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Impact of water availability on arbuscular mycorrhizal fungi and role of these root symbionts in plant adaptation to drought

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List of Abbreviations

A	CO_2 Assimilation rate
AM fungi	Arbuscular Mycorrhizal fungi
ANOVA	ANalysis Of Variance
CESAMM	Center of Study on Arbuscular Mycorrhizal Monoxenics
CIPF	Centre Indépendant de Promotion Fourragère
DAOM	Department of Agriculture Ottawa Mycology
E	Transpiration rate
ERM	ExtraRadical Mycelium
FAO	Food and Agriculture Organization
GINCO	Glomeromycota *IN vitro* COllection
***Gint*AQPF1-2**	*Glomus intraradices* Aquaporin Forward 1-2 (gene)
GintPT	*Glomus intraradices* Phosphate Transporter (gene)
GRPV	Groupe de Recherche en Physiologie Végétale
g_s	Stomatal conductance
HC	Hyphal Compartment
Hoagland ^{lowPi}	Pi-impoverished modified Hoagland solution
ICP-AES	Inductive Coupled Plasma-Atomic Emission Spectrometer
INRAE	INstitut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement
IRGA	Infrared Gaz Analyzer
JMP	John's Mcintosh Project
***mi*RNA**	micro RiboNucleic Acid
MUCL	Mycothèque de l'UCLouvain
MPa	MegaPascal
MSR	Modified Strullu-Romand (medium)
NBT	Nitro Blue Tetrazolium
NLIN	Non-LINear (regression)
PAHs	PolyAromatic Hydrocarbons
PEG-8000	PolyEthylene Glycol 8000
PPF	Photosynthetic Photon Flux
RC	Root Compartment
REML	REstricted Maximum Likelihood
Ri (T-DNA)	plant Root Inducing
ROS	Reactive Oxygen Species

RWC_L	Leaf Relative Water Content
SARDI	South Australian Research and Development Institute
SAS	Statistical Analysis Software
SDH	Succinate DeHydrogenase
T-DNA	Transfer DNA
Tris	Trisaminomethane
TW	Turgid Weight
VPO	Vapor Pressure Osmometer
WUE_i	Instantaneous Water Use Efficiency
Ψ	Water potential

Glossary

Arbuscular mycorrhiza (AM): *most ancient symbiosis (mutualistic) and common mycorrhizal type. They are formed in an enormously wide variety of terrestrial host plants by obligatory symbiotic fungi which have recently been reclassified on the basis of DNA sequences into a separate fungal phylum, the Glomeromycota* (Smith and Read, 2008).

Drought (Meteorological): *meteorological drought is usually defined as a shortage of precipitation (or moisture supply) over some period of time (e.g. weekly, monthly, seasonal or annual timescales). Definitions of meteorological drought, therefore, are location specific since normal precipitation is a function of the climate. Some definitions of meteorological drought focus on the length of time since the last precipitation event (e.g. number of consecutive dry days), while others focus on the magnitude of the precipitation departure from normal.* (Quiring, 2009).

Drought avoidance (plant): Also referred as "dehydration avoidance" (Volaire, 2018): *(= dehydration postponement), strategy allowing plants to maximize water uptake and/or minimize water loss to maintain hydration in leaves.*

Drought recovery (plant): *A plant's capability to resume growth and gain yield after exposure to severe drought stress which causes a complete loss of turgor pressure and leaf* (Luo, 2010). Drought recovery has been proposed to play an important role in plant adaptation to drought and to involve specific physiological mechanisms (Chen *et al.,* 2016).

Drought resistance/tolerance (AM fungi): capacity of an AM fungus to adapt to conditions of drought stress or water shortening. Drought resistance/tolerance of an AM fungal strain can be characterized by its higher capacity to fulfill the different stages of its lifecycle compared to other AM fungal strains.

Drought resistance (plant): *when aerial growth and development remain possible in response to what can be defined as a 'moderate drought', i.e. resulting in the maintenance of growth for the considered species and*

population, and the maintenance of productivity notably for crops (Volaire, 2018).

Drought tolerance (plant): Also referred as "dehydration tolerance" (Volaire, 2018): *strategy allowing plants to tolerate low tissue level of dehydration (> 30% water).*

Life cycle: *the series of stages in form and functional activity through which an organism passes between successive recurrences of a specified primary stage* (www.merriam-webster.com). In AM fungi the different steps of the life cycle can be designated in the following order: *spore germination, growth and morphogenesis of the presymbiotic mycelium, formation of appressoria, penetration of the root epidermis and exodermis, colonization of the root cortex and growth of the extraradical symbiotic mycelium. …. production of viable and infective spores.* (Smith and Read, 2008).

Summary

Drought is probably one of the biggest single threat from climate change which impacts are global, threatening particularly crop production. Under conditions of limited water availability, land irrigation is a common practice while development of crops that are more resistant to drought has become a priority for breeders. However, irrigation can lead to the depletion of water reserves and salinization while both methods require heavy infrastructure and financial resources that often default many farmers. AM fungi are soil microorganisms that establish symbiosis with more than 70% of land plants. Their role in mitigating the effects of drought on plants has been demonstrated in numerous studies. The optimization of AM fungal association with plants through selected inoculation or management of AM fungal communities within the field may thus represent a sustainable and complementary approach to other agricultural practices in order to maintain/optimize crop production under drought conditions. The resistance/tolerance of AM fungi to drought stress, their potential for plant nutrition (especially via their capacity for phosphorus uptake and transport) under drought conditions and for plant recovery following drought are thus important factors to be considered.

In the present study, our main objective was to investigate the impact of decreasing water availability on the arbuscular mycorrhizal fungi and the role of these root symbionts in plant adaptation to drought. Therefore, in a first step we investigated the effects of decreasing water availability on the life cycle, including spore germination and germ tube length,of different AM fungal strains, and on Pi uptake and transport capacity of the model AM fungus *Rhizophagus irregularis* MUCL 41833 under strict *in vitro* culture conditions. Polyethylene glycol (PEG) was used to decrease the water potential (reflecting water availability) of the growth medium. We showed that a decrease in water potential from -0.024 to -0.056 MPa (n.b. -0.033 MPa being field capacity − see Appendix1) induced by the PEG affected differently the germination and germ tube length of various AM fungal strains with *Rhizophagus intraradices* MUCL 49410 and *Rhizophagus clarus* MUCL 46238 being more resistant/tolerant than *Rhizophagus sp.* MUCL 43204 and *Rhizophagus irregularis* MUCL 41833. Spores production and hyphal development as well as Pi uptake by the extraradical mycelium of *R. irregularis* MUCL 41833 were also affected by a decreasing water availability

especially at the highest PEG concentration (−0.056 MPa). This suggested that AM fungi may behave differently under drought stress conditions, requiring a screening procedure to select the more resistant/tolerant and potentially efficient strain to mitigate water stress in plants.

Although the AM fungi was impacted under decreasing water availability, it was still able to develop and to take up most of the available phosphorus in its environment. In a second step we thus evaluated if the AM fungus *R. irregularis* MUCL 41833 could benefit plants during recovery from drought, especially via its effect on Pi mobilization and leaf gas exchange parameters. Maize plants associated or not with the AM fungus were grown in a semi-hydroponic cultivation system under three water regimes (two of which corresponded to a moderate and severe drought) for three weeks followed by a recovery phase induced by circulating a nutrient solution through the plant substrate during 42 h. During recovery, the mycorrhizal plants took up Pi faster than the non-mycorrhizal ones although both plants had similar root biomasses. This indicated that the mycorrhizal plants benefited from a higher Pi supply during recovery even if roots of mycorrhizal and non-mycorrhizal plant had unrestricted access to Pi in the substrate. After 42 h of recovery, stomatal conductance and transpiration were decreased in the mycorrhizal plants as compared to the Controls and this effect was modulated by the water regime before recovery. As CO_2 assimilation rate was not affected, the instantaneous water use efficiency was significantly improved in the mycorrhizal versus non-mycorrhizal plants. It seems that mycorrhizal plants avoided water loss in maize plants during recovery. This could have contributed to a faster hydration of plant tissues as suggested by the improved shoot water contents found in the mycorrhizal plants.

In a conclusion, we demonstrated the effects of decreasing water availability on life cycle and function of AM fungi, suggesting differences in drought resistance/tolerance of strains. The experiment conducted with maize plants revealed for the first time that AM fungi improved instantaneous water use efficiency through a decrease of transpiration during recovery, possibly resulting in higher adaptation of plants to drought. These results plead for developing high-throughput screening strategies to select highly efficient AM fungi and to investigate the mechanisms involved in increasing adaptation of mycorrhized plants to drought. It also pleads for studying the mechanisms involved in the faster recovery of plants associated to mycorrhizal fungi following a drought period.

Author's Contribution

The work presented here was strictly realized during the time course of my PhD. The sections**: Introduction, Outline of the thesis, State of the Art, Material and Methods, General Discussion, Conclusion and Perspectives** were written by myself and have not been published elsewhere.

Chapter 1 is a research paper in preparation. My contribution to this chapter was estimated to 90%. All experiments were performed by myself. The writing of the manuscript was done by myself under the supervision of Pr. Stéphane Declerck.

Chapter 2 is a research paper published in Frontiers in Microbiology (Le Pioufle and Declerck, 2018). My contribution to this chapter was estimated to 90%. All experiments were performed by myself with the exception of the spore counting which was done by François Ferrais, a technician from the laboratory of mycology. The writing of the manuscript was done by myself and both authors read and approved the manuscript.

Chapter 3 is a research paper published in Frontiers in Plant Science (Le Pioufle *et al*., 2019). My contribution to this chapter was estimated to 70%. Experiments were performed by myself, Matike Ganoudi (a PhD student from INRA, Morrocco) and Fatma Ben Dhaou (a master student from Ecole Polytechnique de Sousse, Tunisia). The experiments were designed by myself, Dr. Maryline Calonne-Salmon and Prof. Stéphane Declerck. The writing of the manuscript was done by myself and Matike Ganoudi for the results part and all the authors read and approved the manuscript.

Introduction

Drought is a natural hazard characterized by reduced precipitations over an extended period of time, impacting ecosystems integrity, water supply and crop production in many parts of the world (Mishra and Singh, 2010; NOAA, 2020). Extended drought periods may have major human and economic consequences, especially in developing countries (WMO, 2014) as there is limited access to water management programs or to drought-adapted agricultural practices (Rockström *et al.*, 2010). For instance, in countries such as Angola and Botswana but also in industrialized countries such as the USA and Australia, drought has been associated with a 4 to 6% decrease in the production of cereals over the period 1961-2014 (Mehrabi and Ramankutty, 2017).

An amplification of climate changes is expected for the next decennials and will probably lead to a surge in the occurrence and severity of drought disasters (Dai, 2013; Huang *et al*., 2016) most likely impacting global crop yields (Li *et al*., 2009). Together with an increasing world population that is expected to reach 9.7 billion inhabitants by 2050 (UN, 2019), food security could be endangered. An escalation of drought issues could be prevented by limiting the extent of global warming. To that end, several commitments (i.e. Kyoto's protocol and Paris agreement) have been taken by the United Nations in order to reduce the world's greenhouse gases emissions.

Meanwhile several agricultural practices have been long implemented to ensure sufficient crop production in periods of water shortage. Among them, is the recourse to irrigation. By the year 2012, 21% of the total surface of agricultural land was irrigated, contributing to 40% of the total crop production worldwide (FAO, 2014). However, irrigation may drastically impact the aquifers if used unreasonably (Scanlon *et al.*, 2012). In regions like western United States, China, and West, South, and Central Asia this practice is threatened to be partly abandoned due to a decreasing availability of freshwater for the populations (Elliott *et al*., 2014). Intense and unsustainable irrigation practices may also lead to soil salinization which is responsible for a great loss of agricultural land (Singh, 2015).

Another major agricultural practice to limit the impact of drought on crop production is the use of varieties that are more adapted to drought or more

efficient in water use. After years of selection and traditional breeding technologies, research is increasingly relying on more modern tools (e.g. molecular assisted breeding, genetic engineering) to produce drought-resistant crops (Rasheed *et al.*, 2017). The development of these technologies in developing countries is seen as crucial to improve food security in the context of future global changes (Tester and Langridge, 2010; Azadi *et al*., 2015). However, the access of developing countries to new germplasms is for now largely hindered by their high costs (Azadi *et al*., 2015).

In parallel to these technological advances and agro-environmental practices, an increasing attention has been paid in the last decade to the rhizobiome, in particular to those microorganisms that were reported to increase plant adaptation to drought or improve their water supply (Grover *et al*., 2011; Dodd and Ruiz-Lozano, 2012; Lau and Lennon, 2012; Nadeem *et al*., 2014; Colemann-Derr and Tringe, 2014). Among these are the arbuscular mycorrhizal (AM) fungi, which are ubiquitous soil microorganisms that establish mutualistic symbiosis with more than 70% of vascular plants (Brundrett and Tedersoo, 2018). These plant symbionts supply their hosts with nutrients (mainly Pi but also N − Smith and Read 2008; Chen *et al*., 2018) in exchange for carbon (Bago *et al*., 2003) and lipids (Keymer *et al*., 2017). Particularly, these microorganisms play a major role in Pi nutrition of the plants as they can supply the totality of this nutrient to the plant (Smith *et al.*, 2003) and can access to Pi beyond the depletion zones of roots. Interestingly, these microorganisms have been reported to improve growth, water contents and phosphorus nutrition in plants when compared to non-colonized plants under drought conditions (see reviews by Augé, 2001; Latef *et al.*, 2016; Plouznikoff *et al*., 2016). Mechanisms cited include increased water supply, through improved soil water retention (Augé, 2001; Wu *et al*., 2008) or direct transport via extraradical hyphae (Augé, 2003), and improved tolerance of plants tissues to dehydration through increased antioxidant activity (Smith and Read, 2008).

However, despite being suitable candidates to maintain crop production in conditions of water shortage (Jayne and Quigley, 2014) they have not yet been considered in crop breeding programs (Jacott *et al.*, 2017) and their use in agricultural practices is relatively limited. Indeed, research on the AM fungi still need to be developed especially regarding trivial features such as the resistance/tolerance of AM fungi to water shortage. Indeed, several studies have reported that inoculation with certain AM fungal strains could give a

higher benefit to the plant in condition of drought. For instance, inoculation with AM fungal strains isolated from dry environments and supposedly more drought tolerant were found to develop more extraradical hyphae and better improve water and N contents in plants under drought conditions when compared with other strains (Marulanda *et al*., 2003; Symanczik *et al*., 2018). Augé (2003) found that a higher soil hyphal colonization under drought conditions was correlated with an increased benefit for the plant in terms of water content. Studying the effects of decreasing water availability on the extraradical development and its functions (e.g. water and nutrient uptake and transport from soil to plant) under *in vitro* conditions could thus help us to select strains that have higher capacities to improve plant adaptation to drought *in vivo*. Investigating other fungal traits such as spore germination and spore production could also help us select strains that would colonize plants in dry environments more efficiently.

However, these knowledges are not sufficient to predict if the plant will benefit from its association with the fungus. Thorough investigations are needed to better understand the mechanisms by which AM fungi can improve plant adaptation to drought, especially during periods of drought recovery. In the environment, crops are often subjected to periods of water shortages of variable durations alternating with periods of precipitations during which crops can "recover". Drought recovery has been defined as "*A plant's capability to resume growth and gain yield after exposure to severe drought stress which causes a complete loss of turgor pressure and leaf dehydration"* (Luo, 2010). Recent studies in plant physiology have emphasized the importance of these recovery periods in the adaptation of the plants to drought (Anyia and Herzog, 2004; Hayano-Kanashiro *et al*., 2009; Chen *et al*., 2016; Rivas *et al*., 2016). Meanwhile, the role of AM fungi in plant recovery has been poorly investigated. Studies reported that mycorrhizal plants that were watered had improved water status (Levy and Krikun, 1980; Subramanian and Charest, 1997; Goicoechea *et al*., 2004), P contents (Subramanian and Charest, 1997; Bryla and Duniway, 1997) and increased leaf gas exchange parameters (Levy and Krikun, 1980; Ruiz-Lozano *et al*., 1995) when compared with non-mycorrhizal ones. These improvements can be explained by a higher water and nutrient supply to the plants due to the presence of extraradical hyphae in the substrate. Beyond these mechanisms, it is still not clear whether AM fungi can also benefit plants during recovery through other effects (e.g. metabolic changes in plants, induction of plant Pi transporters). Experiments where the volume of soil limits the contribution of the

extraradical hyphae could help us to determine if Pi uptake, water content and gas leaf exchanges are still improved under these conditions and therefore could highlight possible other mechanisms of action of the AM fungus.

In this thesis, we aimed at investigating the impact of decreasing water availability on the AM fungi and the role of these root symbionts in plant adaptation to drought. To that end, two steps were considered. The first aimed at evaluating the impact of decreasing water availability on the life cycle and Pi uptake and transport capacities of AM fungi and the second at evaluating the effects of AM fungi on plant recovery from drought via their impact on Pi mobilization and leaf gas exchange parameters.

Outline of the Thesis

Within this thesis, different experiments were conducted to address two main objectives:

1. Evaluate the impact of decreasing water availability on life cycle and Pi uptake and transport capacities of AM fungi
2. Evaluate the effects of AM fungi on plant recovery from drought via their impact on Pi mobilization and leaf gas exchange parameters

The successive steps implemented to address these two objectives are schematized in Figure 1.

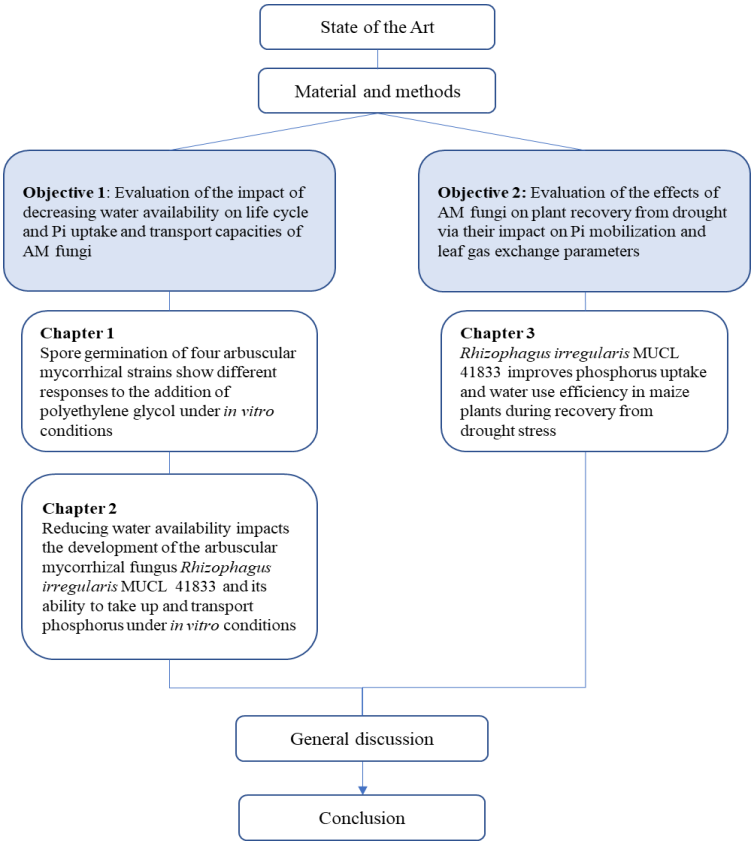

Figure 1 Outline of the thesis

In the **Introduction** section, we presented the subject and the general objectives of the thesis.

In the **State of the Art** section, the different aspects of the thesis were reviewed. In the first part, we revised the significance of drought and its impact on crop production. In the second part, we described the mechanisms of adaptation of plants to drought. In the third part, we presented the AM fungal symbiosis and in the fourth part we reviewed its role in increasing the adaptation of plants to drought. Finally, in the fifth part we detailed the impact of drought on AM fungal development and physiology as well as on AM fungal communities.

In the **Materials and Methods** section we described the biological material and detailed the principal methods and techniques used in the different experiments of the thesis.

The **first and second objective of the thesis** were addressed in **Chapters 1 and 2, and in Chapter 3** of the **Research Results** section, respectively.

The objective of **Chapter 1** was to investigate the impact of decreasing water availability on germination potential and germ tube growth of various AM fungal strains. To that aim, we first tested the impact of increasing concentration (50, 100, 250 and 400 g L^{-1}) of polyethylene glycol of molecular weight 8000 g mol^{-1} (hereafter cited as PEG) on the water potential and polymerization of the MSR medium. We then tested and compared the impact of 25 and 50 g L^{-1} of PEG added to the MSR medium on spore germination and germ tube length of four different AM fungal strains (*Rhizophagus irregularis* MUCL 41833, *Rhizophagus intraradices* MUCL 49410, *Rhizophagus sp.* MUCL 43204 and *Rhizophagus clarus* MUCL 46238).

The objective of **Chapter 2** was to study the effects of a decrease in water availability on the life cycle (spore germination and extraradical mycelium development) and Pi uptake and transport capacity of the model AM fungus *Rhizophagus irregularis* MUCL 41833. The AM fungus was grown in association with *Medicago truncatula* in bi-compartmented Petri plates, separating a root compartment (RC) containing the plant and fungus from a hyphal compartment (HC) in which only the fungus was allowed to grow. The impact of a decreasing water availability in the HC (induced by

PEG at 10, 25 and 50 g L^{-1} MSR medium) on spores number and hyphal length of the extraradical mycelium was evaluated after five weeks of growth, while the Pi uptake by the extraradical mycelium was measured indirectly by monitoring Pi depletion in the HC during 24 h.

The objective of **Chapter 3** was to investigate the role of the AM fungus *R. irregularis* MUCL 41838 on plant Pi uptake and leaf gas exchange parameters during recovery from drought. Maize plants were associated or not with the AM fungus and grown in a semi-hydroponic cultivation system for three weeks under three different water regimes (two of which corresponded to moderate and severe drought). A period of recovery was induced by circulating a Hoagland solution (modified with low concentrations of Pi) during 42 h. The dynamics of Pi uptake in the plant roots was assessed indirectly from the Pi concentration remaining at 9, 21 and 42 h in the Hoagland low-Pi solution. The leaf gas exchange parameters (stomatal conductance, transpiration and CO_2 assimilation rate) were measured at time 0 and at time 42 h of recovery.

In the **General Discussion** section, the major findings of the thesis were summarized and discussed.

Finally, in the **Conclusion and Perspectives** section, the two objectives of the thesis were reviewed and commented. Perspectives and future developments to deepen knowledge on the objectives outlined above were presented.

State of the Art

1. Drought and crop production

1.1. Role of climate change

Since the industrial revolution of the late 18th century, the global temperature of the earth surface has been increased by an average of 0.8°C (IPCC, 2014), marking an unprecedented rise of temperatures since the Holocene (Marcott *et al*., 2013). This increase is mostly attributed to the release of anthropogenic greenhouse gases (i.e. carbon dioxide, methane and nitrous dioxide) in the atmosphere (Crowley, 2000) mainly produced by fossil fuel extraction, land use changes and agriculture (IPCC, 2014).

