

ABA levels of AMF were different from NonAMF plantlets. During peak dehydration, ABA was generally higher in Non-AMF than AMF plantlets, especially in leaf tissue. Apparently, AMF has a role in altering phytohormone concentration during acclimatization, which can help regulate plant performance. The importance of AMF on ABA during water stress was demonstrated in a seedling study of Vigna unguiculata, where ABA fluxes and concentration in the xylem were lower in AMF plants during drought (Duan et al. 1996). They concluded that AMF increased the root system’s ability to scavenge water in drier soil, resulting in less leaf water deficit and hence higher gs. If such a mechanism were operating in the present study, this should allow AMF Chile ancho plantlets to experience less desiccating stress (higher RWC, E and A), and more rapidly allow plantlets to establish. In another study of mycorrhizal chile ancho, greater extraradical hyphae occurred in drought acclimated seedlings and enhanced plant water status (Davies et al. 2002).

Chile ancho plantlets recovered from ex vitro transplant shock during acclimatization, and continued to grow with 100% survival rates. Eight days after transplanting, plantlets had acclimatized and recovered from water stress as indicated by increased RWC, which remained steady through the duration of the experiment, i.e. from days 8 to 48 (Fig. 2a). There was a similar trend in E and gs during post-acclimatization (Fig 2b, c).

The positive effects of AMF on plant growth were not observed during the first days after transplanting, which may be due in part to colonization being in the early stages of establishment and low A levels. Plantlets also came from nutritionally optimal tissue culture systems, so lack of nutritional fitness was not an initial problem. After day 21, AMF and Non-AMF plantlets had increased in overall plant growth. As the plantlets acclimatized and became photoautotrophic, there was a 4.5-fold increase in A from the beginning to termination of the experiment.

Leaf chlorophyll had a similar trend, increasing 2.3-fold from initial ex vitro culture to time of experiment termination. There was a correlation of A to leaf chlorophyll with the highest correlation occurring with AMF plantlets (r = 0.67). AMF enhanced A which was significantly higher at days 5, 7, 21 and 48 than NonAMF plantlets; however, before day 21 there were not consistent differences in A between treatments. Increases in growth may be attributed to enhanced capacity to photosynthesize, more suitable light conditions and the sub-

<table>
<thead>
<tr>
<th>Mycorrhiza</th>
<th>N (g kg⁻¹)</th>
<th>P (g kg⁻¹)</th>
<th>K (g kg⁻¹)</th>
<th>Ca (g kg⁻¹)</th>
<th>Mg (g kg⁻¹)</th>
<th>Fe (µg g⁻¹)</th>
<th>B (µg g⁻¹)</th>
<th>Cu (µg g⁻¹)</th>
<th>Zn (µg g⁻¹)</th>
<th>Mn (µg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>32.6 a</td>
<td>2.2 a</td>
<td>36.1 a</td>
<td>14.4 a</td>
<td>7.4 b</td>
<td>50 a*</td>
<td>40 a</td>
<td>2 a</td>
<td>57 b</td>
<td>29 b</td>
</tr>
<tr>
<td>Absent</td>
<td>25.7 b</td>
<td>1.8 b</td>
<td>28.7 b</td>
<td>14.7 a</td>
<td>8.4 a</td>
<td>45 a</td>
<td>46 a</td>
<td>2 a</td>
<td>82 a</td>
<td>62 a</td>
</tr>
</tbody>
</table>

* Means in each column followed by different letters are significantly different (P ≤0.05) according to Tukey’s multiple range test; n = 4.
stantial increases in chlorophyll that occurred during post-acclimatization. Leaf chlorophyll may vary according to light conditions (Pospisilova et al. 1997) or other factors such as mineral status of the plants, in which N, Mg, Cu and Fe have important roles (Salisbury and Ross 1996, Taiz and Zeiger 1996).

Total biomass production showed significant increases after plantlet acclimatization regardless of the presence of mycorrhiza, which resulted from the development of leaves with higher photosynthetic capacity and increases in leaf chlorophyll content. However, while total plant biomass was similar, changes in carbon partitioning occurred with AMF chile ancho plantlets. AMF plantlets had greater fruit production, leaf area and leaf biomass. Similar trends have been reported in nonmicropropagated AMF chile ancho seedlings, which had increased leaf area (20%) and leaf number (10%) and tended to have higher A and gs (20–40%) than NonAMF plants across a range of P supplied (0, 11, 44 g P mL⁻¹) (Aguilera-Gómez et al. 1999). Mycorrhizal plants frequently have higher photosynthetic rates. In part this is to support greater carbon flow to the root system (from +5 to 20% greater root carbon demand) needed to support the symbiosis (Smith and Read 1997).

