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ORIGINAL ARTICLE

Morphological and physiological responses of the external mycelium of *Rhizophagus intraradices* to water stress

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Abstract

Most studies dealing with mycorrhizal associations and drought have focused on the plants, not on the fungi, and tolerance and adaptations of arbuscular mycorrhizal (AM) fungi to cope with water stress are virtually unknown. This study was conducted to assess how water stress directly affects an AM fungus isolate, particularly through morphological and physiological changes in the external mycelium. We used two-compartment pots separated by mesh and an air gap that allowed us to apply water stress treatments only to the external mycelium. Clover (*Trifolium subterraneum* L.) plants inoculated with *Rhizophagus intraradices* grew at high humidity until external mycorrhizal mycelium developed in the mycelium compartment. Then, we started three watering treatments: high (H, 70% of soil water holding capacity), low (L, 10%), and mixed watering (HLHL, 70–10–70-10%) only in the hyphal compartment. The HLHL treatment was rewetted once to 70% after 42 days. We measured total mycelium length, hyphal length in diameter categories, respiration activity, and protoplasm fragmentation 42 and 76 days after starting the treatments. *Rhizophagus intraradices* mycelium responded to water stress by reducing its length, maintaining larger diameter hyphae, and concentrating protoplasm activity in fragments in the HLHL and L treatments. In both water stress treatments, changes suggested a trade-off between avoiding desiccation and storing resources, and maintaining soil exploration and water uptake capacity.

Keywords Drought · Hyphae · Hyphal diameter · Respiratory activity

Introduction

Arbuscular mycorrhizal (AM) fungi are ubiquitous obligate symbionts of approximately 80% of terrestrial plants (Brundrett 2009) in almost all biomes (Öpik et al. 2010) that develop both inside roots and externally in the soil (Smith and Read 2008). Their intraradical phase is mainly the exchange tissue for nutrients and water between the plant and the fungus, and the extraradical part is the exploring phase foraging for mineral nutrients and water in the soil (Gavito and Olsson 2008).

Water stress is one of the most widespread forms of environmental stress, and the majority of organisms have developed morphological and physiological adaptations to evade or resist desiccation periods. Numerous studies have reported that mycorrhizal plants show improved water relations compared to nonmycorrhizal plants (Smith et al. 2010; Augé et al. 2016) and that AM fungi may help plants to cope with water stress (Augé 2001). Thin mycorrhizal hyphae (2–5 μm) have access to small pores and water in soil that is no longer available to roots (Allen 2007). Water is transported to the intraradical mycelium and transferred to root cells thereby increasing plant water uptake (Ruiz-Lozano 2003). Although there is an ongoing discussion to define if it is water or nutrient transfer from AM fungal hyphae that alleviates drought stress in plants, most studies focus on the development and water relations of the plant and assume that water transfer to plants represents an automatic beneficial trait of AM fungi (Augé 2004; Augé et al. 2016). The other main avenue of research relates to

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the role of AM fungi in soil water retention and availability (Querejeta 2017), where the focus is on the soil.

AM fungi can remove significant amounts of water from soil and contribute up to 20% of plant water uptake (Ruth and Khalvati 2011), but AM fungal species differ in their capacity to improve plant growth and water relations under water stress, and this seems related to their intrinsic growth, uptake and transfer capacities under those conditions (Marulanda et al. 2003; Ruiz-Lozano and Azcón 1995). This suggests that although water transfer to plants would seem an advantageous trait for all AM fungi, not all species or isolates of the same species have the same water uptake capacities, as it is has been measured recently (Zhang et al. 2018), and likely not all have similar resistance to drought. Additionally, carbon supply to AM fungi may be reduced or suspended in water-stressed plants that have closed their stomata and reduced gas exchange in their leaves to avoid desiccation (Augé et al. 2008). Several reports showing lower intraradical colonization in water-stressed plants (Al-Karaki et al. 2004; Ryan and Ash 1996) suggest that drought may reduce carbon flow belowground and limit mycorrhizal development, but other studies have found no response or complex interactions with other environmental factors and differences between AM fungal species (Staddon et al. 2004). Most studies have only measured intraradical colonization, and the effects of drought on the extraradical phase either indirectly through the host plant or to direct exposure have been little explored. AM fungi are considered drought tolerant in most studies because, since the focus is on the plants, those studies rarely reach levels that become water limiting for AM fungi. Climatic manipulation field studies reaching and maintaining very low water potentials for extended periods have shown a reduction in AM extraradical mycelium (Staddon et al. 2003). However, whether this is a direct, or plant-mediated, response remains unclear because both plants and AM fungi are experiencing water shortage.