The International Panel of Climate Change (IPCC) released its first report in 1992, describing several CO_2 emissions scenarios and their consequences on climate evolution. The latest report (IPCC, 2014) suggested that by the end of the 21st century, the cumulative greenhouse gas (GHG) emissions could result in a 2°C increase of global temperature in the most favorable scenario and in a 4°C increase in the worst-case scenario. These fast climate shifts will have serious impacts on biodiversity, ecosystems and habitats of diverse species (see review by Pecl *et al*., 2017) and will also threaten human populations who rely on their environment for food and services (Cardinale *et al*., 2012).

The increase in global temperature is correlated with a progressive increase in dry land areas (Huang *et al*., 2016 − see Figure 2), a tendency that is believed to intensify in the next 100 years (Dai, 2013; Huang *et al*., 2016), with continental parts of Africa, Eurasia and northwest America being the most impacted regions (Huang *et al*., 2016 − see Figure 3). Extreme climatic events like floods and droughts are expected to be more intense and frequent in many regions in Europe (Dankers and Feyen, 2008), California (Dettinger, 2011) and South Asia (Mirza, 2011) with major impacts on crop production (Huang *et al*., 2016).

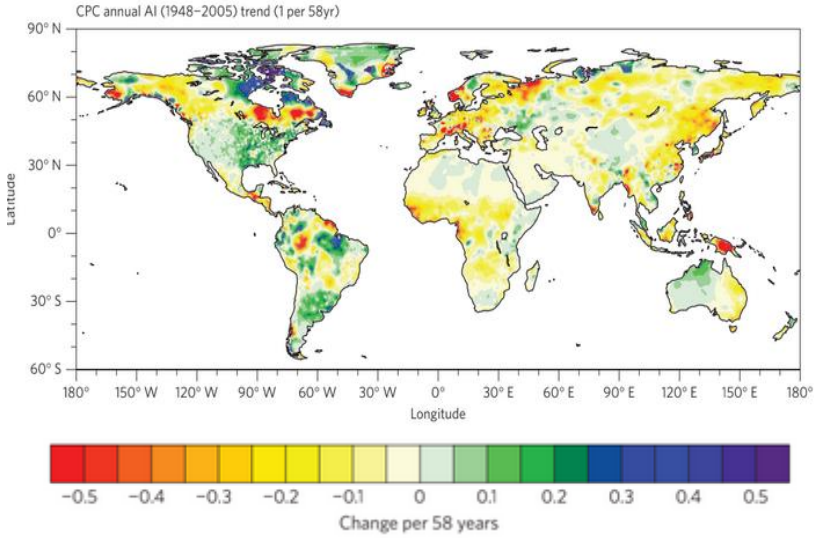

Figure 2 "*Global distribution of the linear trend of aridity index (i.e. the ratio of total annual precipitation to potential evapotranspiration) for 1948-2005*". (Huang *et al*., 2016). A decrease of the aridity index indicates a change towards a dryer climate. Typically, drylands are defined as regions with AI < 0.65 and are further divided into subtypes of hyper-arid (AI < 0.05), arid (0.05 ≤AI < 0.2), semiarid (0.2 ≤ AI < 0.5) and dry subhumid (0.5 ≤ AI < 0.65) regions (Huang *et al*., 2016).

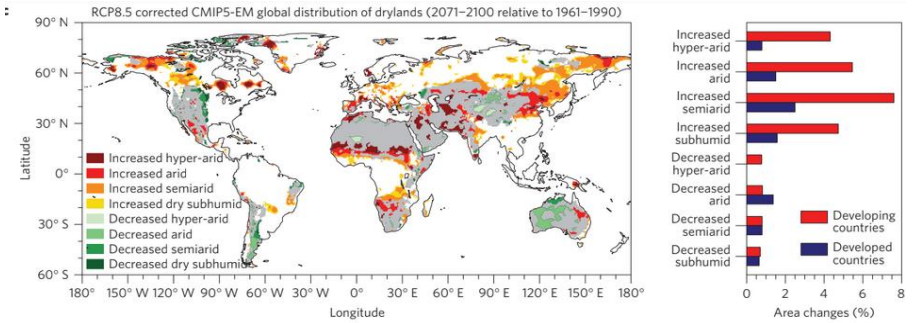

Figure 3 "*Global distribution of future changes in the dryland subtypes for the period 2071-2100 relative to the baseline period (1961-1990)*" (Huang *et al*., 2016). Projections of subtype changes are based on the Fifth Coupled Model Intercomparison Project (CMIP5-EM) bias-corrected with historical data.

1.2. Drought and drought index

According to Wilhite and Glantz (1985), the term “drought” may have different meanings. Among them, “meteorological drought” can be defined as a lack of precipitation or soil moisture relative to a geographic location or a defined period. To measure drought severity, one of the most used index is the Palmer Drought Severity Index (PDSI – Dai, 2011). Briefly, this index measures the difference between the cumulative precipitation observed and the computed precipitation requirements for a given location. For agronomists, which are monitoring crop production, drought definition will often be relative to the water needs during the different stages of growth of crop and be qualified as “agricultural drought”. The Crop Moisture Index (CMI) which was adapted from the PDSI, was thus designed to reflect the abnormal evapotranspiration for a given crop and location (Palmer, 1968).

1.3. Impact of drought on crop production

Drought is a major constraint for crop production. Based on climatic simulations, Rosenzweig *et al*. (2014) and Challinor *et al*. (2014) have predicted a significant decrease in yield of major crops (maize, wheat, rice and soy) especially in tropical areas by the first half of the century. More specifically, the risk of yield losses directly linked to drought episodes is projected to double by 2100 with the highest risks for the African continent (Li *et al*., 2009). In other models, where drought and adaptive capacities of countries were considered, it was estimated that wheat yields will suffer high losses in southeastern USA, South America, northeastern Mediterranean and central Asia, while maize yields will be mostly impacted in South America, northeastern Mediterranean and parts of southern Africa (Fraser *et al*., 2013).

In conclusion, drought issues will represent an increasing threat for crop production in the future. At the same time, the steady increase of the global population, which is estimated to reach 9.7 billion inhabitants by 2050 (UN, 2019) will require more than doubling crop yields to answer food demand (Tilman *et al*., 2011). To ensure sufficient crop production in the future, especially under water limiting conditions, agricultural practices adapted to the region and climate and integrating efficient water management strategies must be developed.

1.4. Role of agriculture in sustaining drought crisis

Agriculture accounts for 24% of the world GHG emissions (IPCC, 2014). About 40% of these emissions are caused by enteric fermentation in livestock while the rest is due to manure deposited on pasture (16%), synthetic fertilizer (12%), paddy rice cultivation (10%), manure management (7%), savannah burning (5%), and other causes (10%) (FAO, 2016). Agriculture thus play a key role in climate change and is partly responsible for a higher prevalence of droughts (Dai *et al*., 2013). But land use changes also affect precipitation leading to local drier areas without affecting global precipitation (Lawrence and Chase, 2010). For instance, in the Amazonian basin, massive deforestation has decreased the amount of evapotranspiration from the canopy resulting in the alteration of water cycling (Spera *et al*., 2016) and unprecedented droughts in the region (Bagley *et al*., 2014). To meet the future food demand, an increase in crop production through conversion of forest into arable land and massive utilization of chemical fertilizers, pesticides is not ecologically sustainable and can accelerate climate change (Tilman *et al*., 2011).

In order to increase crop yields without damaging current ecosystems, some scientists have suggested a transition towards a sustainable intensification of crop production without increasing the surface of areas cultivated (Burney *et al*., 2010; Tilman *et al*., 2011; Lamb *et al*., 2016). However, a change toward intensification requires technological innovations and economic resources that could leave small-scale farmers out of the agricultural transition (Robinson *et al*., 2015). A thorough thinking about how to implement a sustainable intensification, mostly in poor countries, should take place allowing to develop sustainable agricultural methods adapted to each region of the globe.

1.5. Irrigation to mitigate drought

Crop irrigation has been used for millennia to maintain high yields during drought episodes (Edens and Wilkinson, 1998). Over the world, it is responsible for 40% of total crop yield and is used in approximately 20% of the total arable land (FAO, 2014). However, irrigation could be strongly restricted in some areas by a lower freshwater availability under climate change (Eliott *et al*., 2014). The intensive use of this method combined with conventional agriculture can also lead to severe problems. For instance, crop

irrigation represents 70% of the total freshwater withdrawal over the world (FAO, 2018) and if improperly used can imbalance ecology of whole regions (Stockle, 2001). Consequences such as soil drying, decrease in river discharge and increase of temperature have been observed in the Aral-sea basin (Jarsjö *et al*., 2008; Jin *et al*., 2017). Irrigation plays an important role in soil and groundwater salinization (Singh, 2015) and it is estimated that approximately 20% of irrigated land is already damaged by salt (Squires and Glenn, 2011). Combined with high input agriculture, intensive irrigation can also cause soil erosion and soil leaching of nitrate, leading to a long-term nitrate pollution of aquifers as noticed in Spain (Villalba *et al*., 1995) or China (Zhang *et al*., 1996).

However, irrigation when used efficiently, is considered as a cornerstone to enhance crop production and food security in developing countries (FAO, 2003; Burney and Naylor, 2013). Efficient irrigation methods, the so-named "Precision irrigation", has been developed over the years to avoid water waste and leaching. Among them, "deficit irrigation", also named "supplemental irrigation", allows crops to receive supplementary water only during their most sensitive growth stages (Geerts and Raes, 2009), while in "drip irrigation", water loss through soil evaporation is decreased by applying water below the soil surface at slow flow rates (Camp, 1998). These techniques are, however, difficult to implement in poor countries as they require good technical support and financial investments (Wezel *et al*., 2014; Xie *et al*., 2014).

2. Plant adaptation to drought

2.1. Impact of drought on plant physiology

The lack of water affects the photosystem efficiency, decreasing the plant photosynthesis and biomass production (Turner and Begg, 1981). The decrease of water in plant tissues leads to leaves wilting and ultimately to their senescence, thereby decreasing the available foliar surface for photosynthesis (Da Silva *et al*., 2013). Drought has particularly negative effects on crop yields when it occurs at certain sensitive stages of plant growth. For instance, drought during the flowering stage has been shown to affect the number of grains in the ears of maize (e.g. Fischer *et al*., 1983), while during the filling stage it was found to affect the quality of the grains of wheat (Farooq *et al*., 2014) or

legumes (Farooq *et al*., 2017). Drought can also increase crop sensitivity to pests and diseases (Sinha *et al*., 2019).

2.2. Adaptation strategies to drought

Plants have several adaptation strategies to drought which have been confounded in varying terminologies but were unified in a recent work (Volaire, 2018). One of the main adaptation strategy authors refer to in the literature is drought resistance. Drought-resistant plants can be defined as *"... those which in the process of ontogenesis are able to adapt to the effect of drought and which can normally grow, develop and reproduce under drought conditions because of a number of properties acquired in the process of evolution under the influence of environmental conditions and natural selection*." (Henckel, 1964) or similarly as *"when aerial growth and development remain possible in response to what can be defined as a 'moderate drought', i.e. resulting in the maintenance of growth for the considered species and population, and the maintenance of productivity notably for crops (Sinclair, 2011)"* (Volaire, 2018). According to Levitt (1972), plants resist drought either by avoiding or by tolerating dehydration, which can be defined as "drought avoidance" and "drought tolerance" strategies, respectively. These two strategies recently renamed "dehydration avoidance" and "dehydration tolerance" according to Volaire, (2018) are the most studied in the literature but other strategies can coexist (e.g. cavitation tolerance −Volaire, 2018). In drought avoidance strategy, plants modify their physiology and metabolism in order to increase or conserve the water in their tissues (e.g. by decreasing water loss through smaller stomata aperture, osmotic adjustment, increasing water use efficiency or by developing a more extensive root system to improve the access to water). On the contrary, drought tolerant plants will cope with certain level of tissue dehydration and other effects of drought stress by adapting their physiology and metabolism (increased antioxidant activity, dormancy). Finally, other adaptation strategies have been described. For instance, plants can modify their life cycle in order to avoid periods of severe drought stress (drought escape or dehydration escape, according to Volaire, 2018) and depart themselves from their tissues (leaf abscission for instance) in order to favor other parts of the plant (drought abandon strategy) (Xu *et al*., 2010). Recently, the ability of plants to recover from drought (drought recovery) has been suggested as new adaptation strategy with physiological basis differing from those in drought resistance (Chen *et al*., 2016).

2.3. Improving drought adaptation through crop breeding

Selection of crops having drought adaptation traits can be achieved through different methods. In conventional breeding, crop varieties showing desired traits, often related to drought resistance strategies, are bred. Individuals with the desired phenotypes are selected visually or after laboratory tests among the following generations. This technique doesn't require heavy assistance but the obtaining of drought-resistant productive varieties can take 5 to 10 years (Collard and McKill, 2008). In molecular marker-assisted selection, genetic markers − such as quantitative trait loci (QTL) − associated with drought resistance are first identified in the genome of the plants. These markers are then used to select more drought-resistant crops in breeding programs (Collard and McKill, 2008). Finally, in genetic engineered crops, a gene conferring drought resistance is inserted in the plant genome using recombination techniques or more recently using CRISPR-Cas-9 gene editing technology (Scheben *et al*., 2017).

Breeders regularly release drought-resistant crops that show varying success within the field (Tolefson, 2011). Plant breeding, whatever the form it takes, is a necessary step towards improving drought resistance in plants while participating in the sustainability of agro-ecosystems (Cecarelli, 2014; Fita *et al*., 2015). Utilization of germplasms by small scale farmer can yet be hindered by their high price which is due to the lack of resources to fund seed amelioration programs in developing countries (Azadi *et al*., 2015).

2.4. Improving drought adaptation through beneficial microorganisms

A sustainable way to increase drought adaptation of crops is by potentiating the role played by soil beneficial microorganisms, in particular, plant growth-promoting rhizobacteria (PGPR) and AM fungi which are inhabitants of most if not all plants in natural habitats (Grover *et al*., 2011; Lau and Lennon, 2012; Nadeem *et al*., 2014; Miransari, 2014). Microorganisms have the ability to adapt and evolve rapidly towards environmental changes and therefore improve adaptation of plant to drought (Lau and Lennon, 2012). They play an important role in plant nutrition (mainly Pi for AM fungi) and can mitigate the impacts of drought on plants through various mechanisms (Grover *et al*., 2011). PGPR have been for instance shown to improve soil moisture content through higher soil

aggregation by excreting exopolysaccharide (EPS) (Roberson and Fireson, 1992 – see review by Kaushal and Wani, 2016) or to release various compounds such as phytohormones, 1-aminocyclopropane-1-carboxylate (ACC) deaminase or volatile compounds that improve morphology and physiology of roots (see review by Vurukonda *et al*., 2016). The various mechanisms by which AM fungi can benefit plants under drought conditions are presented in detail in section *4. Role of AM fungi in plant protection against drought stresses*.

3. The arbuscular mycorrhizal fungi

3.1. An obligate symbiont

Arbuscular mycorrhizal fungi are soil microorganisms colonizing the roots of approximately 71% of terrestrial vascular plants (Brundrett and Tedersoo, 2018). It is estimated that AM fungi evolved closely with the first plants, *circa* 450 million years ago (Brundrett, 2002), colonizing each terrestrial ecosystem (Brundrett and Tedersoo, 2018). This close evolution resulted in a mutualistic symbiosis, the AM fungi providing the plants with nutrients such as inorganic phosphorus (Pi – Plassard *et al.,* 2019) or nitrogen (Chen *et al*., 2018) in exchange for carbohydrates (Bago *et al*., 2003) and lipids (Jiang *et al*., 2017; Keymer *al.,* 2017).

The AM fungi are placed in the phylum Glomeromycota (Stürmer *et al*., 2018). Within this phylum, the Glomeraceae is the most important family in terms of number of species, followed by the Acaulosporaceae and Gigasporaceae (Stürmer *et al*., 2018 – Figure 4). The Glomeraceae are over-represented in austral/boreal and temperate biomes, while Acaulosporaceae, Gigasporaceae and Ambisporaceae are flourishing more in tropical regions (Stürmer *et al*., 2018).

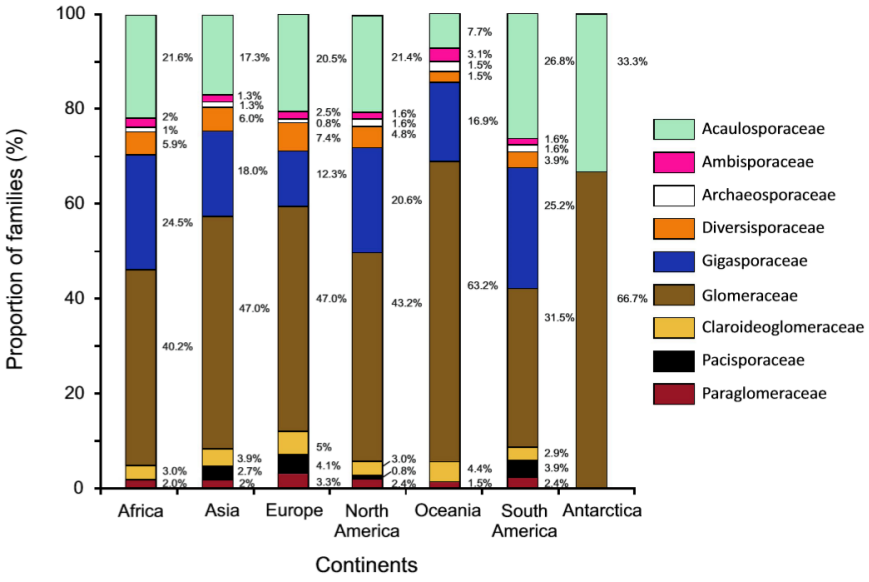

Figure 4 Proportion of AM fungal families in the biogeographical realms based on the number of species. Extracted from Stürmer *et al*. (2018).

3.2. Establishment of the symbiosis

The AM fungi develop coenocytic hyphae and their life cycle is characterized by an asymbiotic, presymbiotic and symbiotic phase.

During the asymbiotic phase, AM fungal propagules (i.e. spores, sporocarps, vesicles, colonized root pieces, and extraradical hyphae) are present in the substrate without exchanging any signals with a plant. These propagules have the ability to colonize roots but whereas hyphae are infective *per se*, spores or vesicles require germination for colonizing roots. Germination of vesicles and spores can occur without the presence of a host.

The pre-symbiotic phase of the fungus starts with the mutual recognition of the plant host and its fungal symbiont. Strigolactones (Akiyama *et al*., 2005) and cutin monomers (Gobatto *et al*., 2012) are released by the plant roots stimulating hyphal branching, whereas Myc-factors (lipochitoligosaccharides − Maillet *et al*., 2011; short chitin tetra- and pentamers − Genre *et al*., 2013) are released by the fungus, inducing a Ca^{2++} spiking signal (Sieberer *et al*., 2012) in the root cortical cells (see detailed explanation in Figure 5). This signal is associated with the activation of the

common symbiotic signaling pathway (CSSP) in plants (Camps *et al*., 2015) and the reprogramming of plant tissues (Dörmann *et al*., 2014). The contact between the root and fungal hyphae is preceded by the formation of a hyphopodium, i.e. an infection structure that will serve to enter the root epidermis. At the same time, the plant plasma membrane starts to invaginate and some cytoplasmic rearrangements occur forming the pre-penetration apparatus (PPA) which channels the progression of infective hyphae into the root cortex (Genre *et al*., 2005). The infective hyphae invaginates in a cell and ramifies to form "arbuscules" or "coils" (Figure 5) which constitute high surfaces of exchange between the plant and the fungus.

The beginning of the symbiotic phase is marked by the exchange of nutrients (essentially Pi) in exchange for carbon compounds through the peri-fungal membrane of the arbuscules or coils (Smith *et al*., 2018). The Pi transport seems to play a pivotal role in the establishment of AM fungal symbiosis as the expression of phosphate transporters is required for the development of arbuscules in roots (Javot *et al*., 2007). Further colonization of the root cortex follows from cell to cell, developing mainly hyphal "coils" and "arbuscular coils" (Paris-type colonization) or progress extracellularly forming mainly arbuscules (*Arum*-type colonization). A continuum between both types of colonization can also be observed (Dickson, 2004). With the exception of the Gigasporaceae family, most fungi also develop intercellular or intracellular thick-walled structures named vesicles that probably serve as storage structures but are also able to colonize new roots (Smith and Read, 2008). During this symbiotic phase, numerous other entry points appear on the surface of the roots increasing therefore the colonization surface of the roots. AM fungi finally develop external hyphae and produce spores, inside or outside of the root, that are able to colonize new hosts.

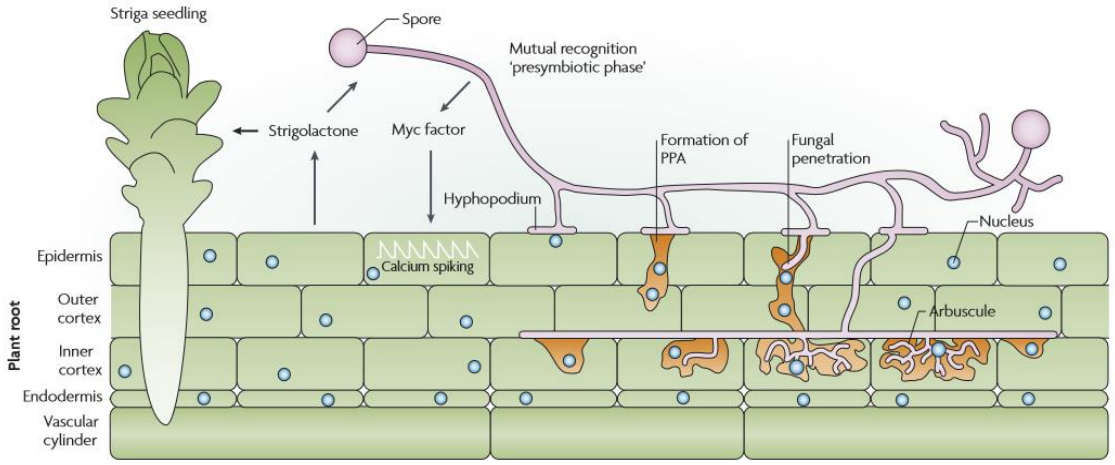

Figure 5 Recognition events and infection of a host by an AM fungus (in this case, *Arum*-type colonization). Signal molecules such as strigolactones are recognized by the AM fungal spores and stimulate germ tube branching. Meanwhile, AM fungi release Myc factors which trigger a calcium spiking in the root cortical cells leading to their reprogramming. Following contact between both symbionts, the AM hyphae forms a hyphopodium at the root epidermis surface. In the meantime, the cytoplasmic membrane of epidermal cells located under the hyphopodium invaginates, guided by the nucleus which migrates toward the inner cortex. This structure is induced in the next cells, therefore forming a channel to the inner cortex of the root called the pre-penetration apparatus (PPA). An infective hyphae originating from the hyphopodium is emitted and progresses through the PPA to reach the inner cortex. Once there, the infective hyphae enters the apoplasm, and grows longitudinally into the root cortex. Hyphae invaginate in the cell forming a specialized structure (here an arbuscule) and starts to exchange nutrients with the plant. New longitudinal hyphae are emitted and progress through the apoplasm to colonize adjacent cells (*Arum*-type colonization). Figure from the review by Parniske, (2008).