Increased LAR, which relates the leaf area to whole plant biomass (Hunt 1982), suggests that AMF plantlets were able to support the carbon cost to maintain active development of AMF, and also favored carbohydrate partitioning toward leaf development. Higher levels of A in AMF plantlets enhanced growth. Consequently, AMF plantlets gradually allocated more assimilates to leaves and fruits, resulting in a decreased root:shoot ratio as compared to NonAMF plantlets. Decreased root:shoot ratios have been reported with other AMF colonized plant species (Azcón-Aguilar and Barea 1997, Smith and Read 1997).

Enhanced AMF plantlet growth in part may have been the result of increased levels in N, P, and K and their influence on higher A. At the termination of the study, AMF plantlets had greater chlorophyll (369 vs. 351 μg m⁻²) and A (18 vs. 14 μmol m⁻² s⁻¹) respectively, compared to NonAMF plantlets. This is a frequent response of non-tissue culture propagated AMF plants, which may be the result of improved P uptake (Fitter 1988) or occur independently of P status (Davies et al. 1993).

Mycorrhiza can enhance nutrient ion uptake of Capsicum annuum L. plants such as P (Gaur et al. 1998, Davies et al. 1993, Sreenivasa 1992, Aguilera-Gómez et al. 1999), Zn (Sreeramulu and Bagaijara 1986), Cu, Mn, and Fe (Sreenivasa 1992). However, the relative influence depends on the fertilization regime. Capsicum species and cultivars, and the AMF species colonizing the roots (Sreenivasa 1992, Aguilera-Gómez et al. 1999, Davies et al. 2000).

Increased uptake of N, P, and K of AMF plantlets were in ranges considered sufficient for producing pepper seedling plants, as compared to the more deficient NonAMF plantlets (Reuter and Robinson 1986, Mills and Jones 1996). In AMF and NonAMF plantlets, levels of Ca, Mg, Fe, B, and Zn were considered sufficient, whereas Cu concentrations were deficient (Reuter and Robinson 1986, Mills and Jones 1996). Mn was sufficient in NonAMF and slightly deficient in AMF plantlets. Levels of Na, Al and Mo were comparable between treatments (data not presented). Hence, differences primarily in leaf mineral N, P and K resulted in greater nutritional fitness of AMF plantlets that was essential for better plant growth.

Capsicum annuum L. can be colonized by different AMF species, including G. macrocarpum, G. fasciculatum, G. albicum, G. deserticola, G. mosseae, G. intraradices, Gigaspora margarita, Acaulospora laevis, and Sclerocystis dusii (Haas et al. 1986, Sreearamulu and Bagaijara 1986, Sreenivasa 1992, Davies et al. 1993, Gaur et al. 1998, Aguilera-Gómez et al. 1999). In this study, chile ancho plantlets were inoculated with a ZAC–19 mixed isolate of G. diaphanum, G. albicum, and G. claroides (Chamizo et al. 1998), which has shown to enhance P uptake and drought resistance of chile ancho seedling produced peppers (Davies et al. 2000, 2002).

In summary, AMF colonization occurred rapidly within three days, and had an early impact on gas exchange, ABA tissue concentration and RWC when plantlets were experiencing desiccation during acclimatization. During post-acclimatization when nutritional stress became the growth limiting factor, AMF plantlets maintained higher N, P and K levels. This indirectly led to higher chlorophyll levels and the higher A, gs, and E observed at the termination of the experiment. AMF also altered plant carbon partitioning (but not total plant biomass) by increasing leaf mass and surface area, increasing the LAR and decreasing the root:shoot ratio. In the kinetics of root colonization, the high frequency of arbuscules observed (37%) indicated very active AMF colonization (Smith and Gianinazzi-Pearson 1988).

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References


Estrada-Luna AA (1999) Mycorrhizal effects on the physiology of micropropagated chile pepper (Capsicum annuum L.) plantlets during acclimatization. Ph. D. dissertation. Texas A&M University, College Station, TX