As the soil water potential drops and water becomes less available, one of the constraints for AM fungal mycelium to perform exploration, uptake, and transfer functions is its own tolerance of desiccation. Maintaining a large network of fine hyphae may be the best way to maximize exploration and surface exposure for nutrient absorption but not to avoid desiccation. The formation of hydrophobic hyphae, hyphal cords, and rhizomorphs to reduce surface exposure and increase long-distance foraging is among the few morphological traits related to water conservation and drought tolerance that have been investigated in other fungal groups (Moeller et al. 2014). The accumulation of melanin pigments is also related to protection from low water potentials in other fungi (Koide et al. 2013).

Because of their extensive development colonizing both the root cortex and soil, AM fungi have one part of their tissue exposed and the other part protected from water fluctuations in the soil. Therefore, the most relevant adaptations to water stress are expected in the extraradical mycelium. De la Providencia et al. (2007) have shown that AM fungal mycelium exhibits healing mechanisms like protoplasm retraction, septa formation, and growth reallocation to isolate sections and maintain the integrity of mycelial networks in response to injury. Growth and resource reallocation also have been observed as a result of aging or starvation (Gavito 2007; Gavito and Olsson 2003), as well as periods of mycelium growth and retraction occurring along circadian environmental changes (Hernandez and Allen 2013). These observations suggest that the AM fungal mycelium is able to make rapid adjustments in response to prevailing resources and environmental conditions.

The objective of this study was to investigate morphological and physiological adjustments of the extraradical mycelium of an AM fungus isolate in order to identify traits related to water stress tolerance. We hypothesized that the AM mycelium has (1) a lower total hyphal length and a lower proportion of fine hyphae (reduced exposure surface to reduce desiccation), and (2) lower respiratory activity and lower protoplasm continuity (concentration of resources in "propagule-like" units) under drought stress than under constant watering.

Materials and methods

We conducted a randomized block experiment with three watering treatments and three sampling times. We used a compartmentalized pot to physically separate one part of the extraradical mycelium from the roots in order to test the direct effect of drought on the extraradical mycelium without the confounding effect of drought on the host plant and a potential shortage of carbon to the fungus. We adapted the experimental units used by Ruth and Khalvati (2011) to measure water depletion by AM fungi to the PVC experimental units with one side compartment used by Munkvold et al. (2004). A 12-cm diameter × 30-cm height PVC tube with a lateral window connecting to another 12-cm diameter tube was used as the plant compartment (Fig. S1). An air-gap barrier was used to prevent roots and water from passing from the plant compartment to the lateral mycelium compartment. Two 12-cm diameter circles were cut from rigid 3-mm-opening plastic mesh and glued together. Both faces of this circle were covered with 25-μm nylon mesh, thereby creating a 5-mm air gap division that was inserted in the window connecting the main, vertical compartment and the lateral, horizontal compartment.

The plant compartment was filled with 3.25 kg of an Andosol loamy soil, sieved through 4 mm mesh, mixed 3:1 with a mixture of fine gravel and coarse sand, and autoclaved twice for 2 h at 1.5 psi. Before packing the soil for each pot, 250 g of inoculum of a *Rhizophagus intraradices* isolate obtained from a semi-abandoned pasture in Ejido Santa Cruz de los Otates, Jalisco, in Mexico, was mixed thoroughly with