3.3. Role of AM fungi in plant nutrition

3.3.1. Indirect effects of AM fungi on plant nutrition

Few studies have pointed to the possible role of AM fungi in increasing the pool of available Pi and N in the soil by promoting mobilization of these elements from minerals and organic substrates (see reviews by Read and Perez-Moreno, 2003 and Neumann and Georges, 2009). Very recently, L.Zhang *et al*. (2018) demonstrated that AM fungi were able to induce phytate mineralization by phosphate solubilizing bacteria through the emission of a fructose-signal perceived by the bacteria.

3.3.2. Direct uptake and transfer of nutrient by the AM fungi

AM fungi play an important role in the direct uptake and transfer of nutrient to the plants in exchange of carbons (i.e. lipids and carbohydrates). A direct uptake and transfer of macronutrients (Pi − Cooper and Tinker, 1978; NH_4^+− Ames *et al*., 1983; SO_4^{2-} Cooper and Tinker, 1978) and of micronutrients (Cu − Li *et al*., 1991; Zn − Cooper and Tinker, 1978) to plants by extraradical hyphae has been demonstrated. Accumulation of other macronutrients such as Ca (Rhodes and Gerdemann, 1978) and K (Georges *et al*., 1992) have also been reported in AM-colonized plants as compared to non-AM colonized plants. The transfer of inorganic phosphorus (Pi) represents, however, the main nutrient transferred to plants (Smith and Read, 2004) as it was shown to contribute to the majority or the totality of the P accumulated in plants (Li *et al*., 1991; Smith and Read, 2004).

3.3.3. Importance of AM fungal Pi nutrition for crop production

Pi availability in soils is relatively low, especially in tropical and sub-tropical soils where low pH and high concentration of Al and Fe oxides induce a high fixation of this element (Fox and Searle, 1978). Application of phosphates (in the form of superphosphate or rock phosphate) is often necessary to ensure crop production (FAO, 2008). However, intensive application of phosphates is not sustainable on the long-term as it impacts the environment and the productivity of soils (Sharma *et al*., 2013). It could also become an issue in the future as the reserves of phosphate rock are depleting (Cordell *et al*., 2009). Moreover, important part of the phosphates applied into

acidic soil is directly fixed (Sharma *et al*., 2013). As discussed previously, mycorrhizal association allows plants to access to remote Pi pools, enhance the solubilization of Pi in soils in relations with phosphate solubilizing bacteria and promote plant growth especially under low Pi availability in soils (Smith and read, 2008). Recently, their role in supplying plant with Pi has also been shown to be essential to observe positive effects on crop yields due to increased CO_2 concentrations in the atmosphere (Watts-Williams *et al*., 2019).

3.3.4. Mechanisms of mycorrhizal Pi uptake

Mycorrhiza develop an extraradical network of fine diameter hyphae (≤ 10µm) that is able to access soil micropores inaccessible to roots and root hairs, and explore a volume of soil that extends far away from the Pi depletion zone of the roots (Sanders and Tinker, 1971; Rhodes and Gerdemann, 1975; Owusu-Bennoah and Wild, 1979). These hyphae actively take up Pi from the soil using high affinity phosphate transporters (Pt) coupled with proton pumps (see below for transporters). Pi is quickly accumulated in the vacuole compartment in the form of polyphosphate (PolyP – Ezawa *et al*., 2004) which represents >60% of total cellular Pi (Hijikata *et al*., 2010). These vacuoles are translocated from the extraradical to the intraradical hyphae via cytosolic flux (Olsson *et al*., 2002) or via microtubules (Uetake *et al*., 2002). The granules of PolyP are then hydrolyzed into inorganic orthophosphate and transferred from the arbuscules to the root cells via the apoplasm (Smith and Read, 2008) (Figure 6).

To achieve direct Pi uptake, translocation in the hyphae and transfer to the plant, a series of Pt have been identified. High affinity Pt were identified in extraradical hyphae of *Glomus versiforme*[1] (Harrison and Van Buuren, 1995), *Glomus intraradices*[2] (Maldonado-Mendoza *et al*., 2001), *Glomus mosseae*[3] (Benedetto *et al*., 2005) and *Gigaspora margarita* (Xie *et al*., 2016). However, putative low affinity transporters and other high affinity Pt

[1] *Diversispora epigaea* according to the updated classification of Schüssler *et al*. (2011).
[2] According to the updated classification of Schüsssler and Walker (2010) the genera changed to *Rhizophagus*. Information on the strain provided by the authors is yet not sufficient to tell which of *Rhizophagus irregularis* or *Rhizophagus intraradices* is the new corresponding nomenclature.
[3] *Funneliformis mosseae* according to the updated classification of Schüssler and Walker, (2010).

orthologs of *S. cerevisiae* might still be discovered (Ferrol *et al*., 2019; Plassard *et al*., 2019). As these transporters have also been identified in arbuscules, they probably also play a role in the regulation of Pi flow to the plant by transferring back Pi from the apoplasm. Numerous plant Pt were found overexpressed in the peri-arbuscular membranes of mycorrhizal plants (e.g. in *Lycopersicon esculentum*– Rosewarne *et al*., 1999; in cereals – Glassop *et al*., 2005; in *Glycine max* – Tamura *et al*., 2012). Other plant Pt were exclusively identified in cells containing arbuscules (e.g. *Solanum tuberosum* – Rausch *et al*., 2001; *Medicago truncatula* – Harrison *et al*., 2002; *Astragalus sinicus* – Xie *et al*., 2013).

3.3.5. Factors affecting mycorrhizal Pi uptake

Various factors can affect mycorrhizal Pi uptake. For instance, Pi nutrition by AM fungi seems to depend on the water fluxes in the soil-plant continuum (Cooper and Tinker, 1981; Kikuchi *et al*., 2016). Indeed, a lower PolyP translocation in the extraradical hyphae was induced by a decreased plant transpiration and associated with a knock-down of the expression of aquaporins in the fungus (Cooper and Tinker, 1981; Kikuchi *et al*., 2016). Also, it is now admitted that mycorrhizal Pi uptake pathway is strongly dependent on the Pi availability in the soil (Smith and Read, 2008). Fungal Pi transporters were for instance inhibited by high Pi concentrations in soils and by high plant P status (Maldonado-Mendoza *et al*., 2001).

In a few experiments conducted under *in vitro* conditions, various stresses were found to modify Pi uptake by the fungus. The short-term exposure to chromium increased Pi uptake by the extraradical hyphae (Gil-Cardeza *et al*., 2017), while polyaromatic hydrocarbons seemed to affect Pi transport from the extraradical hyphae to the intraradical hyphae (Calonne *et al*., 2014) without visible damages to the fungal structures.

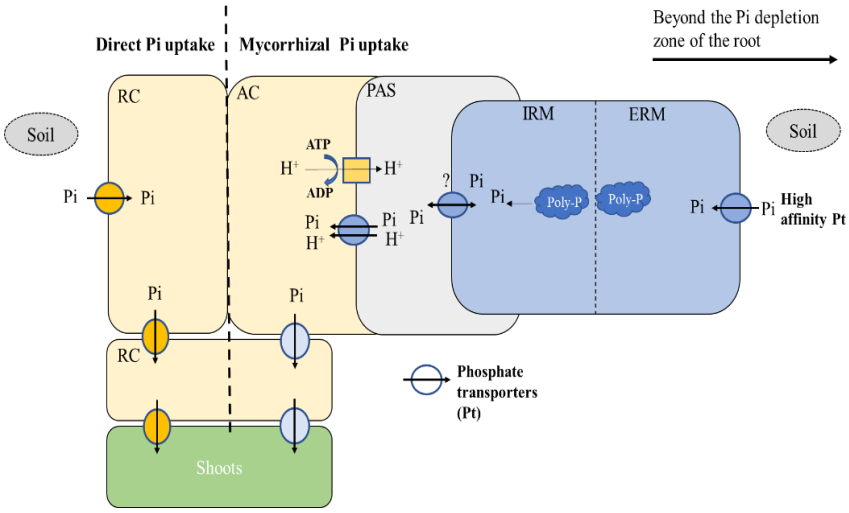

Figure 6 Phosphate transport in mycorrhizal plants. In the "direct uptake pathway" Pi is depleted in soil by direct uptake by plant roots phosphate transporter (Pt) and transported to the shoot. In the "mycorrhizal uptake pathway" the extraradical mycelium reaches soluble phosphate outside the Pi depletion zone created by high plant phosphate uptake concomitant with a low soil-based phosphate diffusion in the root growing zone. Pi is taken up by extraradical hyphae through high affinity Pt coupled with proton pumps and translocated from the extraradical mycelium (ERM) to the intraradical mycelium (IRM) in the form of PolyP. In the fungal cytoplasm, PolyP is hydrolyzed into Pi which is released into the apoplasm or periarbuscular space (PAS), by a still unknown mechanism. Pi is then imported into plant cells by arbuscular mycorrhizal -inducible, phosphate Pt localized in the plant-periarbuscular membrane. This transfer is suggested to be driven by an H^+ energy gradient produced by an H^+-ATPase (Krajinski et al., 2014; Wang et al., 2014). The expression of fungal Pt genes in the intraradical mycelium suggests a possible role in Pi reabsorption from the PAS (Pt transporter with double arrow) (Benedetto et al., 2005; Balestrini et al., 2007; Fiorilli et al., 2013; Xie *et al*., 2016). Figure based on the review of Lanfranco *et al*. (2018) and Calabrese *et al.* (2019). RC: (plant) Root cell; AC: Arbusculated plant cell.

3.4. Role of AM fungi in plant protection against biotic and abiotic stresses

3.4.1. Plant protection against biotic stresses

AM fungi have been reported to play key roles in the protection of plants against pests (e.g. nematodes, insects) and diseases (e.g. fungal and bacterial pathogens) (Whipps, 2004). This has been shown against below-ground (Azcon-Aguilar and Barea, 1997) as well as above-ground (Pozo and Azcon-Aguilar, 2007) pathogens and several mechanisms have been suggested: (i) competition with pathogens or inhibition of their growth within the soil, (ii) fortification of host plants or modification of their morphology, (iii) induction of plant defense system, (iv) promotion of an antagonistic microflora (Whipps, 2004). The efficiency against plant fungal pathogens is, however, dependent on the fungal isolate, the plant species and the environmental factors as revealed by a meta-analysis (Veresoglou and Rillig, 2012).

3.4.2. Plant protection against abiotic stresses

AM fungi have been reported to help plants to withstand abiotic stresses such as drought, salinity, high temperatures, elevated concentrations of trace elements such as Cr, Cd, or contaminations of soils with persistent organic pollutants (Lenoir *et al*., 2016; Plouznikoff *et al*., 2016). In the section below we will exclusively present several effects of AM fungi in relation with their beneficial role under drought stress. Some of these effects are involved in mechanisms of drought resistance, i.e. drought tolerance and avoidance strategies (see Figure 7 − State of the Art - section 2.).

4. Role of AM fungi in plant protection against drought stress

4.1. Direct water supply by the AM fungi

A number of studies have suggested that AM fungi are able to transfer water directly to the plants. Using deuterium, Egerton-Warburton *et al*. (2007) showed for instance, that water couldbe transferred from one plant to another through a common mycorrhizal network. In a split-pot experiment, Ruth *et al*.

(2011) were able to estimate that extraradical hyphae were responsible for 20% of the total water uptake in plants. F. Zhang *et al*. (2018) also suggested that extraradical hyphae absorb proportionally more water during drought than under well-watered conditions. This result can be paralleled with an increased expression of aquaporin genes *Gint*AQP1 and *Gint*AQP2 in the extraradical hyphae of *Rhizophagus irregularis* DAOM 197198 when submitted to osmotic stress induced by Polyethylene glycol (PEG) of molecular weight 6000 (Li *et al*., 2013). Other aquaporin genes identified in *Rhizophagus clarus* and mostly expressed in intraradical hyphae, were found to be involved in water flow and PolyP translocation along the hyphae (Kikuchi *et al*., 2016). Similarly to Pi nutrition, extraradical hyphae have access to higher amounts of water as they have larger access to soil pores (Smith and Read, 2008).

4.2. Effects of AM fungi on soil water retention

AM fungi release in soils various types of glycoproteins defined as "glomalins" or "glomalin related soil proteins" (Wright and Upadhyaya, 1996; Rillig *et al*., 2001, 2004). It is believed that these molecules are released through hyphae degradation rather than excretion by the fungus (Driver *et al*., 2005). These proteins play an important role in soil aggregation together with the tridimensional hyphal network (Deggens, 1997; Allen, 2007). Both features increase soil water holding capacity which results in an increased availability of water for plants (Augé, 2001; Wu *et al*., 2008).

4.3. Effects of AM fungi on root architecture under drought-stress conditions

In dry soils, plants can increase root length and density in order to capture water in deeper layer of soil and smaller cavities (Comas *et al*., 2013). While AM fungi have been shown to enhance root architecture under well-watered conditions in several plants species (see review by Atkinson *et al.*, 1994), so far, their benefits under conditions of drought stress were only studied and demonstrated in citrus plants. AM-fungal colonization has been found to improve root length, density and root hair diameter in *Poncirus trifoliata* plants under well-watered and drought stress conditions, concomitantly with larger concentrations of Indole Acetic Acid (IAA − Wu *et al*., 2016; Zou *et al*., 2017; Liu *et al*., 2018), Methyl Jasmonate (MeJa) (Wu *et al*., 2016; Zou *et al.*, 2017) in roots. In AM-colonized *Citrus tangerina*, these parameters were also altered under well-watered conditions and

associated with higher concentrations of Putrecine, a polyamine known to promote plant growth (Wu *et al*., 2012). Finally, analysis of gene expression patterns revealed that expression of certain genes responsible for IAA synthesis in roots were modulated in function of soil moisture status in mycorrhizal plants (Liu *et al*., 2018).

4.4. Effect of AM fungi on plant P nutrition under drought-stress conditions

AM fungi help plants to face nutrient deficiencies, especially P (see previous section *3.3. Role of AM fungi in plant nutrition*) that can be consecutive to drought stress. It seems also that Pi supply plays an important role in the alleviation of symptoms of drought. For instance, Pi spraying on bean leaves has been reported to increase photosynthetic activity and intrinsic water efficiency during mild drought (dos Santos *et al*., 2004), while higher Pi supply allowed the development of a deeper root systems in pea (Jin *et al*., 2015) and of shoot biomass in wheat (Rodriguez *et al*., 1996). Therefore, it is expected that AM fungi have a positive effect on drought stressed plants through an improved Pi nutrition. Indeed, an increased Pi nutrition has been frequently associated with an increase in shoot and root growth, improved water relations or osmotic adjustment under drought conditions (Augé, 2001; Plouznikoff *et al*., 2016). However, in most of the cases, it is difficult to know to which extent the improved Pi nutrition is responsible for the observed beneficial effects. Those beneficial effects could be due to an increase of water supply or of other nutrients for instance.

4.5. Effects of AM fungi on oxidative stress in plant tissues under drought-stress conditions

Integrity of plant tissues can be threatened by the toxic release of reactive oxygen species (ROS) such as hydrogen peroxide H_2O_2, superoxides ($^{\bullet}O_2$), singlet oxygen (O_2^{-}) and hydroxyl (OH^{-1}) during drought (Cruz de Carvalho, 2008). In numerous studies, AM fungi have been reported to enhance the production of plant ROS scavenging enzymes and to decrease the oxidative stress in drought stressed plants (Smith and Read, 2008; Wu *et al*., 2013; Wu and Zou, 2017). For instance, AM fungi have been reported to increase the production of superoxide dismutase (Ruiz-Lozano *et al*., 1996; Wu and Zhou 2009), catalase and peroxidase (Zhu *et al*., 2011), guaiacol peroxidase (Wu and Zhou, 2009) as well as ascorbate (Subramanian *et al*.,

2006; Wu and Zou 2009; Ruíz-Sánchez *et al*., 2011) and Glutathione (Wu and Zou, 2009; Ruíz-Sánchez *et al*., 2011) in drought stressed plants. In mycorrhizal *Poncirus trifoliata*, higher levels of ROS scavenging enzymes were concomitant with an increased burst of Ca^{2+} signal (Zou *et al*., 2015), of its receptor calmodulin (Huang *et al*., 2014) as well as with an increased H_20_2 efflux (Zou *et al*., 2015; Huang *et al*., 2017) and decreased concentrations of malondialdehyde (MDA, i.e. a marker of lipid peroxidation) in roots (Huang *et al*., 2017).

4.6. Effect of AM fungi on osmolyte concentrations in plant tissues during drought stress

Osmotic adjustment is an important mechanism for drought adaptation in plants. It has been reported that AM-colonized plants accumulated more osmolytes under drought conditions as compared to non-mycorrhizal plants. In AM-colonized citrus plants, increasing contents of K^+, Ca^{2+}, Mg^{2+} (Wu *et al*., 2006) and of fructose and sucrose (Wu *et al*., 2017) were found in leaves and roots while glucose was found increased in roots (Wu *et al*., 2017). Proline, one of the main actor of osmotic adjustment has also been found increased in leaves of AM-colonized plants of *Lactuca sativa* (Ruiz-Lozano *et al*., 1996), *Macadamia tetraphylla* (Yooyongwech *et al*., 2013), *Zenia insignis* (Zhang *et al*., 2019), *Juglans regia* (Behrooz *et al*., 2019) and *Zea mays* (Zhu *et al*., 2011). However, in other studies improved resistance to drought was associated with a decrease of proline content in shoots of AM-colonized plants of *Erythrina variegata* (Manoharan *et al*., 2010), in roots of AM-colonized plants of *Knautia arvensis* (Doubková *et al*., 2013) and *Zea mays* (Zhu *et al*., 2011; Armada *et al*., 2015), in leaves of *Zea mays* (Abdelmoneim *et al*., 2014; Armada *et al*., 2015) and *Poncirus trifoliata* (Wu *et al*., 2017), and in roots and shoots of *Poncirus trifoliata* (Zou *et al*., 2013). In *Poncirus trifoliata* this decrease was associated with a higher activity of enzymes responsible for the proline degradation and a lower activity of those responsible for proline biosynthesis (Zou *et al*., 2013; Wu *et al*., 2017). Both increase and decrease of proline can sign a higher resistance to drought induced by the mycorrhiza. Indeed, the accumulation of proline could reflect a higher osmoregulation in the plant while a decrease could indirectly result from a greater water status in AM-colonized plants.

4.7. Effects of AM fungi on water relations in plant tissues under drought stress

Water status of plants cover a number of parameters that reflect movement and storage of water in the different parts of the plant. Many studies have contributed to demonstrate the role of AM fungi on water status describing for instance the impact of the fungus on water potential and water content in leaves, on root hydraulic conductivity in stems, etc.

4.7.1. Hydraulic conductivity

The role of AM fungi in improving root hydraulic conductivity (L) is known for a long time (Graham *et al*., 1984). In the last decades, the mechanism of regulation of L by AM fungi has been more extensively described, especially regarding the cell-to cell water transport. Increased L was indeed associated with higher expression of plant plasma membrane aquaporins (PIP) in *Phaseolus vulgaris* (Aroca *et al*., 2007) and *Zea mays* (Barzana *et al*., 2014; Quiroga *et al*., 2017) which are thought to be induced by absissic acid (ABA − Aroca *et al*., 2007) and MeJa (Sanchez-Romera, 2016). Interestingly, aquaporin expression was differently modulated by AM fungi among drought tolerant and drought-resistant maize plants (Quiroga *et al*., 2017). In maize, increased L has also been associated with an increased apoplastic flow of water (Barzana *et al.,* 2012). But this type of water flow was found to be regulated by AM fungi and salicylic acid (SA) as application of the hormone only increased this water uptake pathway in non-mycorrhized plants (Quiroga *et al*., 2018). Finally, an improved L could also originate from an increased water supply through AM fungal extraradical hyphae, from a more efficient root architecture or a better conservation of water in tissues of mycorrhizal plants (Augé, 2000).

4.7.2 Tissue hydration: water potentials and relative water content.

Measurement of water potential and water contents in plant tissue is one of the most direct way to measure the impact of drought on tissue hydration. In AM-colonized plants, both parameters were mainly found increased under drought stress conditions but not under well-watered conditions (Augé, 2001). During recovery, mycorrhizal maize plants were also found to rehydrate faster (Subramanian *et al*., 1997; Zhao *et al*., 2015). Leaf relative water contents and

water potential were found increased at the same time as stomatal conductance (Dell'amico *et al*., 2002; Augé *et al*., 2015), transpiration (Augé, 2001) and photosynthesis (Dell'amico *et al*., 2002; Wu and Xia, 2006; Zhu *et al*., 2012).

4.8. Effects of AM fungi on leaf gas exchange parameters and instantaneous water use efficiency

Based on the review by Augé (2001), stomatal conductance (g_s) and transpiration (E) were often increased in AM-colonized plants when compared to non-colonized ones, whatever the water regime. Moreover, in a meta-analysis of the literature, stomatal conductivity was found to be 24% higher in mycorrhizal plants whatever the moisture conditions and this effect was more important under moderate drought than under well-watered conditions (Augé *et al*., 2015). These results could reflect lower drought stress in AM-colonized plants possibly through an increased water supply or a more efficient use of water in plant tissues. However, as these parameters were also increased under well-watered conditions, direct modulation through hormone signals for instance is not to be overlooked (Lozano and Aroca, 2010).

Water use efficiency (WUE) is defined as the ratio of assimilated CO_2 by a crop and its water loss (Bacon, 2007). Depending on the context, different types of WUE can be measured. The biomass WUE (WUE_b) can be measured from the gain in biomass and the water consumption of a crop over a long period (Ruiz-Lozano and Aroca, 2010). The instantaneous water use efficiency (WUE_i) is the ratio of CO_2 assimilation rate (A) over E while the intrinsic water use efficiency is the ratio of A over g_s (Bacon, 2007).

AM fungi were found to increase water use efficiency (WUE_i and WUE_b) of plants under well-watered and drought-stressed conditions (see review by Augé, 2001). Most of these studies measured a WUE_b which was determined through different ways. For instance, as the ratio of fresh weight (Boyer *et al*., 2015) or of dry weight (Zhao *et al*., 2015) over the consumption of water or as the ratio of yield over seasonal evapotranspiration (Bolandnazar, 2007). Comparatively, fewer studies measured WUE_i. An increase of WUE_i was reported for instance in AM-colonized plants of *Lactuca sativa* (Ruiz-Lozano *et al*., 1995), *Boswellia papyrifera* (Birhane *et al*., 2012), *Zea mays* (Zhu *et al*., 2012) and *Hordeum vulgare* L. cv. Pallas (Li *et al*., 2014) under drought stress and in AM-colonized plants of *Lactuca sativa* (Ruiz-Lozano *et al*., 1995) during recovery from drought. In these cases, as in others (see review by Ruiz-Lozano and Aroca, 2010), the increase of WUE_i was concomitant with an increase of CO_2 assimilation rate and of E and g_s which indicates that

WUE_i was increased through a higher photosynthesis efficiency. To our knowledge, an increased WUE_i in mycorrhizal plants was not associated with a decrease in g_s or E. It has been shown that the effects of AM fungi on WUE_i was dependent on the AM fungal species considered (Ruiz-Lozano *et al.*, 1995).

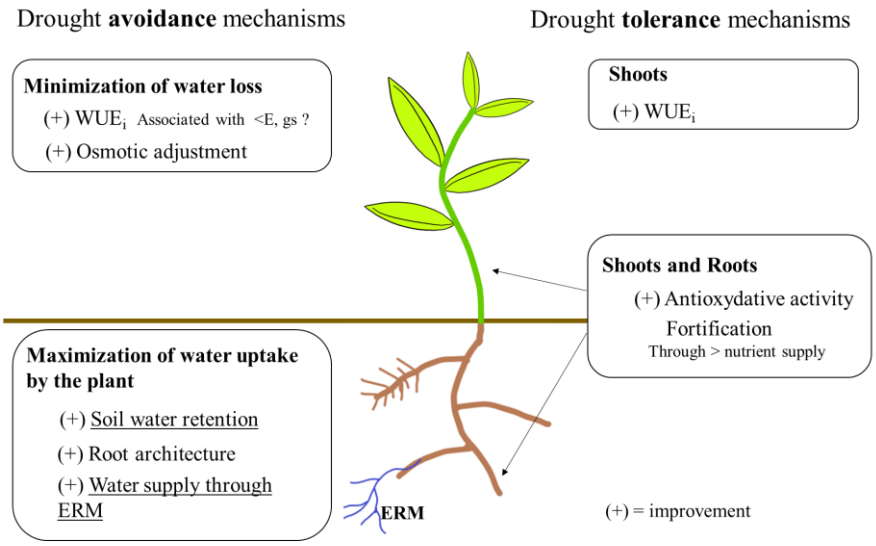

Figure 7 Improvement of drought resistance in plants (here through drought avoidance and drought tolerance strategies) mediated by AM fungi. AM fungi promote both strategies of adaptation in plant via different mechanisms (those underlined are related directly to the AM fungi). Physiological mechanisms in drought avoidance strategy include water uptake maximization and water loss minimization mainly in shoots. Instantaneous water use efficiency (WUE_i) is the ratio between CO_2 assimilation rate and transpiration (A/E). An improvement of this ratio is driven by an increased efficiency of the photosynthesis (Ruiz-lozano and Aroca, 2010). The improvement of WUE_i was usually associated with an increase of both A and E or only of A but never with a decrease of E or g_s. It is thus not clear whether its improvement takes part in an avoidance or tolerance strategy. ERM= extraradical mycelium.

5. Impact of drought on AM fungi and their community structure

5.1. Impact of drought on AM fungal structures

The roles of AM fungi in increasing the resistance of plants against drought stresses have been highlighted above. Drought conditions may also impact the AM fungi as reviewed by Augé (2001). This author indicated that drought decreased mycorrhizal colonization in half of the studies considered and increased it mostly in field studies where longer periods of drought were surveyed. Its hypothesis is that longer periods of drought have a higher impact on Pi levels in soils and Pi taken up by the plant which in turns stimulate root colonization. In contrast, only a restricted number of papers focused on spore germination, hyphal growth and ramifications, sporulation or other parameters (Augé, 2001). More information on these parameters (such as the germination potential of AM fungal strains) could help to improve inoculation strategies by selecting more drought adapted strains. Moreover, some of these parameters may play an important role in drought alleviation in plants. For instance, Augé *et al*. (2003), showed that a higher leaf water potential of bean plants at the end of a dry period was more correlated with soil hyphal density than with root hyphal colonization, root density, soil aggregation, soil glomalin concentration, leaf phosphorus concentration or leaf osmotic potential. According to this study, soil hyphal density was also associated with lower soil hydration which suggested that soil hyphae may help plants to extract more water from dry soils. An indication of the effect of drought on these parameters could also lead to a better understanding of the functional role of each specie in AM fungal communities.

We tried to summarize the literature on the effect of drought or decreased water availability on spore germination (Table 1). To our knowledge, germination potential of AM fungi spores was not investigated anymore after the year 1991, therefore Table 1 reflects the literature cited in Augé (2001). Most of these studies reported a decrease in spore germination under water potentials below field capacity (−0.033 MPa) with the exception of *Gi. margarita* (Augé, 2001). However, high moisture levels also impact spore germination which seems to indicate that different strains have variable optimal water potential for spore germination (Sylvia and Schenck, 1983 − Table 1).

Based on the review by Augé (2001) and Table 1, spore production/sporulation and hyphal length were in most cases affected by soil dryness but in a number of studies these parameters were also unchanged (e.g. Sieverding and Toro, 1988; Wu *et al*., 2008; Silva *et al*., 2015) or even increased (e.g. Bildusas *et al*., 1986; Davies *et al*., 1992; Bethlenfalway *et al*., 1988; Khalvati *et al*., 2005; Wu *et al*., 2008). These contrasting effects could be explained by different adaptations of AM fungi species to a certain water potential/drought level but also by the host specificity and the duration of drought stress. As for sporulation and hyphal length, AM fungal identity, host specificity and duration of drought can explain this variation. Root colonization variability is strongly dependent on plant fitness and in some cases a decreased root biomass due to drought can result in a higher frequency of AM fungi colonization (Smith and Read, 2008).

Table 1 Impact of drought and decreased water availability on AM parameters under various experimental conditions. Corresponding water potential (ψ) in growth substrate or medium is indicated when available. AM fungal strain were named accordingly to the nomenclature of Stockinger *et al*. (2009), Schüssler and Walker (2010) and Schüssler *et al*. (2011).

Stress	Experimental conditions	AM fungal species	Effect	References
Spore germination				
Moisture level < 40% of field capacity (FC)	Pot culture	*Diversispora epigaea*	(–) germination	Daniels & Trappe, 1980
Sand + PEG400, ψ < –1 MPa		*Gigaspora gigantea*	(–) spore germination	Koske, 1981
ψ < or > – 0.1MPa ψ < or > – 0.01MPa ψ < or > – 0.01MPa	Filter membrane	*Rhizophagus clarus*, *Glomus macrocarpum*, *Claroideoglomus etunicatum*	(–) spore germination	Sylvia & Schenck b, 1983
ψ < –1.4 MPa	Pot culture	*Funneliformus caledonium*, *Scutellospora calospora*, *Acaulospora laevis*	(–) spore germination, germ tube length	Tommerup, 1984
Mannitol. ψ < –0.21 MPa	*In vitro*	*Funneliformis mosseae*	(–) germination, germ tube growth	Estaun, 1990
–0.01 MPa ≥ ψ ≥ –2.20 MPa	Pot culture	*Gigaspora margarita*	(0) spore germination, (+) germ tube length	Douds & Schenck, 1991
		*Glomus intraradices**	(–) spore germination, (–) germ tube length	
		Acaulospora longula	(–) spore germination, (0) germ tube length	
		Funneliformis mosseae	(–) spore germination, (–) germ tube length (NB: no stats for spore germination)	

To be continued →

Stress	Experimental conditions	AM fungal species	Effect	References
Extraradical hyphae, spore production, root colonization and other parameters (since the review by Augé, 2001)				
20 days drought cycle	Pot culture	*Rhizophagus fasciculatus*	(+) extraradical hyphae index	Davies *et al*., 1992
Soil drying for 7 days (8 cycles)	Pot culture	*Glomus intraradices* *	(+) hyphal length	Khalvati *et al*., 2005
25% PEG6000, in M medium	*Monoxenic culture*	*Rhizophagus irregularis*	(+) expression of fungal *Gi 14-3-3*, (0) root col	Porcel *et al*., 2006
	+ Pot culture		(+) expression of fungal *Gi* BiP	Porcel *et al*., 2007
ψ = –0.44 MPa	Pot culture	*Diversispora epigaea*	(0) hyphal length and Bradford reactive soil protein (BRSP), (–) root col	Wu *et al*., 2008
		Funneliformis mosseae	(+) hyphal length, (0) BRSP, (–) root col	
		Rhizophagus diaphanum	(0) hyphal length, BRSP, root col	
Soil drying 12 days	Pot culture	*Diversispora epigaea*	(–) root col	Wu & Zou, 2009
ψ = –0.44 MPa	Pot culture	*Diversispora epigaea*	(–) hyphal length, active hyphae	Wu *et al*., 2011
		Funneliformis mosseae	(–) hyphal length, active hyphae	
		Rhizophagus diaphanum	(0) hyphal length, (–) active hyphae	
Glycerol in M medium	*Monoxenic culture*	*Rhizophagus irregularis*	(–) mycelium biomass, spore production, spore size	Hammer & Rillig, 2011
25% FC	Pot culture	*Glomus sp*	(0) root col	Doubkova *et al*., 2013
		Native AM fungal community	(–) root col	
25% PEG6000, in M medium	*Monoxenic culture*	*Rhizophagus irregularis*	(+) expression of fungal *GintAQPF1, 2*	Li *et al*., 2013

To be continued →

Stress	Experimental conditions	AM fungal species	Effect	References
50, 25% FC	Pot culture	*Gigaspora albida*, *Fuscutata heterogama*	(–) spore number	Silva *et al* ., 2015
		Claroideoglomus etunicatum	(0) spore number	
ψ = –0.14 MPa. ψ = –0.38 MPa	Pot culture	*Rhizophagus intraradices*	(+) root col	Zhao *et al* ., 2015
Soil drying	Pot culture	*Funneliformis mosseae*	(–) root col	Sun *et al* ., 2017
50% FC	Pot culture	*Diversispora versiformis*	(–) root col	Zou *et al* ., 2017
55% FC	Pot culture	*Funneliformis mosseae*	(–) root col	Huang *et al* ., 2017
35–55% FC	Pot culture	*Rhizophagus irregularis* *Rhizophagus arabicus*	(–) hyphal length, arbuscules	Symanczik *et al* ., 2018
50% FC	Pot culture	*Funneliformis mosseae*	(–) hyphal length, root col	F. Zhang *et al* ., 2018
		Paraglomus occultum	(–) hyphal length, root col	
40% FC	Pot culture	-	(–) hyphal density	Mai *et al* ., 2018
10% FC	Split pot experiment	*Rhizophagus irregularis*	(–) hyphal length, (0) hyphae diameter, (+) protoplasm fragmentation	Leyva-Morales *et al* ., 2019

* According to the updated classification of Schüssler and Walker (2010) the genera changed to *Rhizophagus*. Information on the strain provided by the authors is yet not sufficient to tell which of *Rhizophagus irregularis* or *Rhizophagus intraradices* is the corresponding new nomenclature.

Positive (+), negative (–) and no (0) impact on fungal parameter.
Root col : root colonization

5.2. Impact of drought on AM fungal physiology

Drought has been shown to affect different AM fungal genes. Under *in vitro* conditions, application of 25% PEG in the M liquid growth medium was found to increase the expression of the fungal aquaporin genes *Gint*AQPF1 and 2 (Li *et al.,* 2013) in the extraradical mycelium of *Rhizophagus irregularis* suggesting that AM fungi could improve drought resistance in plants by increasing the water supply. Other fungal genes such as the *Gi* 14-3-3 gene coding for an ubiquitous eukaryotic protein involved in numerous cellular processes (Porcel *et al.,* 2006) and *Gi*BiP gene coding for a molecular chaperone (Porcel *et al.,* 2007) have been found up-regulated under conditions of drought. The role of these proteins in the symbiosis is not clear but authors suggest that they could play multiple role in stress-related signals in the host plant.

So far, no studies have reported on the impact of drought on the extraradical hyphae and its capacity to transport nutrients and transfer it to the plants. It is probable that nutrient transfer could be hindered by an impact of drought on fungal structure and physiology. Indeed, studies conducted under other abiotic stresses reported various disturbances in AM fungi such as increased oxidative stress, lipid membrane modifications and activation of the antioxidant system (Lenoir *et al.,* 2016). It is not excluded that nutrient transporters in extraradical hyphae respond to drought constraints as shown above for the aquaporin genes (Li *et al.,* 2013), and that nutrient translocation and transport depend on the water movements as recently shown by Kikuchi *et al*. (2016) with Pi.

Finally, the growth of AM fungi and their function could be hampered by a lower transfer of photosynthates from the plants, due to a deleterious effect of drought on plant physiology.

5.3. AM fungal communities in dry environments

AM fungal communities in dry environments are often dominated by species belonging to the *Glomeraceae* family (see Table 2). A variation of water supply (in terms of precipitation or artificial water supply) is generally leading to a shift in the structure and composition of an AM fungal community. For instance, in a same AM fungal community, AM fungal diversity was enhanced by watering (Sun *et al*., 2013) while it was decreased by a subsequent dry season (Da Silva *et al*., 2014). In another study comparing different AM fungal communities along a precipitation gradient, species richness of AM fungi was positively correlated with higher precipitation (Zhang *et al*., 2016). The proportion and the dominance of some genera was also affected by variation in water availability. For instance, AM fungal communities were found dominated by unidentified taxa under dry conditions but by *Glomus*[4] genera when soil moisture was increased (Yang *et al*., 2010). *Acaulospora* were found to dominate in dry forests but not in moist forests (Da silva *et al*., 2014). The abundance of *Glomus* species was variably affected as it was decreased (Zhang *et al*., 2016) or increased under dryer conditions (Querejeta *et al*., 2009). These results suggest that under conditions of decreased water availability, a specific population of AM fungal species, apparently more diverse and more stress-tolerant (such as *Acaulosporaceae*, Chagnon 2013), can develop. As these results were based on the identification of spore morphotypes it is probable that the number of species discovered is underestimated (Herman, 2000). It is only until recently, that sequencing methods were used to have a more complete and reliable picture of the AM fungal communities in dry environments (Symanczik *et al*., 2014; Zhang *et al*., 2016; Chen *et al*, 2017).

[4] Many AM morphotypes described as belonging to the *Glomus* genera by the several authors in Table 1 might belong to *Rhizophagus* genera according to the new classification of Schüssler and Walker (2010).

Table 2 Characteristics of AM fungal communities in dry environments and/or impact of variation in water supply on their structure. Studies were based on determination of spore morphotypes, genetic marker analysis or sequencing analysis (SQ). N.B. Numerous morphotypes ascribed to the *Glomus* genera below might belong to the *Rhizophagus* genera, according to the phylogenetic study of Schüssler and Walker (2010).

Biome/Ecosystem	Vegetation & Plant species	Experimental conditions	Climate, Annual precipitation (mm)	Variation in water supply	AM fungal community parameters	References
Savannah	Natural and managed forest	Field	Semi-arid, 600-1200	–	*Glomus* (4 morphotypes described), *Scutellospora* (2), *Gigaspora, Sclerocystis, Acaulospora* (1 morphotype	Diallo *et al*., 1999
Desert	*Mitragyna parvifolia*	Field	Arid	–	*Glomus* dom. (based on number of spores and species richness of the genera)	Panwar & Tarafdar, 2006
Semi-savannah	Grass, shrubs, trees	Field	Arid, 600-800	–	Spore density lowest during rainy season *Glomus (28 morphotypes), Acaulospora (7), Scutellospora (4), Entrophospora (2), Gigaspora (2).* *Glomus* and Gigasporaceae dom. (based on spore number)	Dandan & Zhiwei, 2007
Desert	Trees and shrubs	Field	Arid, 180	–	*Glomus* dom. (number of sites where detected, based on 30 sites)	Bills *et al*., 2008
Forests	Oak trees	Field	Semi-arid, 298	Increased rainfall the next year	(–) *Glomus* (+) *Scutellospora*	Querejeta *et al*., 2009
Prairie grasslands	*Agropyron cristatum (L.)* Gaertn, *Stipa comata* Trin.	Field	Semi-arid, 450-500	Moisture deficiency	Unidentified AM fungal species dom.	Yang *et al*., 2010
				Moisture sufficiency	*Glomus viscosum*, *Glomus mosseae*, and *Glomus hoi dom.(genetic marker analysis)*	

To be continued →

Biome/Ecosystem	Vegetation & Plant species	Experimental conditions	Climate, Annual precipitation (mm)	Variation in water supply	AM fungal community parameters	References
Desert	Date palm trees	Field	Arid –	–	*Glomus* (72% morphotype occurence) *Scutellospora* (8%), *Racocetra* (8%)	Al-Yahya'ei *et al*., 2011
Desert steppe	Grasslands	Field	Temperate to semi-arid, 209.12	–	*Glomus* (4 recorded taxa), *Acaulospora and Funneliformis* (3), *Claroideoglomus* (1), *Diversispora* (1), *Scutellospora* (1).	Bai *et al*., 2012
Desert steppe	Grasslands	Field	Temperate to semi-arid, 387.2	Enhanced watering treatment	(–) AM fungal diversity, spore % of rare species *Entrophospora, Scutellospora* , and *Gigaspora,*	Sun *et al*., 2013
				(In the same AM fungal community)	(–) spore % *Claroideoglomus etunicatum* , (+) spore % of *Ambispora gerdemannii*	
Semi-deciduous forests	> 50 species	Field	Semi-arid, 1000	Dry forest	*Acaulospora* and *Glomus* dom.	Da Silva *et al*., 2014
				Transitional zone	Highest AM fungal diversity, *Glomus* dom.	
				Moist forest (MF)	*Glomus* dom.	
				Dry season	Higher AM fungal diversity compared to wet season (except MF, contrary)	
Desert	Asteraceae	Field	Semi-arid, 0.2-26.6	–	*Glomus* dom.	Dhar *et al*., 2015

To be continued →

Biome/Ecosystem	Vegetation & Plant species	Experimental conditions	Climate, Annual precipitation (mm)	Variation in water supply	AM fungal community parameters	References
–	*Sorghum bicolor*	Pot culture	–	Application of drought cycles and drought stress (In the same AM fungal community)	Shift in the abundance of AM fungal species in a native assemblage (isolated from desert), less *Diversispora.* (SQ).	Symanczik *et al* ., 2015
Savannah	*Mimosa tenuiflora*	Field	Semi-arid	–	Most frequent species on all sites: *Gigaspora albida* > *Rhizoglomus clarum* >*Gigaspora decipiens* > *Claroideoglomus etunicatum*	De Souza, 2016
Alpine steppe	*Stipa pirpurea, Potentilla bifurca L.,*	Field	Arid to semi-arid, 59.96-382.49	Decreasing precipitation (In different AM fungal communities)	(–) AM fungal species richness, hyphal length density (effect of plant species coverage involved) (–) *Glomus,* (+) *Diversispora*. (SQ).	Zhang *et al* ., 2016
Cold steppe	Grasslands	Field	Temperate, 385.5	– Precipitation increment (In the same AM fungal community)	Glomeraceae dom. (+) AM fungal biomass, root col. (0) OTU richness, diversity indexes Changes in AM fungal taxon composition but not phylogenetic composition. (SQ).	Chen *et al* ., 2017

Positive (+), negative (–) and no (0) impact on fungal parameter.
Dominance (Dom.)

Material and Methods

1. Biological material

1.1. Arbuscular mycorrhizal fungi

Four AM fungal strains were used for the experiments (Table 3): ***Rhizophagus irregularis*** (Błaszk., Wubet., Renker and Buscot) C. Walker and A. Schüßler comb. nov. MUCL 41833, ***Rhizophagus intraradices*** (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler 2010 MUCL 49410, ***Rhizophagus sp.*** MUCL 43204 and ***Rhizophagus clarus*** (T.H. Nicolson & N.C. Schenck) C. Walker & A. Schüßler MUCL 46238. The strains were supplied by the Glomeromycota *in vitro* collection (GINCO – https://www.mycorrhiza.be/ginco-bel/catalogue.php) on the Modified Strullu–Romand (MSR) medium (Declerck *et al*., 1998 − see composition in Appendix 2) associated with Ri T-DNA transformed carrot (*Daucus carota)* roots.

Table 3 Geographic origin and host plant/habitat of the *Rhizophagus* strains

Scientific name	**Accession number**	**State, Country**	**Host Plant/ Habitat**	**Climate***
R. irregularis	MUCL 41833	Canary Islands, Spain	Unknown	Arid, steppe, hot
Rhizophagus sp.	MUCL 43204	Ontario, Canada	Meadow	Cold, without dry season, warm summer
R. clarus	MUCL 46238	Cuba	*Poa annua*	Tropical, savannah
R. intraradices	MUCL 49410	Florida, USA	*Citrus*	Temperate, without dry season, hot summer

* Köppen-Geiger climate classification (Peel *et al.*, 2007)

The four AM fungal strains were used to test the impact of decreasing water availability on germination and germ tube elongation under *in vitro* conditions (Research results - Chapter 1). Strain originating from different geographical zones and climates were selected in order to test the hypothesis that strains from drier climates are better adapted to drought stress, and by extension are less impacted by decreasing water availability.

The fungal strain *Rhizophagus irregularis* MUCL 41833 was also used for the *in vitro* experiments in association with *Medicago truncatula* (Research results - Chapter 2) and for the greenhouse experiments in association with maize plants (Research results - Chapter 3). This AM fungus is a worldwide distributed organism easily cultivable *in vitro* (Savary *et al*., 2018). In particular, the strain MUCL 41833 produces an extensive hyphal network bearing thousands of spores (e.g. Voets *et al*., 2009). For this reason it has been used as a model organism in numerous studies conducted *in vitro* in association with transformed roots of carrot (Declerck *et al*., 1998) or whole plants such as banana (Koffi *et al*., 2013, 2015; Oye Anda *et al*., 2015), potato (Voets *et al*., 2005), barrel medic (Voets *et al*., 2009) or rubber tree (Sosa-Rodriguez *et al*., 2013). It has also been widely used in greenhouse and field experiments in association with banana (Oye Anda *et al*., 2016), maize (Garcés-Ruiz *et al*., 2017), barrel medic (Buysens *et al*., 2016; Calonne-Salmon *et al*., 2018) or potato (Buysens *et al*., 2017; Alaux *et al*., 2018).

1.2. Plants

Excised roots as well as whole plants were used for the experiments:

Carrot (*Daucus carota* L. clone DC2) Ri T-DNA transformed roots. They were provided by the Glomeromycota *in vitro* collection (GINCO) on the MSR medium in association with *R. irregularis* MUCL 41833 to produce AM fungal inoculum.

Barrel medic (*Medicago truncatula* Gaertn. cv. Jemalong A17) seeds. They were provided by SARDI (Australia). The seeds were associated with *R. irregularis* MUCL 41833 to set up the Mycorrhizal Donor Plant (MDP) *in vitro* culture system (see below) and to study the impact of a decrease of water potential on hyphal development, spore production and phosphorus uptake of the AM fungus (Research results - Chapter 2).