3 kg of the soil/gravel mixture. The inoculum consisted of soil from a pure culture containing *Medicago sativa* colonized roots, mycelium, and spores. The barrier circles were glued to the PVC window, and the 15-cm long lateral tube was filled with 800 g of the same soil/gravel mixture (but without mycorrhizal inoculum) and attached to the plant compartment. Both compartments had drainage holes to prevent water accumulation, and the lateral compartment had an open rim on the top to allow evaporation and insertion of the TDR probes to control water content. The end of the lateral compartment additionally had four holes to insert the soil borer at the times of soil sampling, which were covered with tape until that moment. Ten *Trifolium subterraneum* seedlings were transplanted to each plant compartment, and the pots were transferred to a growth room with 250–350 μmol m^{-2} s^{-1} PPFD in a 12-h photoperiod, mean day temperature of 26.3 ± 1.2 °C, mean night temperature of 24.4 ± 1.1 °C, and 60 ± 20% relative humidity. The pots were watered as required using a Fieldscout TDR 100 Soil Moisture Meter to maintain the soil at 70% (23% moisture content, − 0.35 MPa) of its water holding capacity (35% moisture content, − 0.1 MPa) (Fig. 1a). This level provided adequate watering and aeration to minimize growth of saprotrophic air-borne fungi during the establishment of the plant in the central compartment and the mycorrhizal mycelium in the lateral compartment. There were 15 inoculated pots that were later split into five replicates for each of the three watering treatments and three replicates of pots without mycorrhizal inoculum at each watering level that were used to measure background hyphae. These values were subtracted from the total hyphal length since there is no way to distinguish the new mycorrhizal mycelium from the nonmycorrhizal and dead hyphae remaining in the autoclaved soil. After 3 months, all plants received 50 mg N kg^{-1} soil as ammonium nitrate in water solution only in the plant compartment to ensure that the plants were well nourished.

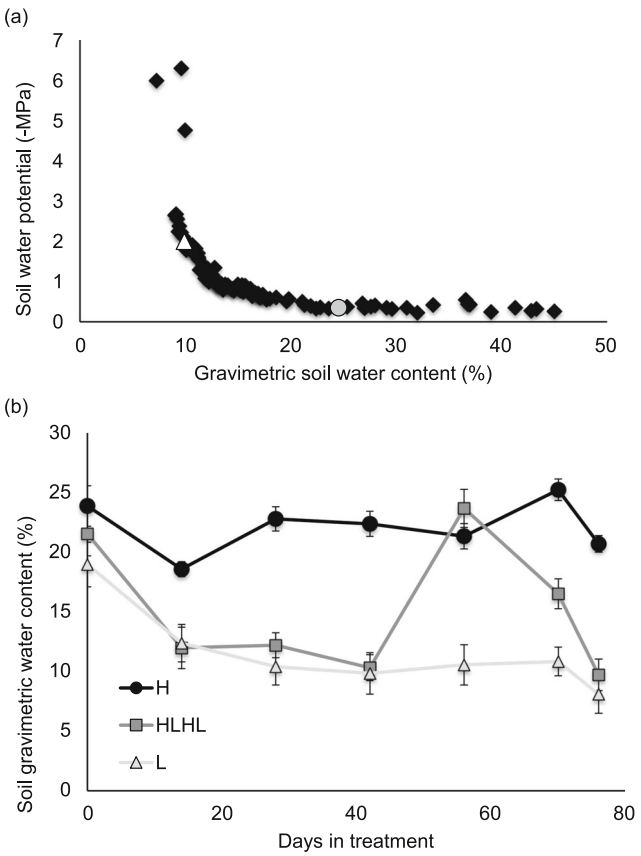

Fig. 1 Soil water retention-release curve for the soil used in the experiment indicating the water potentials selected for the high (gray circle) and low (white triangle) watering treatments and their corresponding gravimetric water contents (**a**). Soil water content (mean ± S.E., $n = 5$) measured with TDR probes in the hyphal compartment during the watering treatment period (**b**)

Watering levels to provide high and low availability of water in the soil were obtained through a soil moisture release curve (Decagon 2010) of the soil/gravel mixture made with a dew point potentiometer WP4T (Decagon, Pullman, WA). Based on this curve (Fig. 1a), the low availability level was established at 10% of the soil water holding capacity, with values between − 1.5 and − 2 MPa, that indicate high water stress conditions. The watering treatments were initiated when the mycelium was well established in the lateral compartment, 4 months after planting the seedlings, as confirmed by sampling, extraction, and quantification of mycelium as explained below.

Five of the mycorrhizal pots were randomly assigned to the high watering treatment (H) and continued with watering at − 0.35 MPa in both compartments, and five were assigned to the low watering (L) and five to a mixed high-low-high-low watering (HLHL) treatment. The L and HLHL watering treatments maintained the − 0.35 MPa in the plant compartment and switched to the new watering regime only in the lateral hyphal compartment. These two treatments simulated a continuous long drought period (L) and a drought period with a single rewetting event (HLHL), and were used to explore mycelium tolerance to extended drought and its capacity to recover with occasional rain. We withheld watering until reaching between − 1.5 and − 2 MPa in the L treatment and maintained this level for the rest of the experiment. The HLHL treatment received no water until reaching between − 1.5 and − 2 MPa, maintained this level for 3 weeks, and was rewetted once to − 0.1 MPa. Then, it was allowed to dry again until reaching − 1.5 and − 2 MPa, and maintained at this level for the rest of the experiment. The nonmycorrhizal pots we included within each treatment to obtain background hyphal length values were handled identically.

Soil samples were taken at days 0, 42, and 76 after initiating the watering treatments. Samples at day 0 were used to establish the initial values in all pots after being under the same watering. Samples taken at day 42 allowed the 3 weeks needed to reach the desired water potential in the L treatment plus 3 weeks at this level to allow changes in mycelium

development to be quantified. At day 43, after sampling, the hyphal compartments of pots in the HLHL treatment were rewetted to − 0.1 MPa and allowed to dry until they reached − 1.5 MPa again, 3 weeks later. After that time, they were watered to maintain − 1.5 MPa for 3 weeks more. The L treatment continued watering as required to maintain − 1.5 MPa until the end of the experiment. At all sampling times, soil borers (1-cm diameter × 10-cm length) were pushed horizontally across the hyphal compartment. The soil withdrawn was replaced with new soil to minimize disturbance.

Soil samples were processed immediately after sampling. Hyphae were extracted from 5-g soil by adding 250 ml water and blending the water suspension for 30 s. After allowing sedimentation for 30 s, the suspension was passed through two layers of 20-μm mesh to retain the mycelium. Vital staining was used to assess respiration activity of succinate-dehydrogenase, as in Schaffer et al. (1994). Finally, the vital-stained mycelium was filtered through a nitrocellulose membrane to be retained and quantified under the microscope.

The membrane filters were placed on slides with lactoglycerol and cover slips and were quantified with the gridline intersection method as in Jakobsen et al. (1992), counting one 4-mm^2 grid per field in 50 fields for each sample. Total intersections, intersections with formazan deposits indicating respiration activity, and intersections with active protoplasm fragmentation (evident gaps in formazan deposits, Fig. S2) were registered. Total hyphal length was multiplied by the proportion of intersections with activity and fragmented protoplasm. Slides were maintained in the dark and scored within 48 h to avoid degradation of formazan deposits. After counting, mycelium images were obtained from fields containing hyphae with an Olympus camera.

Twenty images from each sample were edited to remove debris and trace the hyphae using brushes of the appropriate pixel size to distinguish hyphae of each diameter category in Adobe Photoshop CS3 Extended Version 10.0 Software. Nine hundred (100 per treatment, 300 per sampling time) clean, edited images (Fig. S3) were analyzed with Win-Rhizo software (WinRhizo, Ontario) to measure the length of hyphae in each diameter category. After correcting for pixel size and magnification in the microscope, hyphal diameter categories were grouped into five final categories: 2.5–7.5, 7.6–12.5, 12.6–17.5, 17.6–22.5, and > 22.5 μm.

Total hyphal length, active length, fragmented-protoplasm length, and the proportion of hyphal length in each category were analyzed with repeated measures ANOVAs considering the watering treatments and sampling dates. Data were transformed as required to meet ANOVA assumptions. Statistically significant differences among means were examined with Tukey mean comparisons. All analyses were conducted in Statistica software V. 12.5.