Seeds were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 10 min and rinsed three times (each time during 10 min) in sterilized (121°C for 15 min) deionized water. Seeds were then germinated in Petri plates (90 mm diameter) containing 35 ml of MSR medium without vitamins and supplemented with 10 g L^{-1} sucrose and 3 g L^{-1} Gelrite® (Merck & Co., Kenilworth, NJ, United States). The Petri plates were incubated at 27°C in the dark. The seeds germinated within two days and plantlets were ready to use after four to five days.

Maize (*Zea mays* L. cv. ES Ballade) seeds. They were supplied by the Centre Indépendant de Promotion Fourragère (CIPF, Belgium). Maize plants associated with *R. irregularis* MUCL 41833 produce dense hyphal networks and allow the fast colonization of plants (Garcés *et al.*, 2017, Calonne-Salmon *et al.*, 2018). Thus, the seeds were used in our studies to produce trap plants for AM fungal inoculum production in the greenhouse (Research results - Chapter 3). Recently, the same germplasm used for inoculum production has been used to study the dynamics of Pi absorption by the maize/AM fungus associates under hydroponic conditions (Garcés *et al.*, 2017). Therefore, it was used as a model plant to study the effect of water shortening on the Pi absorption by the maize/AM fungus associates and on the physiology of the plants under semi-hydroponic conditions (Research results - Chapter 3).

Seeds were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 15 min and rinsed three times (each time during 10 min) with sterilized (121°C for 15 min) deionized water. The seeds were then placed on wet filter paper (N°1, Whatman, United Kingdom) and germinated in plastic boxes (Aarts Plastics, Netherlands) in the dark at room temperature (~20°C). The seeds germinated in three days. and were immediately transferred to a sterilized (twice at 121°C for 15 min with 24 h interval) substrate made of vermiculite (PullRhenen, Netherlands), sand (quartz N°4, Euroquartz, Belgium) and lava stone (DCM, Belgium) (w:w:w, 1:1:1). The seeds were placed in contact with ~100 spores of *R. irregularis* MUCL 41833 for root colonization (Research results - Chapter 3).

2. Application of decreased water availability in MSR medium

Polyethylene glycol (PEG 8000 − hereafter cited as PEG) was used to decrease the water availability in liquid and gelled MSR medium (Research results - Chapter 1 and 2).

2.1. Preparation of liquid MSR medium added with PEG

The liquid MSR medium with PEG was used to determine the water potential (Research results - Chapter 1 and 2) and to study the Pi depletion in the hyphal compartment (Research results - Chapter 2). To determine the water potential, aliquots of 5, 10, 25 and 40 g of PEG powder (necessary to reach final PEG concentrations of 50, 100, 250 and 400 g L^{-1} of MSR in 100 ml of MSR medium) were weighted in a sterilized 250 ml Erlenmeyer using a spoon cleaned with EtOH 99%. The Erlenmeyers were then immediately sealed with aluminum foil. MSR medium without Gelrite, sucrose, and vitamins was autoclaved (121°C for 15 min) and left to cool down at ambient temperature. Under laminar flow, 100 ml of liquid MSR medium was poured in each of the Erlenmeyers containing the PEG powder. The solutions were mixed using a sterilized magnetic stirrer until complete dissolution of the PEG. The Erlenmeyers containing the solutions were then sealed and ready to use (Figure 8). To study the Pi depletion in the hyphal compartment a similar protocol was used to produce liquid MSR medium at concentrations of 10, 25 and 50 g L^{-1}.

2.2. Preparation of gelled MSR medium added with PEG

The gelled MSR medium added with PEG was used to study the impact of decreasing water availability on the germination of AM fungal strains (Research results - Chapter 1 and 2) and on the extraradical development and sporulation of AM fungi (Research results - Chapter 2).

Before that, gelled MSR medium added with increasing concentrations of PEG (i.e. 50, 100, 250 and 400 g L^{-1}) were prepared to assess the impact of the molecule on the polymerization of the medium (Research results - Chapter 1). It was determined that concentrations above 100 interfered with the polymerization of the medium (the gel broke easily) and rendered it opaque

which would have prevented any quantitative or qualitative observations of fungal structures. To prepare the gelled MSR medium added with PEG, aliquots of 5, 10, 25 and 40 g of PEG powder (necessary to reach final PEG concentrations of 50, 100, 250 and 400 g L^{-1} of MSR in 100 ml of MSR medium) were prepared using the same protocol as presented for the medium without Gelrite. MSR medium with 3 g L^{-1} of Gelrite, without sucrose and vitamins was autoclaved (121°C for 15 min) and left to cool down to a temperature comprised between 45 and 50°C. Meanwhile, a heating-stirring plate (IKA C-Mag HS 7, Germany) was disposed under laminar flow, cleaned with EtOH 99% and set at a temperature of 50°C. One hundred ml of MSR medium was poured in the Erlenmeyer containing the PEG powder. The solution was then immediately left to agitate on the heating block until complete dissolution of the PEG. During this step, the temperature of the solution was kept between 45 and 55°C by adjusting the heat Figure 8). This temperature range was adequate to avoid polymerization of the medium before use and stands below the fusion temperature of PEG (e.g. 60 °C for PEG 8000-Majumdar *et al*., 2010).

A similar protocol was used to prepare gelled MSR medium added with PEG at concentrations of 25 and 50 g L^{-1} for the germination tests in the Chapter 1 and then at concentrations of 10, 25 and 50 g L^{-1} for the experiments in Chapter 2.

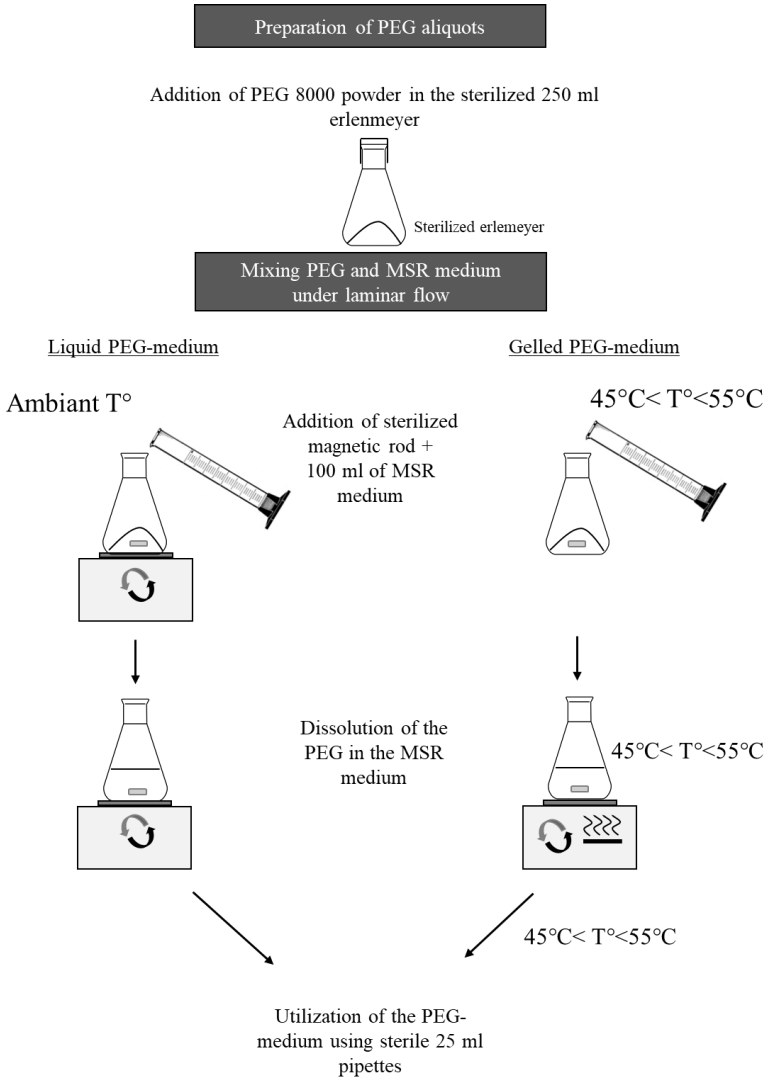

Figure 8 Scheme presenting the different steps for the preparation of MSR medium with PEG, in the absence or presence of Gelrite.

2.3. Measurement of the water potential in the liquid MSR medium using the vapor pressure osmometer

The water potential of the solutions of MSR medium without Gelrite and containing different concentrations of PEG was assessed using a vapor pressure osmometer (VAPRO® 5520, Wescor Inc., Logan, UT, United States – see Research results - Chapter 2 and 3). This device is equipped with a thermocouple psychrometer that measure the difference in temperature corresponding to the state equilibrium of the sample. This temperature difference is depending on the vapor pressure, a colligative property of the solution and therefore changes with the osmolarity.

The vapor pressure osmometer has been regularly used to assess the water potential of solutions containing PEG (Michel and Kaufmann, 1973; Steuter *et al*., 1981; Van der weele *et al*., 2000) and is considered more efficient than the devices using the freezing point depression method (Money *et al*., 1989).

Briefly, the apparatus was placed in a room that was not subjected to high temperature changes. The device was switched on to allow the temperature to equilibrate. To calibrate, 10 µl of the standard solution (of 1000 mM) was pipetted on a paper disc placed in the sample holder, using a precision pipette (Figure 9). The chamber was closed and the osmolarity of the standard was read after 80 seconds. If the value was comprised within a range of 5 mM, the calibration was accepted. The same procedure was repeated with lower concentrated standards solutions (290 and then 100 mM). At the end of the calibration with the 100 mM solution, the sample was not removed and another reading was done using the CLEAN TEST procedure to check for the contamination of the chamber. After this procedure, the samples were ready to be analyzed similarly as for the standards.

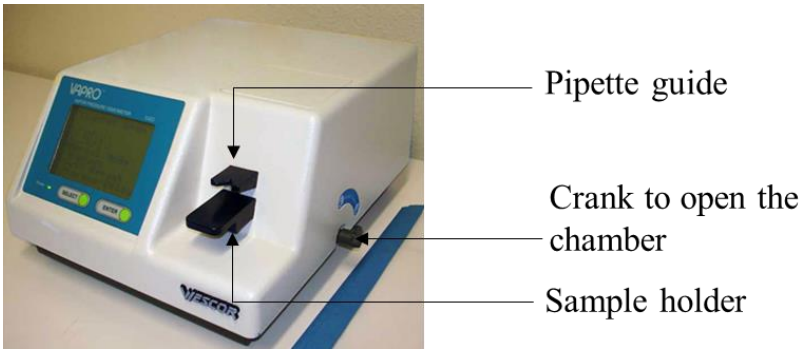

Figure 9 Vapor pressure osmometer (VAPRO® 5520, Wescor Inc., Logan, UT, USA). The sample was loaded on a paper disc placed on the sample holder.

3. *In vitro* cultivation systems of AM fungi

3.1. Root organ cultivation of *R. irregularis* MUCL 41833 for mass production of inoculum

Rhizophagus irregularis MUCL 41833 was mass-cultured in association with Ri T-DNA transformed carrot roots, the so-named root organ culture (ROC) system.

3.1.1. Root organ culture of carrot

Ri T-DNA (clone DC2) transformed roots of carrot were grown *in vitro* following the method described by Cranenbrouck *et al*. (2005). Briefly, each two weeks, two 5 cm-long pieces of carrot roots with actively growing apexes were isolated from an *in vitro* culture with scalpel and forceps, and gently placed in opposite directions to each other on a Petri plate containing 35 ml of MSR medium. The apexes of the roots were cautiously pushed into the gel to facilitate their regrowth. The Petri plates were incubated in the dark at 27°C upside down (the geotropism of DC2 roots is negative) to allow the roots to grow into the medium. This process was repeated until a sufficient number of roots was reached.

For the association with *R. irregularis*, two 5 cm-long pieces of carrot roots were similarly plated on the root compartment (RC) of a 90 mm bi-compartmented Petri plate containing 25 ml of MSR medium (see below for

explanation of the bi-compartmented cultivation system). The other compartment (named hyphal compartment - HC) was filled with 25 ml of MSR- (MSR medium without sucrose and vitamins). After association of the AM fungus with the roots, a profuse mycelium extended in the HC producing thousands of spores. The HC was devoid of roots (roots were regularly trimmed using scalpel and forceps), facilitating harvesting of the AM fungal inoculum.

3.1.2 Preparation of the AM fungal inoculum

An initial culture of *R. irregularis* MUCL 41833 containing a sufficient number of spores in the HC was supplied by the GINCO to inoculate the carrot roots. The preparation of the inoculum followed a modified protocol of Cranenbrouck *et al*. (2005). Briefly, the gel of the HC was cut in six equal size pieces, each containing an approximate of 100-150 spores. Each piece was placed in a 90 mm Petri plate, chopped finely using a sterilized scalpel and covered with 10 mM filter-sterilized sodium citrate buffer (Doner and Bécard, 1991). The Petri plates were placed on a plate shaker during 1 h for the dissolution of the gel.

3.1.3. Association of the carrot roots with R. irregularis MUCL 41833

An average of 100 spores of the above-prepared inoculum was transferred with a micropipette into the vicinity of Ri T-DNA transformed carrot roots in the RC of bi-compartmented Petri plates containing 25 ml MSR medium. The Petri plates were sealed with Parafilm and incubated in an inverted position at 27°C in the dark. Spores started to germinate within a few days and first contact points and colonization of the roots were noticed after one to two weeks. New hyphae extended from the root, covering the RC and crossing the plastic barrier separating the RC from the HC within six to eight weeks. After 12 weeks, a profuse hyphal development with the production of a sufficient number of spores was obtained in the HC and further used as inoculum (Figure 10).

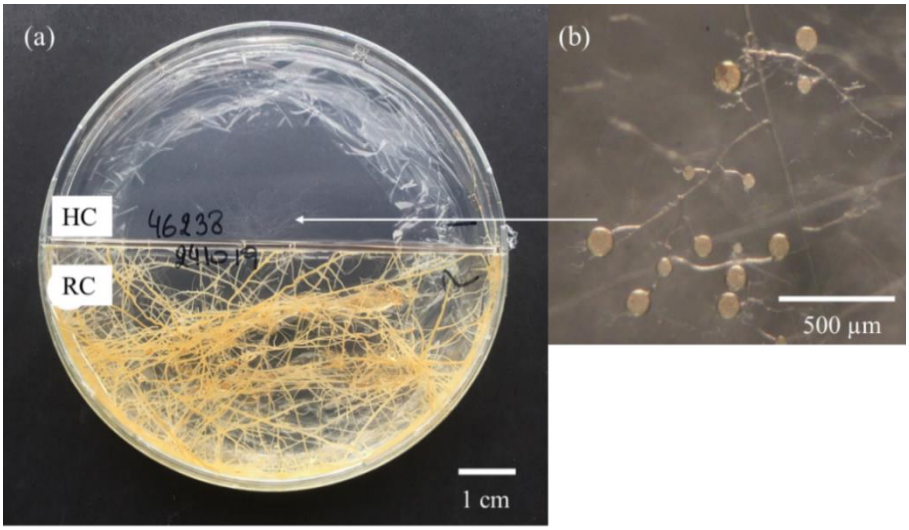

Figure 10 (a) Example of a root organ cultivation (ROC) system for the cultivation of AM fungi. Ri T-DNA (clone DC2) transformed roots of carrot are associated with *R. clarus* MUCL 46238 in the Root compartment (RC). The extraradical hyphae extend in the hyphal compartment (HC) **(b)** Extraradical mycelium of *R. clarus* MUCL 46238 observed under compound microscope (45×).

3.3. *In vitro* germination tests

The impact of decreased water availability on the germination of different AM fungal strains (Research results - Chapter 1 and 2) was tested under *in vitro* conditions using 24-well plates containing gelled medium added with PEG at different concentrations.

Gelled medium at 0, 10, 25 and 50 g L^{-1} (only 0, 25 and 50 in Chapter 1) concentrations of PEG was prepared according to the protocol presented in Material and Methods - section 2. Approximately 2 ml of the gelled MSR medium was then poured in each well of the 24-well plates using a 25 ml pipette. The plates were left open under laminar flow to dry for around 30 minutes. Spores of two months-old *in vitro* cultures of each AM fungal strain were isolated from the MSR medium following solubilization of the gelled medium with 10 mM filter-sterilized sodium citrate buffer (Doner and Bécard, 1991). Spores were separated with forceps under binocular microscope and placed single with a micropipette in each well of the corresponding 24-well tissue culture plates. They were then sealed with cellophane (Toppits®, Melitta

Group, Germany) and incubated in the dark at 27°C. Spore germination was estimated at regular intervals under compound microscope at 40× magnification. Germ tube length was evaluated according to the protocol described in Material and Methods - section 5.

3.2. Mycelium donor plant *in vitro* culture systems

The mycelium donor plant (MDP) *in vitro* culture system was developed by Voets *et al*. (2009) for the rapid and homogeneous colonization of young plantlets (i.e. the so-named receiver plants) grown in contact with a dense extraradical hyphal network extending from a donor plant. Colonized plantlets were then used for producing whole plant *in vitro* systems (see section below and Research results - Chapter 2).

Under a laminar flow, a 90 mm bi-compartmented Petri plate was opened, the lid being placed upside down on the bench. Using a heated tip of a forceps, a 5 mm opening was formed in the middle, on the upper part of the border of the plastic wall of the RC. Another opening was formed on the border of the lid of the Petri plate (Figure 11a). The Petri plate was then closed and kept under the laminar flow.

Using a sterilized cork-borer, a piece of gel containing an approximate of 100 spores of *R. irregularis* MUCL 41833 was sampled from a ROC and placed in the middle of the RC of the Petri plate. The RC was then filled with MSR medium without sucrose and vitamins until it reached the lower level of the opening. Similarly, the HC was filled with 25 ml of MSR medium without sucrose and vitamins. One four-days-old seedling of *M. truncatula* was placed carefully on the surface of the medium in the RC with the shoot protruding outside via the opening (see above). The Petri plate was first sealed with Parafilm® (Bemis, USA), then with silicon grease around the stem of the plant to avoid any contamination of the MSR medium (Figure 11b). The system was then covered with a dark plastic sheet to protect *M. truncatula* roots and AM fungi from the light, while the shoot developed under light conditions. The MDP *in vitro* culture system was placed in a growth chamber under controlled conditions (22/18°C (day/night), 70% relative humidity, photoperiod of 16 h day^{-1} and an average photosynthetic photon flux of 225 µmol m^{-2} s^{-1}).

After 3 weeks, the medium in the RC decreased due to the absorption of the medium by the roots. The RC was thus supplied every week with

approximately 10 ml of fresh MSR medium without sucrose and vitamins using a 25 ml sterile pipette (VWR, USA). The medium was kept liquid at 45-50°C and brought to the plants with the pipette through a hole of 1cm diameter that was made in the lid above the RC using a heated cork-borer (Figure 11b). The medium then solidified. The hole was sealed each time after bringing nutrients to the plants by sticking a new double layer of sterilized plaster above it (Micropore ™, 3MTM, Maplewood, MN, USA). After eight to 12 weeks, hyphae started to cross the partition wall separating the RC from the HC. At that time, two new openings were made in the lid and the cap of the RC (see above for details) and two four-days-old *M. truncatula* plants were transferred in the HC of each system to allow the fast colonization of young plants (Figure 12a, b). Roots of the young plants were plated on the MSR medium and shoot of each plant protruded outside of the system via an opening, similarly plastered with silicon grease to avoid contamination. The new plants were homogeneously colonized within 12 days and ready to use (Figure 12c).

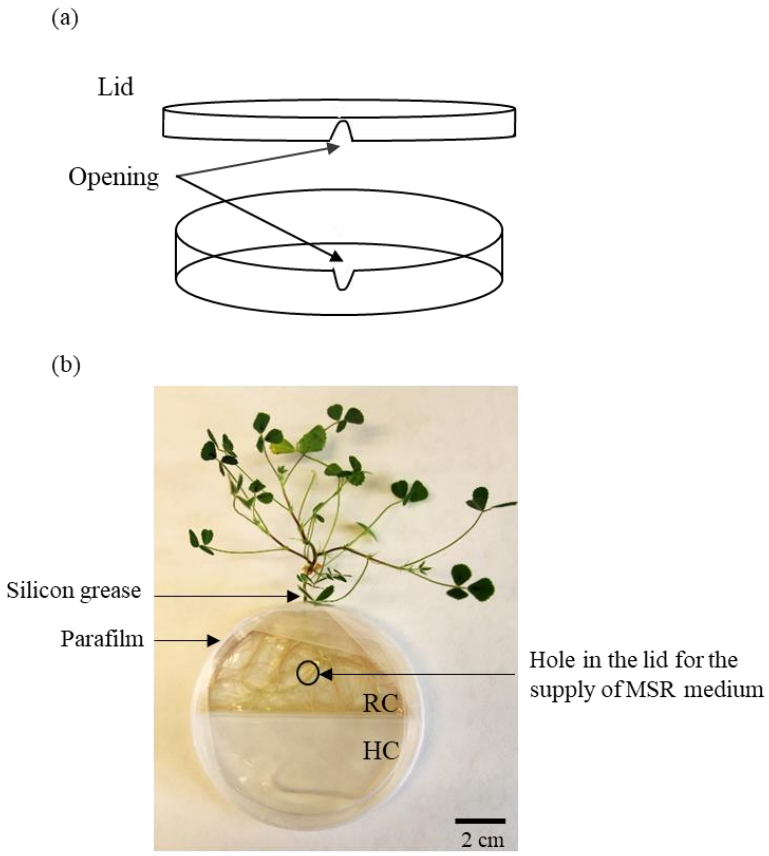

Figure 11 (a) Schematic representation of the modification of the Petri plate for the construction of the MDP *in vitro* culture system (**b)** Example of a Mycelium Donor Plant *in vitro* culture system before adding plants to the hyphal compartment (HC). RC= Root compartment.

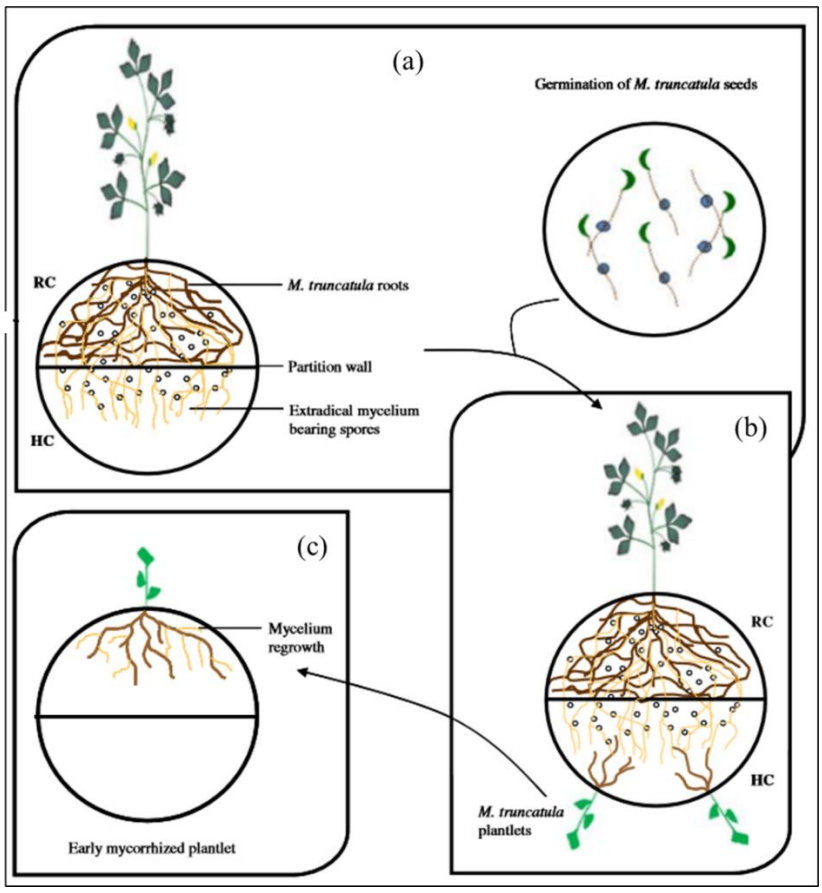

Figure 12 Schematic representation of the early and fast mycorrhization of *M. truncatula* plants (adapted from Voets *et al*., 2009). After eight to 12 weeks, the HC was colonized with extraradical hyphae **(a)**. Four-days-old *M. truncatula* plants were plated into the HC of the MDP *in vitro* culture system **(a, b)**. Transfer of mycorrhized *M. truncatula* plants to new Petri plates after 12 days **(c)**.

3.4. Whole plant *in vitro* systems for the study of the development of extraradical mycelium

These systems were produced to study the impact of decreasing water availability on the development of extraradical mycelium (Research results - Chapter 2).

Early mycorrhized *M. truncatula* plants were produced in the MDP *in vitro* culture systems and plated in new bi-compartmented Petri plates as described in the previous section. After nine weeks, a profuse ERM was produced in the RC and started to cross the partition wall separating the RC from the HC. After colonization of the HC, the gel bearing the mycelium was partially removed leaving a 2 mm strip along the partition wall to homogenize a front of growth of mycelium. The HC was then supplemented with 25 ml of fresh MSR medium lacking sucrose and vitamins and solidified with 3 g L^{-1} of gelrite in absence ($Control^{-PEG}$ treatment) or supplemented with 10, 25, and 50 g L^{-1} PEG (PEG^{10}, PEG^{25}, and PEG^{50} treatments, respectively). After four weeks, the hyphal length and spore production in the HC was determined (see protocol in Material and Methods - section 5.1.2.).

3.5. Whole plant *in vitro* systems for the monitoring of Pi depletion in the liquid MSR medium without sucrose and vitamins

These systems were produced to study the impact of decreasing water availability on the Pi depletion by extraradical hyphae (Research results - Chapter 2).

Whole plant *in vitro* systems were prepared as described in the previous section. After nine weeks, an extensive extraradical mycelium started to develop in the HC. The gelled MSR medium was entirely removed from the HC and replaced by 15 ml of liquid MSR medium without sucrose and vitamins. A hole of 1cm diameter was made in the lid above the HC using a heated cork-borer to add the nutrient solution (Figure 13). The HC was supplied weekly through this hole with approximately 15 ml of liquid MSR medium without sucrose and vitamins using a 25 ml sterile pipette, until a profuse hyphal regrowth was obtained in the HC (after seven weeks). The whole plant *in vitro* culture systems were then separated in four homogenous

groups based on the surface covered by the hyphae in the HC. Hyphal surface was estimated following the method described by Voets *et al*. (2009) by tracing on a transparent sheet the growing front constituted by the hyphal network. This measure confirmed no significant differences (P = 0.6395) between treatments. Four treatments, each with six replicates, were considered: MDP *in vitro* culture systems without PEG in the HC ($Control^{-PEG}$ treatment) or supplemented with 10, 25, and 50 g L^{-1} PEG (PEG^{10}, PEG^{25}, PEG^{50} treatments, respectively). A Control (six replicates) without mycelium in the HC ($Control^{-AMF}$ treatment) was also considered by cutting and removing the ERM from the HC. Liquid MSR medium was retrieved from the HC and the compartment was rinsed two times with sterilized deionized water. Fifteen ml of fresh liquid MSR medium was added to the $Control^{-AMF}$ and $Control^{-PEG}$ treatments, whereas liquid MSR medium containing 10, 25, and 50 g L^{-1} PEG was added to the other treatments. The Pi concentration in the HC at the start of experiment was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES− see protocol in Material and Methods - section 5.3.). The value was 0.59 mg L^{-1} and total P content 0.09 mg.

For Pi concentrations analysis in the HC, 0.5 ml of MSR medium was sampled every 2 h during 24 h from the HC of each system. The samples were then prepared for ICP-AES analysis (see protocol in Material and Methods - section 3.5.). Data of Pi concentration obtained in ppm were subsequently converted into mg L^{-1} and standardized as in Garcés-Ruiz *et al*. (2017). Data were finally transformed into % of Pi remaining in the medium which is the percentage of the Pi concentration remaining in the medium of the HC at time 2, 4, 6, 8, 12, 18, 24 h relative to the Pi concentration in the medium of the HC at time T0:

$$\%\ of\ Pix = ([Pix]t + [Pic]t - [Pic]t0)/([Pix]\ t0 \times 100$$

where:

[Pix] = Pi concentration in the medium of the HC for the sample x considered

t = time considered (2, 4, 6, 8, 12, 18 or 24 h after the start of the experiment)

t0 = 0h before the start of the experiment

[Pic] = Mean Pi concentration measured over 6 replicates in the Control$^{-}_{AMF}$ treatment

At the end of the sampling phase, the ERM in the HC was harvested from the liquid MSR medium and succinate dehydrogenase (SDH) activity was evaluated (see protocol in Material and Methods - section 5.1.3.). The SDH staining method enabled to evaluate the proportion of metabolically active hyphae (Saito, 1995; Vierheilig *et al*., 2005). Following harvest, shoots and roots were separated and dry weights evaluated after 48 h at 45°C in an oven. The dried shoots and roots material were analyzed for P concentration via ICP-AES (see protocol in Material and Methods - section 5.3.).

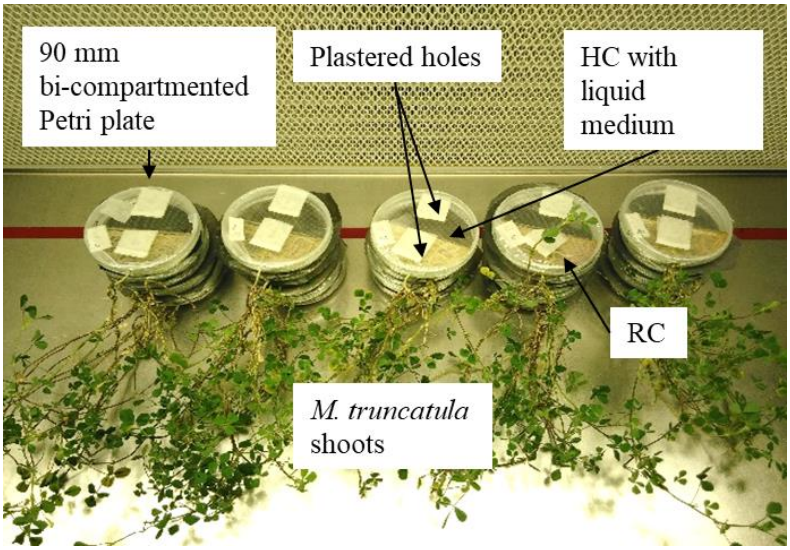

Figure 13 Whole plant *in vitro* culture systems associating *M. truncatula* with *R. irregularis* MUCL 41833 after addition of liquid MSR medium without sugar and vitamins in the HC. Two holes were made in the lid of the Petri plates to allow the addition of medium in the RC and HC using a 25 ml pipette. The holes were closed with sterilized plaster to avoid contaminations.

4. Greenhouse assay

A semi-hydroponic circulatory system was used to monitor the dynamics of Pi uptake of maize plants associated with *R. irregularis* MUCL 41833 during recovery from drought stress (Research results - Chapter 3). This system was first used and described by Garcés Ruiz *et al*. (2017). In brief, the system consists in circulating a nutrient solution in a closed circuit through the root system of a plant and evaluating the dynamics of Pi uptake.

4.1. Preparation of the substrate for the fast colonization of maize plantlets

Three 5 L pots containing a sterilized (121°C for 15 min) substrate composed of vermiculite (PullRhenen, Netherlands), sand (quartz N°4, Euroquartz, Belg) and lava stone (DCM, Belgium) (w:w:w, 1:1:1) were prepared to produce mycorrhizal (M) plants. In each pot, maize seedlings were placed with their roots in contact with an approximate of 100 spores of an *in vitro* culture of *R. irregularis*. Three other pots containing a similar substrate but without AM fungal inoculum were used to produce non-mycorrhizal (NM) plants. All the pots were maintained 4 months under greenhouse conditions (21°C/18°C (day/night), a relative humidity (RH) varying from 40 to 70%, a photoperiod of 16 h day^{-1} and a photosynthetic photon flux (PPF) of 120 µmol m^{-2} s^{-1}) to produce maize plants with strong root systems whether colonized or not with the AM fungus. The shoots of the plants were finally cut before transfer of new maize plantlets.

4.2. AM-colonization of the maize plantlets

Three-days-old disinfected maize seedlings were planted in the 5 L pots containing or not the AM fungus extending from the roots of the cut maize plants. The plants were watered twice a week with 1400 ml of deionized water and received additionally 500 ml of Pi-impoverished modified Hoagland solution ($Hoagland^{lowPi}$ – see Appendix 3 for chemical composition). The plants were kept in a grow chamber set at 21/21°C (day/night), a RH of 75%, a photoperiod of 16 h day^{-1} and a PPF of 300 µmol m^{-2} s^{-1}.

4.3. Preparation of the material for the semi-hydroponic circulatory system

A structure to hold the containers for the plants was crafted from squared styrofoam panels of approximately 80 cm width and 10 cm height. Nine holes for the placement of the containers were cut out of the foam and the panel was placed over a support grid that allows the passage of the tubing system (see Figure 15a in Material and Methods - section 4.8.). The containers were crafted from 500 ml plastic wash bottles (500 mL − VWR, USA) that were cut at the base. A nylon mesh of 100 μm pore size (Prosep BVBA, Belgium) was then glued inside at approximately 5 cm above the neck of the bottle to prevent the roots to grow out of the bottle (see Figure 15b in Material and Methods - section 4.8.). The bottles were then placed in inverted positions in the holes of the styrofoam panel (see Figure 15a,b in Material and Methods - section 4.8.). Mycorrhized and non-mycorrhized maize plants were transferred into the bottles containing 25 g of perlite. Non-vegetated Control bottles were filled similarly with the same amount of perlite. The bottles were covered with aluminum foil to prevent the apparition of algae in the substrate.

4.4. Transfer of plantlets in perlite and acclimatization of maize plantlets

After three weeks, the maize plants were gently removed from the pots and roots carefully rinsed from the substrate with deionized water. Three plants from the M and NM treatments were randomly selected and root colonization evaluated to make sure that AM-colonization was high enough (usually above 70% − see protocol for root staining and colonization rate in Material and Methods - section 5.2.2.). The other plants were transferred to the greenhouse into individual containers that consisted of 500 ml wash bottles filled with 25 g of dry perlite (see above). Nine containers without plants were also included as non-vegetated (NV) Controls. Every two days, all the plants and the NV Control containers received the same volume (i.e. between 60 and 100 ml) of Hoagland^{lowPi} solution. Deionized water was further added until saturation of the substrate (estimated until the first drops of water were released from the opening at the bottom of the containers). Plants were acclimatized for 24 days.

4.5. Preliminary experiment: estimation of the water holding capacity of the substrate

The 100% water holding capacity of 25 g of perlite was estimated according to Meddich *et al*. (2000): twenty-five g of perlite was immerged in deionized water and left to drain until stabilization of the weight of the substrate. The difference between the dry weight of the perlite and its weight at saturation gives the volume of water necessary to reach 100% of saturation of the perlite (133.34 ml in our experiment).The volume of water corresponding to 50% of water holding capacity was thus :133.34 × 0.5 = 66.67 ml while for 25% of water holding capacity was : 133.34 × 0.25 = 33.34 ml.

4.6. Implementation of the water regimes

After acclimatization of the plants in the perlite, the M and NM maize plants had the same height (P = 0.923). They were separated in three homogenous groups and grown under three water regimes (WR): well-watered (WW^{M} and WW^{NM}), moderately-watered (MW^{M} and MW^{NM}) or poorly-watered (PW^{M} and PW^{NM}), the two later corresponding to moderate and severe drought conditions, respectively. Every two days, the bottom of the plant containers were opened and deionized water was added accordingly to the water regimes: well-watered plants were watered until saturation of the substrate. Saturation was estimated until the first drops of water were released from the opening at the bottom of the containers. Depending on the presence of the plant, its growth, and the climatic conditions in the greenhouse, the volume of water added in the WW pots fluctuated greatly. Moderately-watered and poorly-watered maize plants (MW and PW water regimes, respectively) were watered with 60 and 30 ml of deionized water corresponding approximately to the volume of water necessary to reach 50 and 25% of saturation (or of water holding capacity / field capacity) in 25 g of perlite. MW and PW pots were thus brought to a minimum of 50 and 25% of field capacity every two days.

4.7. Experimental design

Maize plants associated or not with *R. irregularis* MUCL 41833 were submitted to different water regimes in the greenhouse. In this environment, the condition for the growth of the plants are semi-controlled. Indeed, T°,

humidity, light intensity and photoperiod were set and controlled but some factors were found to vary a lot and could potentially affect plant growth. For instance, plants placed closer to the entrance could experience lower T° due to the opening of the door but also received more light during the day due to the natural solar exposition. To avoid biases and high heterogeneity the following experimental design was built: It was first decided to place all the plants in front of the window in order to give the maximum sun exposition. Therefore, five tables capable of holding each 9 pots were placed along the window. A minimum of 3 mycorrhizal (M) and non-mycorrhizal (NM) plants each were then placed on each of the 5 tables. Nine NV Controls were filled in the empty positions and the position of the pots was randomized for each table. Six treatments with six replicates each were considered: M and NM plants were assigned to the three different water regimes (well-watered, moderately or poorly watered water regime). Each of the treatment was represented at least one time per table (Figure 14). Three replicates of the NV were assigned to each of the three water regimes. Finally, position of the plants were randomized a last time on each table. The factor “table” was considered in the model to perform the statistical analysis.

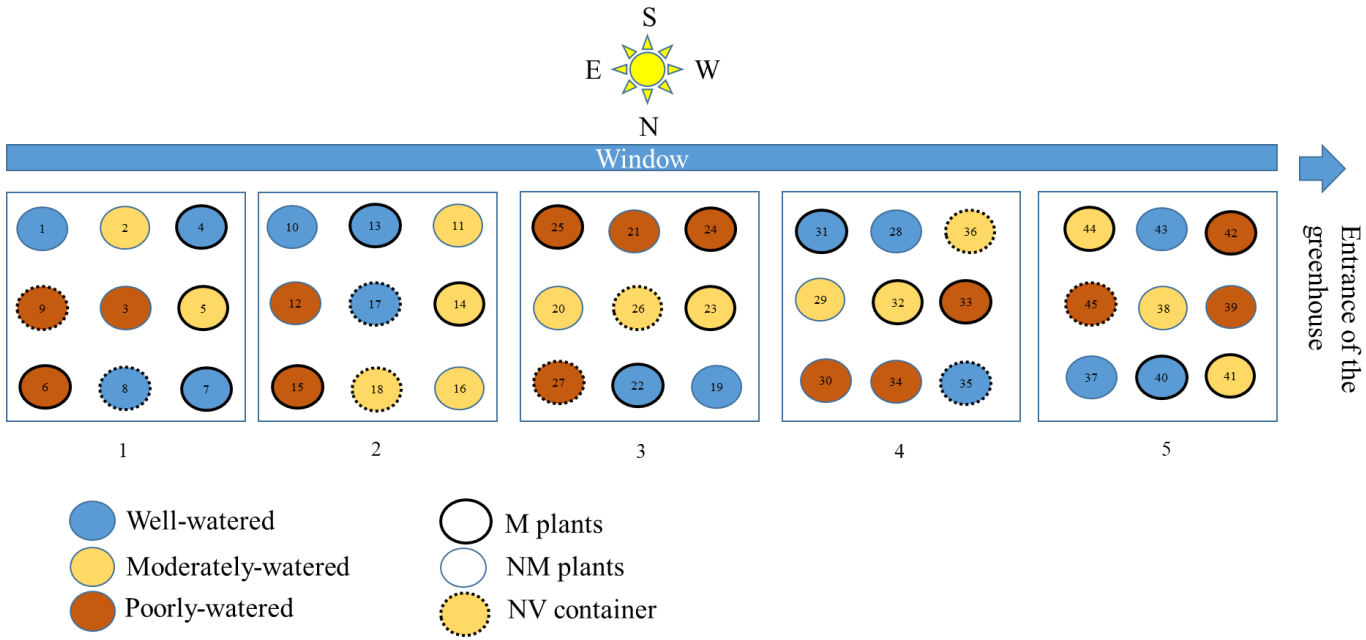

Figure 14 Experimental design of the greenhouse experiment with mycorrhizal (M) and non-mycorrhizal (NM) maize plants grown in a semi-hydroponic cultivation system. Each treatment is represented at least one time per table (labelled 1 to 5). NV= non-vegetated containers. Environmental factors potentially affecting maize growth (sun exposition and entrance of the greenhouse) are represented

4.8. Set up of the semi-hydroponic circulatory system

After one week of application of the water regimes on the acclimated maize plants the circulatory system was started a first time with Hoagland^{lowPi} solution for 42 h without any analysis. During this week, plants did not show any clear signs of wilting. The water regimes were thus applied for two more weeks before setting the circulatory system a second time. After these two weeks, MW and PW plants showed clear signs of drought as leaf wilting was observed in MW and PW plants but not in WW plants. Furthermore, photosynthetic activity and plant growth parameters were significantly lower in MW and PW plants when compared with WW plants (Appendix 4).

4.8.1. Flushing

Before the pump was started, a "flushing step" was conducted in order to equally saturate the substrate with the nutrient solution. Each container received 200 mL (4 aliquots of 50 mL) of Hoagland^{lowPi} solution. The solution was drained from the containers and discarded to a recipient using a silicon tube connecting the neck of the bottle with a multichannel peristaltic pump with a flow rate set at 44 mL min^{-1}.

4.8.2. Operation of the circulatory system

Each plant container (i.e. bottle) was connected via a silicon tube to a peristaltic pump and a 1 L glass bottle containing a Hoagland^{lowPi} solution (Figure 15b). Before starting the peristaltic pump, 15 ml of the Hoagland^{lowPi} solution was sampled from the glass bottle to later determine the concentration of Pi at time 0 h (T_0).

The pump was started at a flow rate of 7.4 mL min^{-1} allowing the nutrient solution to be pumped on the above part of the container and to percolate on the root system of the plant back to the nutrient solution bottle (Figure 15b).

4.8.3. Solution sampling

To determine the Pi concentration in the solution all peristaltic pumps were switched off. The tip of the silicon tubes providing the nutrient solution above the plant containers were then placed in a 15 ml falcon tube (VWR,

USA). The pumps were simultaneously started again and stopped when 15 ml of solution was collected in the tubes at times 9, 21 and 42h.

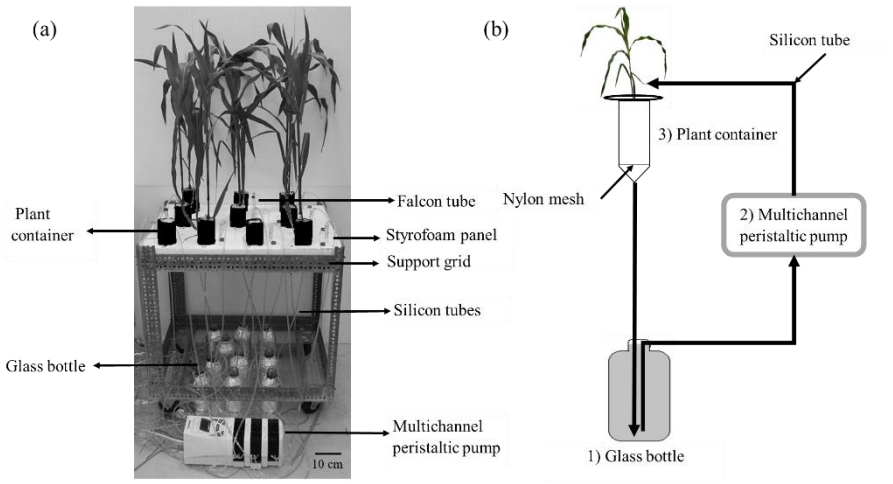

Figure 15 (a) Complete set-up of the semi-hydroponic circulatory system showing the position of the different elements. Both figures are adapted from Garcés-Ruiz *et al*. (2017). Aluminum foil around plant containers (i.e. bottles) were used instead of black plastic foils in the study of Garcés *et al*. (2017). **(b)** Schematic representation of the semi-hydroponic circulatory system. The dynamics of Pi depletion was assessed from a Hoagland^{lowPi5} solution circulating through containers with mycorrhizal (M) or non-mycorrhizal (NM) maize plants. The nutrient solution in the glass bottle (1) was pumped with a peristaltic pump (2) to the upper part of the container (3) via silicon tubes. The solution percolates through the plant container back into the glass bottle. Dark arrows indicate the direction of the flow of the nutrient solution in the tubing.

[5] Pi-impoverished modified Hoagland solution with Phosphorus concentration of 6.245 mg L^{-1} . For preparation see Appendix 3

4.9. Timeline of the experiment

To summarize, the maize seeds were germinated for three days, then associated or not with the AM fungus in 5 L pots for 21 days. The M and NM plants were then transferred in the semi-hydroponic cultivation system and acclimatized for 24 days. Three water regimes (WW, MW and PW) were then applied for one and two weeks respectively, both periods being separated by 42 h of recovery. At harvest, the plants were grown in the semi-hydroponic cultivation system for 49 days and were 73 days old (see Figure 16).