Results

The L and HLHL treatments desiccated slowly and achieved the desired water content 40 days after initiating the watering treatments but differed clearly from the H treatment after 15 days (Fig. 1b). Total mycelium length density increased steadily with time in the H treatment (Fig. 2a). Drought

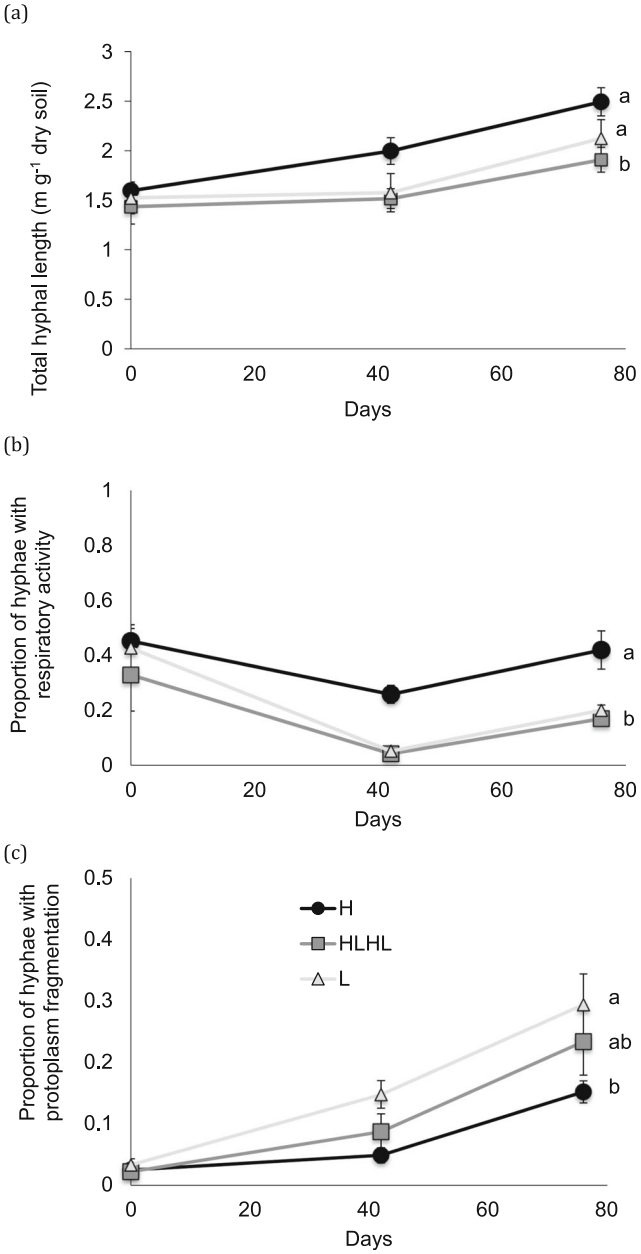

Fig. 2 Total mycelium length density at the time the watering treatments started and after 42 and 76 days (**a**). Proportion of mycelium length with respiratory activity (**b**), and proportion of mycelium length with protoplasm fragmentation (**c**). The H treatment is represented with circles, the L treatment with triangles, and the HLHL treatment with squares. The HLHL treatment was rewetted once to − 0.35 MPa on day 43. There were no differences among watering treatments at time 0. Lines with the same letters did not differ significantly at $p < 0.05$ according to repeated-measures ANOVAs (means ± S.E., $n = 5$)

significantly reduced hyphal length in the L and HLHL treatments after 42 and 76 days with no evidence for new growth after rewetting in the HLHL treatment. Mycelium length density increased in all treatments after 76 days.

Respiratory activity in fungal hyphae was reduced to one third in both drought treatments during the first period (0–42 days), and rewetting in the HLHL treatment made no difference after the second period (42–76 days, Fig. 2b). However, activity decreased slightly in all treatments during the first period and increased slightly again in the second period (42–76 days). Protoplasm fragmentation increased with time in all treatments as a result of mycelium aging (Fig. 2c) but was clearly highest in the L treatment, and the HLHL treatment had intermediate values between the H and L treatments.

Hyphal mean diameter became clearly larger in both drought treatments than in the H treatment, which maintained a relatively constant mean diameter in all sampling dates (Fig. 3). A close examination of hyphal diameter showed the departure of the different categories from the initial condition (Fig. 3a–c). The only significant change after 42 days was an increase in length in the 12.5–17.5-μm category in both water-stressed treatments that were identical in watering until this time (Fig. 3b). Another change that was visible but was still not significant after 42 days was a reduction in the thinnest hyphae in the water-stressed treatments. This reduction became significant after 76 days (Fig. 3c). The proportion of hyphal length in the intermediate diameter categories (7.5–17.5 μm) increased in both drought treatments, whereas the proportion of the thickest hyphae had either small (17.5–22.5 μm) or no (> 22.5 μm) changes, after 76 days (Fig. 3c).

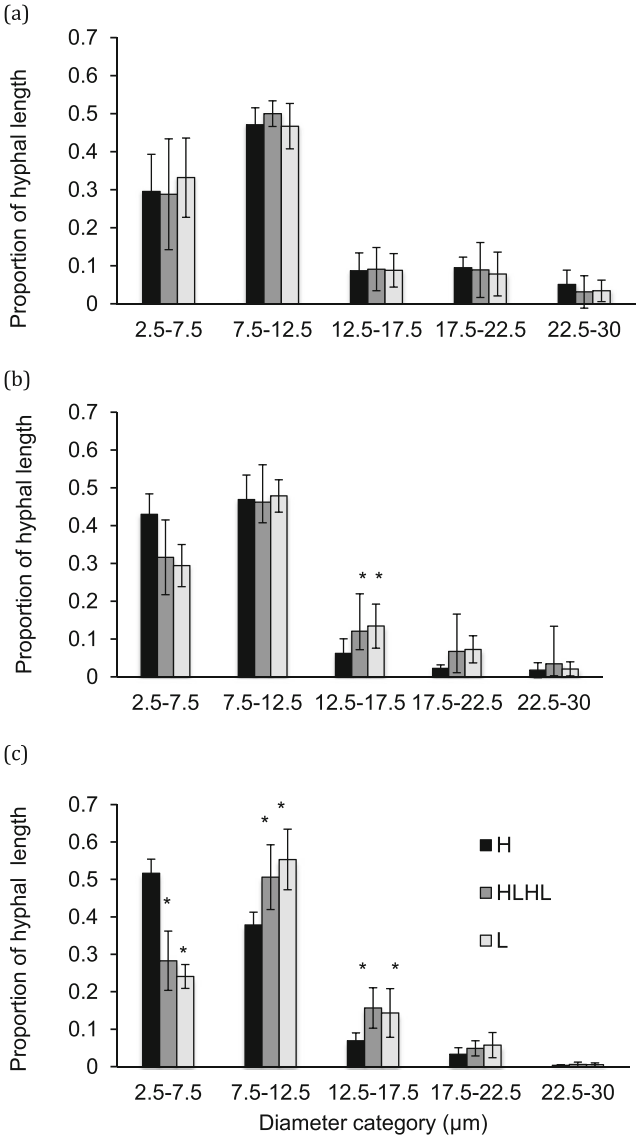

Fig. 3 Means (± S.E., $n = 5$) for the proportion of hyphal length in each diameter category at the time the watering treatments started (**a**) and after 42 (**b**) and 76 (**c**) days. Asterisks topping the bars indicate significant differences between that treatment and the high watering treatment within each diameter category

Discussion

This study documented the morphological and physiological adjustments in the mycelium of an AM fungus isolate in response to drought conditions. Changes seemed to improve survival by two main pathways: (1) reducing the surface exposed to desiccation by maintaining large-diameter hyphae and (2) concentrating respiration activity and resources in small sections that may act as propagules under more severe or prolonged drought conditions. The adjustments were achieved through the inhibition of new hyphal growth, especially the production of fine hyphae, a reduction in respiration activity, and the retraction of protoplasm to form concentration points in thick hyphae. Protoplasm retraction to cope with stress has been reported previously in other isolates and conditions (De la Providencia et al. 2007; Giovannetti et al. 2000). Addy et al. (1997) also reported protoplasm activity concentration into scattered units from which mycelium could rapidly reinitiate growth and reestablish functional mycelium networks after winter freezing for several months, which combines temperature and drought stress.