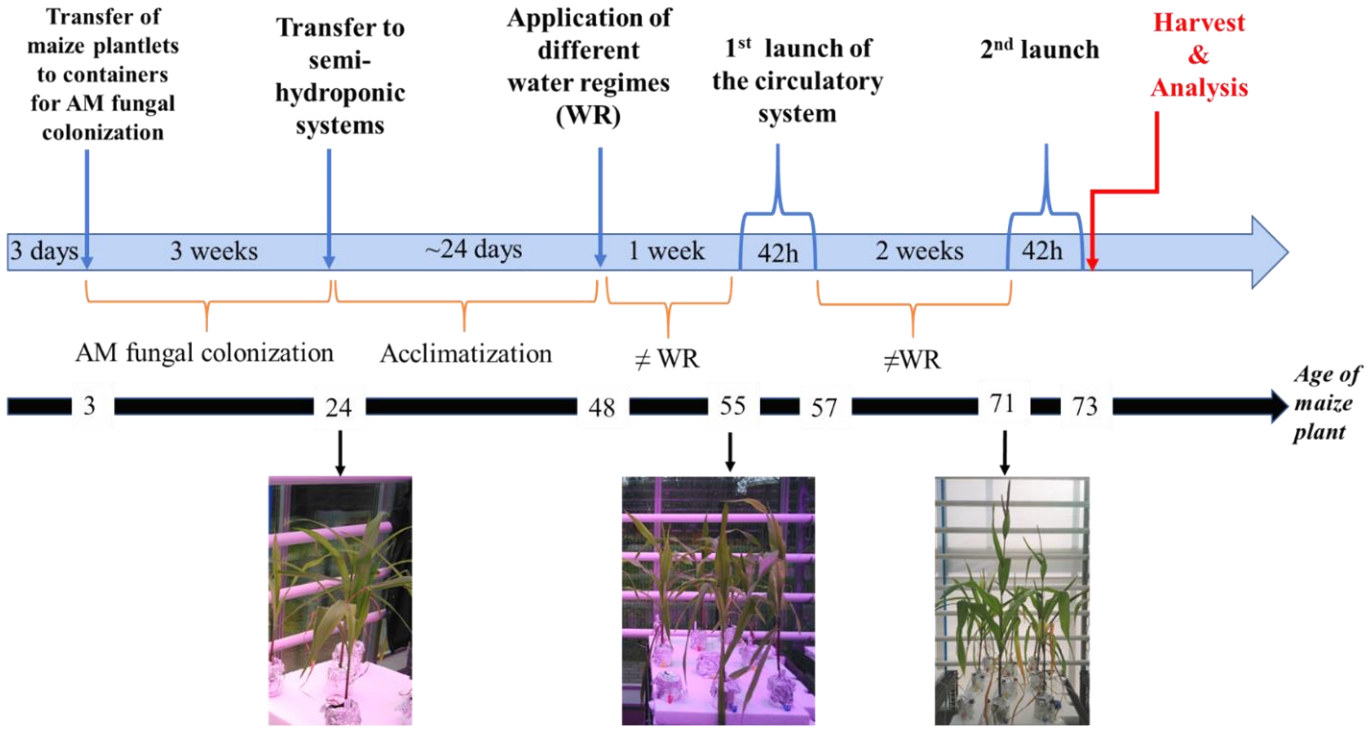

Figure 16 Timeline of the experiment in the greenhouse with maize plants. Represented are the main events of the experiment (blue arrows and brackets) and the duration of the different growth phases of maize (main thick blue arrow) under different growth conditions (orange brackets). The main thick black line indicates the age of the maize plants during the experiment and is illustrated with pictures of maize plants at ages 24, 55 and 71 days.

5. Measurements and evaluation of parameters

5.1. Evaluation of fungal parameters

5.1.1. Measurement of germ tube length

Germ tube length of *R. irregularis* MUCL 41833, *Rhizophagus intraradices* MUCL 49410, *Rhizophagus sp.* MUCL 43204 and *Rhizophagus clarus* MUCL 46238 was evaluated under a compound microscope (Olympus BH2-RFCA, Japan) at 40× magnification (Research results - Chapter 1) and again evaluated in *R. irregularis* MUCL 41833 under a binocular microscope (Olympus SZ, Olympus Optical, Germany) at 30× magnification (Research results - Chapter 2) by counting the number of intersections of the germ tube with a 1×1mm grid (Figure 17).

The grid was printed on a transparent plastic sheet and placed under the 24-well plate. The plate was put inverted under the compound microscope and the grid was positioned in order to have the spore in the center of a $1mm^2$ square (Figure 17). The germ tube length was estimated from the number of intersections with the grid using the formula of Newmann (1966):

Hyphal length (mm) = (NI × π ×A) / 2H

where

NI= Number of intersections with the grid,

A= Total area observed (A= area of the well of a 24-well plate = 1.9 mm^2)

H = Total length of the straight lines in the well 2H = 77.4 mm for a 1.9 mm^2 grid with squares of dimension 1 ×1 mm

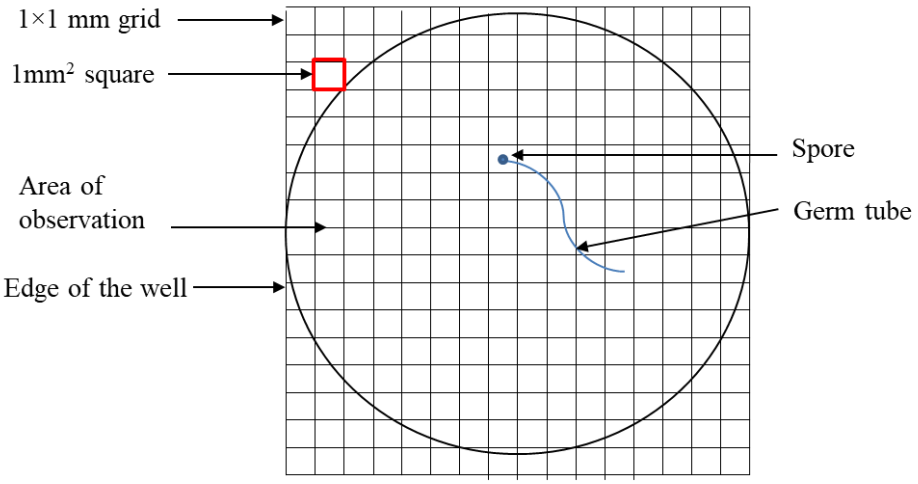

Figure 17 Representation of a germinated spore observed with a 1×1 mm grid placed under the 24-well plate.

5.1.2. Assessment of spore number and hyphal length

The number of spores and hyphal length of *R. irregularis* MUCL 41833 were evaluated in the HC of a whole plant *in vitro* culture systems (Research results - Chapter 2). For the sake of consistency and in order to limit the number of observations per Petri's plate, the measurements were restricted to three $1cm^2$ squares located in the middle of the HC, perpendicular to the partition wall and parallel to the growth direction of the hyphae (Figure 18). Hyphal length and number of spores were counted under the binocular microscope (Olympus SZ, Olympus Optical, Germany) at 40× magnification. Hyphal length (L) was estimated using Newman's formula modified by Marsh (1971):

$$L\ (cm) = N \times (11/14) \times \text{grid unit (cm)}$$

Where:

N is the number of hyphal intersections with the grid

11/14 is the ideal size of the grid sides that gives direct estimation of length in cm and grid unit = 1cm.

The number of spores was summed over the three squares (Declerck *et al*., 2003).

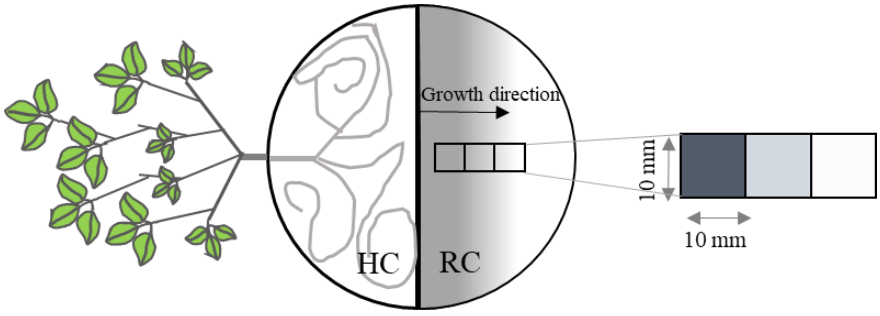

Figure 18 Localization of the area considered for the evaluation of the number of spores and length of hyphae in the HC of a whole plant *in vitro* culture system. Darker to lighter colors represent higher to lower density of hyphae and spores, respectively.

5.1.3. Evaluation of succinate dehydrogenase activity in hyphae

Succinate dehydrogenase activity was evaluated in the extraradical hyphae developing in the hyphal compartment under *in vitro* conditions (Research results - Chapter 2).

Principle

Succinate dehydrogenase (SDH) is an enzyme active in the inner mitochondrial membrane of Eukaryotes. It was first observed by McDonald and Lewis (1978) who used a histochemical staining method developed by Pearse (1968): Succinate is oxidized into fumarate by the SDH. This reaction releases two protons which reduces Nitro Blue Tetrazolium (NBT) into formazan, a blue dye (Figure 19). This technique has been used to visually detect metabolic activity in AM fungal in roots (Kough *et al*., 1987) as well as directly in hyphae (Saito, 1995; Zocco *et al*., 2011).

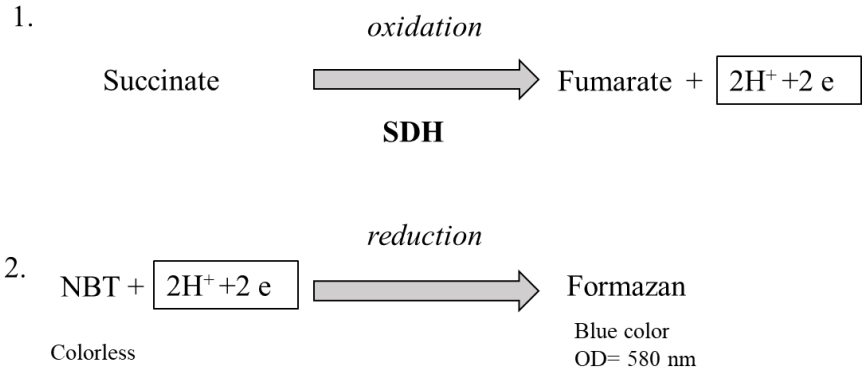

Figure 19 Chemical reaction responsible for the formation of the dark-blue coloration in tissues presenting succinate dehydrogenase (SDH) activity. 1. The SDH oxidizes the succinate into fumarate and releases two electrons and two protons which are captured by a coenzyme (not shown). 2. NBT which is colorless, captures the protons and electrons and is reduced into formazan, a blue compound.

Preparation of the sample

Composition of the root staining solutions and methodology is based on the protocol of Zocco *et al*. (2011). Hyphae were harvested and incubated overnight at room temperature in the dark in a freshly prepared staining solution containing Tris–HCl (50 mM), $MgCl_2$ (0.5 mM), sodium succinate (25.1 mM) and NBT (1.22 mM). The samples were then washed with deionized water, counterstained in 0.1% acid fuchsin in lactic acid for 15 min and transferred to lactoglycerol for de-staining. Hyphae samples were mounted on microscope slides with lactoglycerol. The precipitation of the substrate after enzymatic reaction was assessed under a bright field compound microscope (Olympus BH2-RFCA, Japan) at 400× magnification (Figure 20). For each sample, an approximate of 100 hyphal intersections was observed. According to Van Aarle *et al*. (2002), intersections of hyphae were classified as active or non-active following substrate precipitation or not.

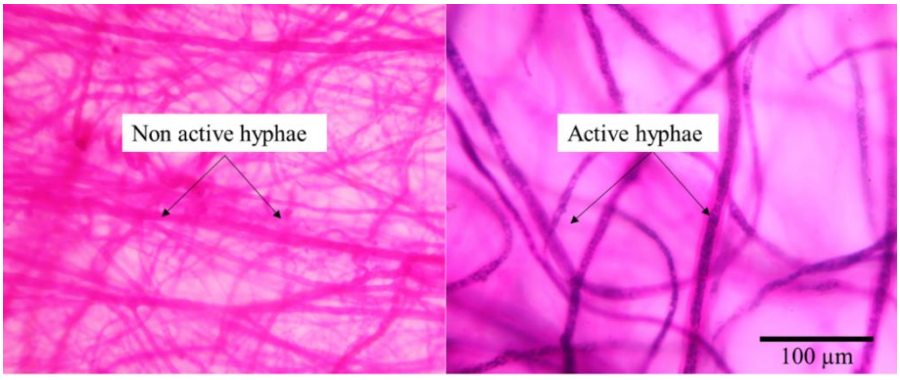

Figure 20 Histochemical staining of succinate dehydrogenase in arbuscular mycorrhizal hyphae. Dark vesicles indicate the presence of the SDH. Hyphae are observed under compound microscope (400×).

5.2. Assessment of plant parameters

5.2.1. Evaluation of plant biomass

Medicago truncatula and maize plants were dried before assessing their biomass and/or P contents and concentrations (Research results - Chapter 2 and 3).

Shoots and roots of *Medicago truncatula* plants were separated and their fresh weight was directly evaluated. Dry weights were evaluated after 48 h at 45°C in an oven. The dried shoots and roots material were ground for Pi assessment using ICP-AES (see protocol in Material and Methods - section 5.3.).

Maize shoot and roots fresh weights of each plant were evaluated and a leaf sample (approx.1x7cm) was cut from the 5th leaf to evaluate leaf relative water contents (RWC_L – see below). Shoots and roots were oven-dried at 50°C for seven days for evaluation of shoot and roots dry weights.

The root systems were subsequently separated in two parts to evaluate AM fungal root colonization and P concentration in tissues. After evaluation of dry weight, 500 mg of shoot and roots were ground separately for P analysis. Samples were digested following the procedure described in Garcés-Ruiz *et al*. (2017). Phosphorus concentration was quantified using ICP-AES.

RWC_L was determined at harvest from a sample of maize leaf (see above). After determination of the FW, the leaf sample was placed in a Petri dish containing water at 4°C in the dark for one night and turgid weight (TW) was estimated. Leaf samples were then oven-dried at 50°C for 4 hours and dry weight (DW) was estimated. The RWC_L was calculated using the equation of Barrs and Weatherley (1962): RWC_L (%) = (FW–DW)/(TW–DW) ×100.

5.2.2. AM fungal root colonization in maize

Preparation of the sample

Maize roots were stained for the evaluation of AM fungal colonization (Research results - Chapter 3) using the modified protocol of Vierheilig *et al*. (1998). Briefly, maize roots were cut in small pieces and incubated at 70°C for 1 h in a solution of KOH 10%. The KOH solution was removed and roots washed with HCl 1%. The roots were then incubated at 70°C for 1 h in a staining solution of ink 2% (Parker Blue Ink, USA) and HCl 1% (Walker, 2005). The roots were rinsed before observation. Roots that were not immediately observed were maintained in a solution of lactoglycerol (lactic acid/glycerol/water, 1:1:1) for longer storage.

Estimation of hyphal colonization

Percentages of total (%TC), arbuscules (%AC), and vesicules/spores (%VC) root colonization were estimated under a compound microscope (Olympus BH2–RFCA, Japan) at 100× magnification using a modified method described by McGonigle *et al*. (1990). Five pieces of stained roots were mounted parallel to each other on microscope slides (Figure 21) and 3 to 4 slides were prepared per sample. Around 100 intersections of the vertical line of the microscope eyepiece with the root axis were observed (Figure 21). At each intersection the presence or absence of each fungal structure (arbuscules, vesicles, spore, hyphae) was noted to determine the percentages of the respective structures. The total root colonization percentage was calculated by integrating only one of the fungal structures observed at each intersection.

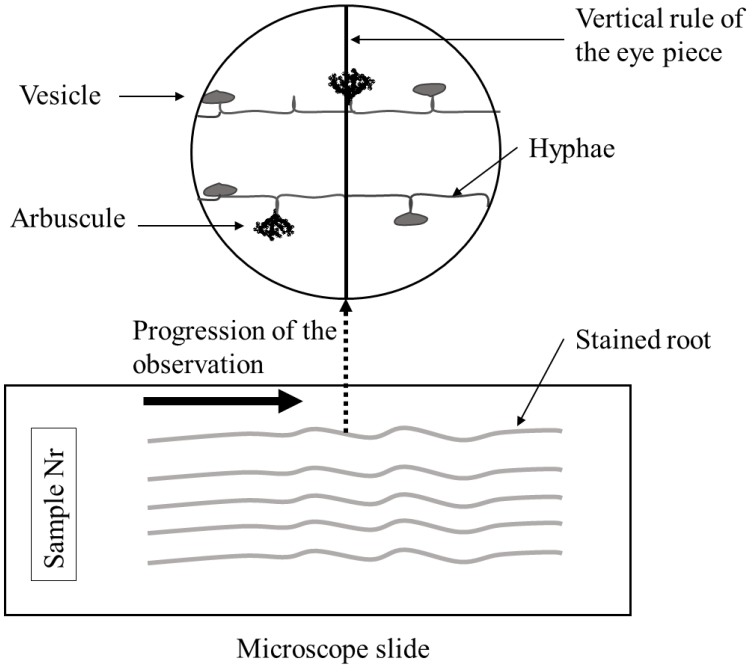

Figure 21 Observation of mycorrhizal structures (arbuscules, vesicles/spores, hyphae) in stained roots using the method described by McGonigle *et al*. (1990). The objective of the microscope was moved horizontally along the root and 100 intersections of the root with the vertical axis of the ocular was observed.

5.2.3. Assessment of leaf gas exchange parameters using the Infrared Gas Analyzer

An Infrared Gas Analyzer (IRGA) was used to measure the photosynthetic activity, transpiration rate and stomatal conductance from maize leaves (Research results - Chapter 3).

Principle

The IRGA technology has been used for decades to determine non-destructively gas exchange parameters from maize leaves (Meidner, 1961; Bird *et al*., 1977; Cramer *et al*., 1993; Bellasio and Grifitths, 2014; Wang *et al*., 2018). It measures CO_2 and H_2O concentrations by infrared absorption and a laser-trimmed humidity sensor, respectively, in the air flowing in and out of a chamber containing the leaf sample. Using several sets of formula, the CO_2 assimilation rate (A) is deduced from the difference in CO_2 concentration between in and out while the transpiration (E) and stomatal conductance (g_s) are calculated from the differences in H_20 concentrations (see instruction manual for more details). Besides that, the IRGA records other useful parameters such as the surface temperature and the photosynthetically active radiation (PAR) incident on leaf surface.

Measurement

In our experiments, we used the portative model (IRGA-LCi Photosynthesis system, ADC BioScientific Ltd) with the narrow chamber (5.8 cm^2 area) to measure the leaf gas exchange parameters in the 4 or 5^{th} fully expanded leaf (from maize plants bearing 7 to 9 expanded leaves) (Figure 22). To produce an air flow into the leaf chamber, the IRGA needs a fresh air supply whose CO_2 concentration is stable. During the measurements, the air probe was placed close to an open window in the corridor of the greenhouse. As the light intensity could affect photosynthetic activity in leaves (Bellasio and Grifitths, 2014), the measurements were done under day mode (with a photosynthetic photon flux (PPF) of 120 µmol m^{-2} s^{-1}) on maize leaves that were not shaded by other leaves. Finally, to avoid noise of the measurement, the leaf chamber jaw was hold on a tripod.

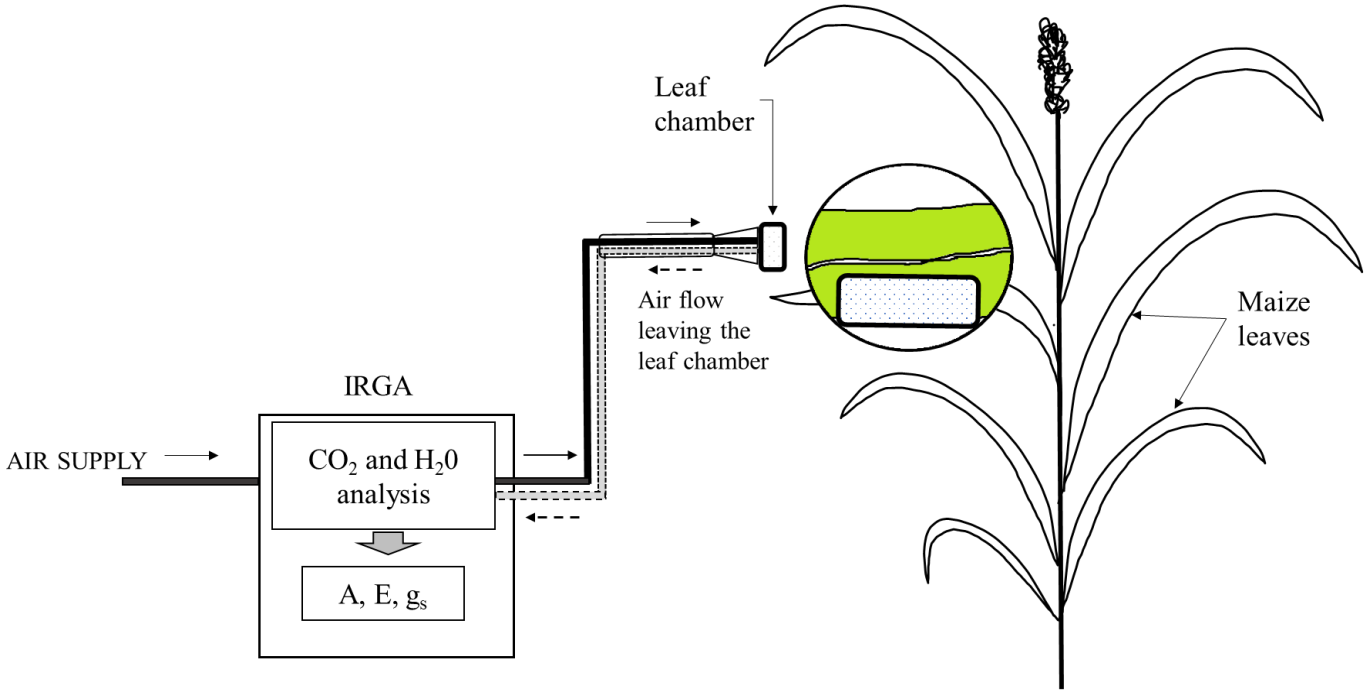

Figure 22 Representation of the measurement of CO_2 assimilation rate (A), transpiration (E) and stomatal conductance (g_s) from maize leaves using the Infrared Gas Analyzer. The air flowing in and out of the leaf chamber was analyzed for CO_2 and H_20 concentration. The narrow leaf chamber was placed parallel to the axis of the maize leaf without covering the central vein.

5.3. Analysis of P concentrations using the inductively coupled plasma atomic emission spectroscopy (ICP-AES).

P concentrations were analyzed in liquid MSR medium and *M. truncatula* plants (Research results - Chapter 2) as well as in Hoagland^{lowPi} solution and maize plant samples (Research results - Chapter 3).

Principle

The determination of the P concentration using ICP-AES is based on the analysis of the absorption spectrum of a gas-phase atom. The solution containing the element is nebulized and injected into an Argon plasma (ionized Argon particles). This plasma ionizes the atoms which reach higher energy levels. Atoms come back to lower energy state by releasing radiation of specific wavelength and intensity which are analyzed in the optical chamber (Figure 23).