The set of adjustments reported here suggests that there is a trade-off between survival and protection from desiccation versus maintenance of absorption capacity and growth. In accordance with our results, Zhang et al. (2018) found that although mild water stress enhanced mycorrhizal hyphae water uptake, it reduced hyphal length in the two isolates tested. In their study, the reduction in mycelium might be attributed to reduced C supply from the host, since the plants were also exposed to drought. In our study, we applied the water stress treatment locally only to mycorrhizal hyphae, and even though this might have initially triggered gene regulation responses

and "alarm" signals in the plant (Bárzana et al. 2015; Xu et al. 2018), these were likely temporary because the host plant compartment was maintained with independent high watering in all treatments. Independent high watering to the host plant and low watering to the connected AM mycelium opens the possibility for water translocation from the plant to the AM fungi, as has been observed in other drought studies (Querejeta et al. 2003). That may have helped the AM mycelium to cope with water stress in the hyphal compartment, but hyphae were still growing exposed to a highly desiccating environment and likely required some adjustments to reduce exposure.

The isolate we tested had been recently isolated from a cattle-grazing field in a tropical dry forest ecosystem that experiences high intra- and inter-annual rainfall variability, drought spells during the rainy season, and severe seasonal drought in the dry season (Maass and Burgos 2011). Its growth stopped, however, and its activity dropped to one fifth, after 3 weeks under severe water stress. The mycelium showed signs of recovery 4 weeks later in the treatment with one rewetting, but the experiment did not follow longer-term responses so as to ensure the mycelium recovered completely if no further drought was imposed or if undergoing repeated water stress periods. Single and repeated drought periods of varying intensity and duration are common in the ecosystem from which this AM fungus was isolated and in other ecosystems. The effects of prolonged and repeated drought spells have been relatively well studied for plants from this ecosystem and revealed highly diverse adaptations and tolerance thresholds among species to cope with water stress (Méndez-Alonso et al. 2012; Paz et al. 2015; Pineda-García et al. 2011). Retracting protoplasm and concentrating respiratory activity in smaller areas within the mycelium seem an equivalent adaptation to nutrient resorption and leaf shedding by trees, a widespread mechanism of plant water conservation in seasonal environments. In AM fungi, the fungal wall likely remains for months in soil when protoplasm retracts and metabolic activity gets concentrated until the stress factor disappears and protoplasm continuity is reestablished. Petri-dish monoxenic culture studies allowing direct visual observation of mycelium in transparent growth media have revealed that protoplasm retraction concentrates near roots where nutrient exchange is high between the intraradical and extraradical phases of the mycelium and C essential for fungal growth and metabolism is nearby (Bago et al. 2002; Gavito and Olsson 2008).

It seems unlikely that substantial decay had occurred during the experiment even under the severe water stress reached in the drought treatments and fungal walls remain even when protoplasm has retracted. Therefore, hyphae that died or were emptied of protoplasm most likely were included in the total hyphal length measurements, which may explain why total mycelium length did not differ much among treatments during the experimental period. qPCR quantification including only living mycelium detected a loss of extraradical mycelium biomass of ectomycorrhizal *Lactarius* during seasonal drought in field conditions (Castaño et al. 2017). Querejeta et al. (2009) also found significantly lower viable ectomycorrhizal and AM fungal mycelium associated with oak trees in a severe drought year than in an above-average rainfall year. In our experiment, hyphal respiratory activity was surprisingly low (20–40%), even before initiating the drought treatments, and protoplasm fragmentation did increase (although to a low extent) in the high watering treatment suggesting that even in the absence of water limitation, aging and resource allocation modify growth and a large proportion of mycelium becomes inactive.

Our results add evidence suggesting that soil humidity has a direct influence on AM hyphal length (Gonzales-Dugo 2010) and drought pressure reduces hyphal length independently from drought effects on the host plant. Drought tolerance thresholds and functional trade-offs like those we observed may have important implications for mycorrhizal functioning and resource movement in soil which deserve further exploration with more AM fungal isolates, drought manipulation conditions, and field studies.

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Analyzed data: RLM, MEG
Wrote the manuscript: RLM, MEG

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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