Preparation of the samples

Preparation of plant samples

Oven dried, grinded shoots and root samples (500 mg samples were considered for maize and 200 to 540 mg samples for *M. truncatula*) were placed in 25 ml Erlenmeyer (Pyrex, United States), covered with a watch-glass and heated overnight at 60°C in 4 ml of nitric acid 65%. After an evaporation step at 120°C the samples were dissolved in 2 ml solution of aqua regia (3/4 HCl 38% for 1/4 HNO_3 65%). The solutions were neutralized with approximately 15 ml of purified MilliQ (MilliporeTM), filtered, transferred into a 50 ml volumetric flask and diluted to the mark with purified MilliQ water. The solutions were homogenized before analysis by ICP-AES (ICAP 6500, Thermo-Scientific, United States).

Preparation of liquid samples of Hoagland ^{lowPi} and MSR medium

Samples were directly diluted (1/10 and 1/4 for MSR medium and Hoagland^{lowPi} solution, respectively) and digested with 20 µl of nitric acid (65%, Suprapur®, Merck, Germany) before analysis by ICP-AES.

ICP-AES analysis specifics

The Pi content was determined with axial viewing of the emitted radiation. A peristaltic pump was used to introduce the solutions into the ICP-AES at a flow rate of 1.5 mL min^{-1}. Operating parameters for the instrument included forward power 1150 W, coolant gas flow rate 12 L min^{-1}, auxiliary gas flow rate 1 L min^{-1} and nebulizer gas flow rate 0.6 L min^{-1}. Pi quantification was analyzed under a wavelength of 177.495 l nm^{-1}. The limit of detection was <100 ppb. (Garcés-Ruiz *et al*., 2017).

Analysis of the data

Data obtained (in ppm) were converted in mg L^{-1} for liquid samples and mg kg^{-1} for plant samples. Analysis of Pi concentrations were conducted at regular intervals to monitor Pi depletion in Hoagland ^{lowPi} solution and MSR medium (Research results - Chapter 2 and 3). To monitor the Pi depletion, Pi concentrations were first normalized after the mean Pi concentration in the Controls without hyphae and without plants and transformed into % of Pi remaining in the solution which is the percentage of the Pi concentration remaining in the solution at time t relative to the Pi concentration in the solution at time t0:

$$\%\ of\ Pix = ([Pix]t + [Pic]t - [Pic]t0)/([Pix]\ t0 \times 100$$

where:

[Pix] = Pi concentration in the MSR medium or Hoagland ^{lowPi} solution for the sample x considered

t = time considered (x h after the start of the experiment)

t0 = 0h before the start of the experiment

[Pic] = Mean Pi concentration measured over 6 replicates in treatments without hyphae or without plants.

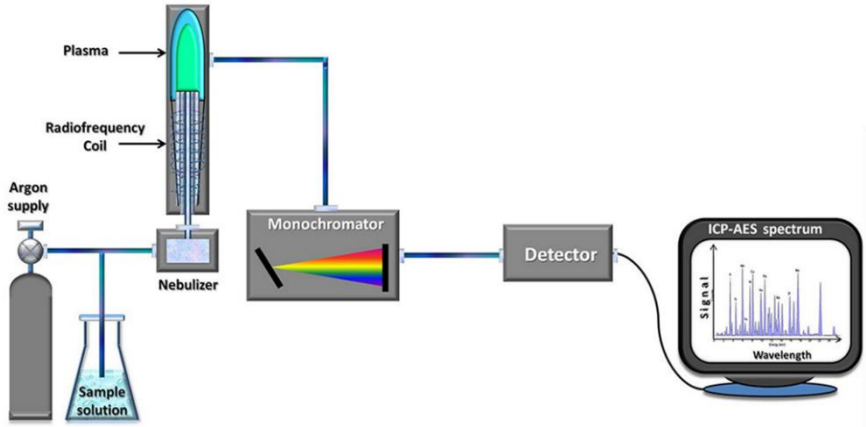

Figure 23 Schematic representation of the analysis of the concentration of an element using inductively coupled plasma atomic emission spectroscopy (ICP-AES) method (Adapted from : http://www.viaduct-diadrasis.net/img/methods/how_does/icpaes.jp). The sample solution is nebulized and mixed with argon gaz. The mix is then brought at high temperatures using a radiofrequency coil to turn the gas phase into a plasma. The absorption spectrum of the ionized molecules is analyzed using a monochromator coupled with a detector.

Research Results

Chapter 1

Spore germination of four arbuscular mycorrhizal strains show different responses to the addition of polyethylene glycol under *in vitro* conditions

Article in preparation

Olivia Le Pioufle and Stéphane Declerck

Preface

In vitro cultivation is an adequate method for the study of arbuscular mycorrhizal (AM) fungi under strict led conditions. The earliest *in vitro* experiments with AM fungi were conducted under axenic conditions, testing the impact of environmental factors on spore germination. Later, cultivation systems associating excised roots or whole plants in mono or bi-compartmented conditions extended the range of possible studies. In particular, the development of bi-compartmented Petri plates, enabling a physical separation of a AM fungal-colonized host plant located in a root compartment from a hyphal compartment only accessible to the extraradical mycelium, allowed to study the mycelium architecture and its transport capabilities under several stress (e.g. salinity, presence of hydrocarbons) conditions. The effects of drought on the physiology and development of AM fungi using *in vitro* systems is, however, complicated. Indeed, solid or liquid Modified Strullu-Romand (MSR) medium contains high amounts of water. It is thus necessary to develop strategies that mimic water deficit under *in vitro* cultivation systems. In this chapter, we presented a methodology using polyethylene glycol (PEG – molecular weight 8000 g mol^{-1}) to decrease the water potential in liquid or solid MSR medium. We discussed the possibilities and limits of this method and compared the impact of PEG on spore germination of four different AM fungal strains.

Abstract

Many studies have reported the benefits of arbuscular mycorrhizal (AM) fungi on plant resistance to decreased water availability. Conversely, the impacts of water deficit on the development and physiology of AM fungi remains poorly explored. Here, an *in vitro* cultivation system was used to investigate the effects of Polyethylene glycol (PEG 8000 - hereafter cited as PEG), used to decrease water availability in growth media, on spore germination and germ tube lengths of four AM fungal species. Prior to this, the impact of PEG on the water potential of the Modified Strullu-Romand (MSR) medium was explored as well as its effects on the gel polymerization. Four concentrations of PEG (50, 100, 250 and 400 g L^{-1}) and a Control without PEG were tested on the water potential of liquid MSR medium using a vapor pressure osmometer. When compared with the absence of PEG, the water

potential of the MSR medium decreased significantly at 250 and 400 g L^{-1} of PEG but not at 50 and 100 g L^{-1} of PEG. However, the addition of PEG at concentrations higher than 50 g L^{-1} opacified the gelled MSR medium and prevented its polymerization. Therefore the germination and germ tube length of spores belonging to four AM fungal strains (*Rhizophagus irregularis* MUCL 41833, *Rhizophagus intraradices* MUCL 49410, *Rhizophagus sp.* MUCL 43204 and *Rhizophagus clarus* MUCL 46238) were tested with two lower concentrations of PEG (25 and 50 g L^{-1}). The germination and germ tube length of spores belonging to the AM fungal strains were increasingly impacted with PEG concentrations ranging from no impact in the absence of PEG to decreased germination percentages and germ tube lengths at 50 g L^{-1} of PEG. The strains MUCL 49410 and MUCL 46238 seemed more tolerant than MUCL 41833 and MUCL 43204 as their response were the least affected. The water potential PEG could thus potentially be used to discriminate AM fungal species/strains on their resistance/tolerance to decreased water availabilities and possibly on their subsequent effects on plant physiology (e.g. transport of nutrients or water from soil to plant). It is suggested that the method used to measure the water potential lacked sensitivity, especially at PEG concentrations lower than 100 L^{-1} and/or that PEG could modify other characteristics of the growth medium (e.g. viscosity).

Introduction

Very little is known on the impact of decreased water availability on the development (e.g. mycelium architecture, spores production…), physiology and functions (e.g. modulation of host defense, transport of nutrients) of arbuscular mycorrhizal (AM) fungi. Interestingly, it has been shown that plants associated with AM fungi originating from dry environments, and thus theoretically more drought resistant, had higher leaf growth (Ruiz-Lozano *et al*., 1995), better water use efficiency (Ruiz-Lozano *et al*., 1995), improved leaf gas exchange and water status (Querejeta *et al*., 2006) as well as transpiration, P and N nutrition (Symanczik *et al*., 2018) as compared with AM fungi from temperate climates. While the mechanisms still remains to be fully explored, the ability of some AM fungal strains isolated from arid environments to enhance soil water uptake by plants has been related with the amount of extraradical mycelium developed in the soil (Marulanda *et al*., 2003).

The *in vitro* culture of AM fungi in mono-compartmented Petri plates provides a practical way to produce contaminant-free cultures, while their growth in bi-compartmented Petri plates allows to visualize the development of extraradical mycelium (ERM) networks in the absence of any direct influence of roots and to study processes such as phosphorus transport to plants. Interestingly, with bi-compartmented Petri plates it is possible to apply a chemical stress solely on the ERM located in a root-free compartment. Such system thus represent an innovative avenue to investigate the effects of decreased water availability on various aspects of the AM fungus and thus plant-fungus relationships.

Drought stress can be applied using various chemicals. Among them, polyethylene glycols (PEGs), also known as polyethylene oxides in polymer chemistry are synthetic polymers composed of an assemblage of n-ethylene glycol monomers giving them variable properties. PEGs are highly hydrophilic and also highly soluble in organic solvents (Dinç *et al*., 2010). Because they are inert and increase the viscosity of the solution (Jerome *et al*., 1968; Michel and Kaufmann, 1973), they are widely used in the industry (e.g. thickener in cosmetic products, ink solvent). PEGs also decrease the water potential of aqueous solutions (Michel and Kaufmann, 1973; Steuter *et al*., 1981). As they cannot enter the cell wall pores at molecular weights higher than 6000 g mol^{-1} (PEG 6000) (Carpita *et al*., 1979) they are non-toxic to

plants and better mimic the effect of drying soil on plant tissues by causing cytorrhysis (loss of water from cell wall and protoplast) instead of plasmolysis (loss of water the from protoplasm only) (Verslues *et al.*, 2006).

For these reasons PEGs have been largely used in aqueous solutions to simulate drought stress in plant physiological studies (Michel and Kaufmann, 1973). They have been used in nutrient solutions for plant growth in pot culture (Ruiz-Lozano and Azcón 1997; Hernández-Sebastià *et al*., 2000; Vivas *et al*., 2003; Türkan *et al*., 2005; Hosseini *et al*., 2019) as well as in liquid (Bajji *et al*., 2004) and solid (Dami and Hughes, 1995, 1997; Van der weele *et al*., 2000; Verslues *et al*., 2004) culture medium for *in vitro* studies.

A limited number of studies have reported the impact of PEGs on mycorrhizal fungi. For instance, Mexal and Reid (1973) and Zhang *et al*. (2011) used PEGs to test the impact of a decreased water potential on the development of ectomycorrhizal fungi, while in AM fungi, Porcel *et al*. (2007) and Li *et al*. (2013) used PEGs in liquid growth medium to investigate the expression of genes coding for the 14-3-3 protein and for aquaporins, respectively. Therefore, it seems that PEGs could be successfully used to study the effects of decreased water availability on the growth of *in vitro* cultured AM fungi, in particular in gelled medium. However, the introduction of PEGs in gelled medium can raise two problems. Firstly, as polymers, it is not excluded that PEGs can interfere with the polymerization of the gelling agent added to the growth medium. Secondly, sterilization via autoclaving of the growth medium containing PEGs is not possible since their fusion temperature is relatively low (e.g. 60 °C for PEG 8000) (Majumdar *et al*., 2010). As mentioned previously, PEG has been successfully added into gelled medium using a technique of "infused plates" (Van der weele *et al*., 2000; Verslues *et al*., 2004). Briefly, plates containing the gelled medium are infused over night with an overlay solution added with PEG (Van der weele *et al*., 2000; Verslues *et al*., 2004). To measure the water potential in PEG gels the authors advise to use a psychrometer or a vapor pressure osmometer (VPO) (Van der weele *et al*., 2000; Verslues and Bray, 2004). While the first apparatus gives a direct estimation of the total water potential (ψ), in the second apparatus, the difference in vapor pressure of a sample is measured, translated into a corresponding osmolarity and finally converted by the experimenter into an osmotic ψ (ψ_o) using the following equation (Money, 1983) : ψ_o= iCRT. Authors considered thus that the water potential in a gelled medium added with PEG was mainly constituted of an osmotic potential.

Indeed, it is probable that water potential of gels is mainly constituted by osmotic forces as the matric component in the gelled medium accounted for only 1-2% of the total ψ measured (Beruto *et al.*, 1995). Moreover, most of the studies considered that PEGs decrease the water potential of aqueous solutions by decreasing the osmotic potential (Michel and Kaufmann, 1973; Michel, 1983; Money, 1989). Therefore numerous studies so far have used a VPO to determine the ψ / ψ_o in PEGs solutions under *in vitro* conditions (e.g. Wang *et al*., 2005; Caruso *et al*., 2008).

The present study aimed at evaluating the effect of decreasing water availability on spore germination of four different AM fungal strains under *in vitro* conditions using PEG. To do so we first evaluated the effects of different concentrations (0, 50, 100, 250 and 400 g L^{-1}) of PEG of molecular weight of 8000 g mol^{-1} (PEG 8000) on the water potential of liquid MSR medium by determining the osmolarity and osmotic potential using a VPO. We then prepared gelled MSR medium at the same PEG concentrations using a novel protocol with PEG added after autoclaving of the medium. Based on the results of polymerization of the medium at these concentrations, we finally tested the impact of PEG at concentrations 25 and 50 g L^{-1} on the germination rate and germ tube length of four AM fungal strains (*R. irregularis* MUCL 41833, *R. intraradices* MUCL 49410, *R. sp* MUCL 43204 and *R. clarus* MUCL 46238).

Material and methods

Biological material

The AM fungi *R. irregularis* (Błaszk., Wubet., Renker and Buscot) C. Walker and A. Schüßler comb. nov. MUCL 41833, *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler 2010 MUCL 49410, *Rhizophagus sp.* MUCL 43204 and *Rhizophagus clarus* (T.H. Nicolson & N.C. Schenck) C. Walker & A. Schüßler MUCL 46238, were supplied by the Glomeromycota *in vitro* collection (GINCO – https://www.mycorrhiza.be/ginco-bel/catalogue.php). The fungi were grown in bi-compartmented Petri plates (90 mm diameter) associated with transformed roots of carrot (*Daucus carota* L.) (see Material and methods – section *3.1. Root organ cultivation of R. irregularis MUCL 41833 for mass production of inoculum*).

Assessment of water potential of liquid MSR medium supplemented with PEG.

Liquid MSR medium without Gelrite, sucrose and vitamins but supplemented with increasing concentrations of PEG was prepared to determine the decrease of water potential in the MSR medium.

Aliquots of 5, 10, 25 and 40 g of PEG 8000 (hereafter cited as PEG) powder (necessary to reach final PEG concentrations of 50, 100, 250 and 400 g L^{-1} of MSR medium respectively, in 100 ml of MSR medium) were weighted in a sterilized 250 ml Erlenmeyer using a spoon cleaned with EtOH 99%. The Erlenmeyers were then immediately sealed with aluminum foil. In parallel, MSR medium without Gelrite, sucrose and vitamins was autoclaved (121°C for 15 min) and left to cool down at ambient temperature. Under laminar flow, 100 ml of liquid MSR medium was poured in each of the Erlenmeyers containing the PEG powder. The solutions were mixed using a sterilized magnetic rod until complete dissolution of the PEG. The Erlenmeyers containing the solutions were then sealed and ready to use for water potential measurements.

A vapor pressure osmometer (VAPRO® 5520, Wescor Inc., Logan, UT, USA) which measures the dew point temperature of the sample and translates it into osmolarity was used to assess ψ_o which is an estimation of the total ψ. The apparatus was calibrated and the readings were made in the morning (from 8 to 12 h) to avoid any large modifications in the temperature of the room. Briefly, 10 µl of the liquid MSR medium in absence of PEG or with PEG concentrations of 50, 100, 250 and 400 g L^{-1} was pipetted on a small paper filter disc (Wescor Inc ., Logan, UT, USA) placed in the measuring chamber. The chamber was closed and osmolarity recorded after 80 s. Osmotic potentials were calculated from the osmolarities similarly as in Money (1983) using the following formula: ψ_o= iCRT , where *i* is the dimensionless van 't Hoff index, *C* is the molar concentration of solute, R is the ideal gas constant, and T is the temperature in kelvins. For each concentration of PEG, a minimum of three replicates were considered. Response curve of water potential to PEG concentrations was finally fitted using a quadratic model in the JMP statistic software (JMP® Pro 14.0.0) from SAS. Quality of fit between the model and the data was evaluated by computation of the R^2.

Impact of PEG on the polymerization of the gelled MSR medium

MSR medium without sucrose and vitamins and containing 3 g L^{-1} of Gelrite with different concentrations of PEG (50, 100, 250 and 400 g L^{-1}) were prepared to test the effect of this molecule on the polymerization and transparency of the medium.

Aliquots of 5, 10, 25 and 40 g of PEG powder necessary to reach a final concentration of 50, 100, 250 and 400 g L^{-1} of PEG, respectively, were prepared using the same protocol as presented for the medium without Gelrite. MSR medium with 3 g L^{-1} of Gelrite, without sucrose and vitamins was autoclaved (121°C for 15 min) and left to cool down to a temperature comprised between 45 and 50°C. Meanwhile, a heating-stirring plate (IKA C-Mag HS 7, Germany) was disposed under laminar flow, cleaned with EtOH 99% and set at a temperature of 50°C. One hundred ml of medium was poured in the Erlenmeyer containing the PEG powder. The solution was then immediately left to agitate on the heating block until complete dissolution of the PEG. During this step, the temperature of the solution was kept between 45 and 55°C by adjusting the heat and the rotating speed of the magnetic rod. Using a sterile 25 ml pipette, 30 ml of each MSR medium at different concentrations of PEG was poured in four separate 90 mm Petri plates and left to solidify under laminar flow. Visual observation of Petri plates after three days and in the following days did not reveal any contamination.

Spore germination and germ tube growth of AM fungal strains MUCL 41833, 49410, 43204 and MUCL 46238 in response to PEG addition in the gelled MSR medium

MSR medium without sucrose and vitamins and containing 3 g L^{-1} of Gelrite without PEG (Control) or supplemented with 25 or 50 g L^{-1} PEG (PEG^{25} and PEG^{50} treatments, respectively) was prepared following the same protocol as described above. After the dissolution of the PEG in the medium, 2 ml of the medium was immediately pipetted in each well of a 24-well tissue culture plate (VWR, Radner, PA, USA). One plate per AM fungal strain and per PEG concentration was considered. The plates were left open to dry under laminar flow for 1 h, closed and checked after two days for contamination.

Spores of a two-month-old *in vitro* culture of each AM fungal strain were isolated from the MSR medium following solubilization of the gelled medium with 10 mM filter-sterilized sodium citrate buffer (Doner and Bécard, 1991). Spores were separated with forceps and placed single with a micropipette in each well of the corresponding 24-well tissue culture plates. They were then sealed with cellophane (Toppits®, Melitta Group, Germany) and incubated in the dark at 27°C. A minimum of 23 spores and a maximum of 42 spores were considered for each strain and PEG treatment. Spore germination was estimated at regular intervals for 50 days and hyphal length of germinated spores was evaluated 30 and 50 days after the start of the experiment under compound microscope at 40× magnification.

Statistical analysis

Data analysis was performed with JMP software (SAS Institute Inc., Cary, NC, United States) with a 5% α-threshold. Water potentials were tested using a one-way analysis of variance (ANOVA1) followed by a post hoc Tukey test. Levene's test was run prior to the ANOVA analysis to confirm homogeneity of variance in the distribution. For each fungal strain, germination percentages at 15, 30 and 50 days were submitted to a Chi-square test, while germ tube lengths at 30 and 50 days were submitted to a Wilcoxon test. To compare germination percentages among AM fungal strains at 15, 30 and 50 days and for each PEG treatment, the mean values in PEG^{25} and PEG^{50} were expressed as percentages relative to the Control and submitted to a Chi-square test. To compare germ tube length among AM fungal strains at 30 and 50 days, for each PEG treatment the values in PEG^{25} and PEG^{50} were normalized to the cControl and submitted to a Wilcoxon test. All pairwise comparisons following chi-square and Wilcoxons tests were analyzed using Bonferroni's correction.

Results

Measurements of water potentials of liquid MSR medium supplemented with increasing concentrations of PEG

Water potentials of liquid MSR medium supplemented with increasing concentrations of PEG are presented in Figure 24. When compared to the Control (without PEG), the water potential was significantly decreased at 250 and 400 g L^{-1} of PEG (−0.82 and −1.79 MPa, respectively), while it remained almost unchanged at 50 (−0.13 MPa) or 100 g L^{-1} (−0.21 MPa) of PEG. It was significantly lower in the MSR medium at 250 and 400 g L^{-1} of PEG when compared with the MSR medium at 50 and 100 g L^{-1} of PEG and significantly lower in the MSR medium at 400 g L^{-1} of PEG when compared to the MSR medium at 250 g L^{-1} of PEG. Water potential was decreased in a similar range as described by Michel (1983) (see Figure S1 at the end of Chapter1). Data could be fitted to a quadratic model ($y = -0.151x^2 + 4.45.10^{-5}.\ x - 1.04.10^{-5}$, where x is PEG concentration) with a high coefficient of determination ($R^2 = 0.991$).

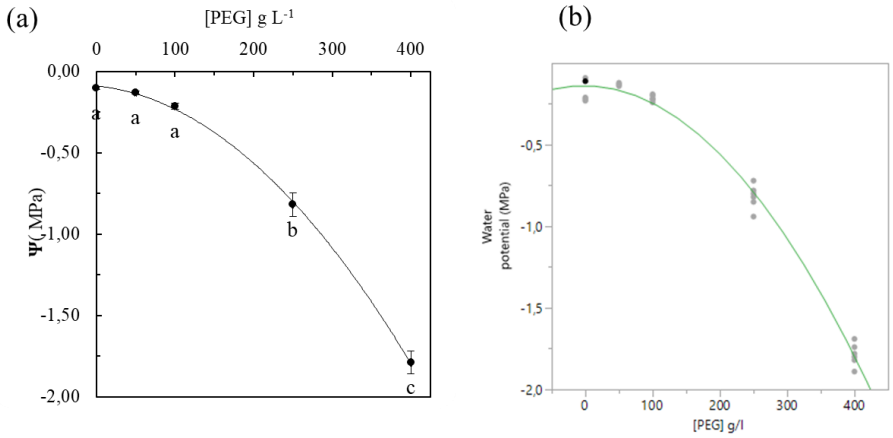

Figure 24 Water potentials (Ψ) of liquid MSR medium in absence (0 g L^{-1}) or supplemented with increasing (i.e. 50, 100, 250 and 400 g L^{-1}) concentrations of PEG, measured with a vapor pressure osmometer. **(a)** Mean water potentials with standard deviations. Different letters stand for significant differences between treatments using pairwise comparisons (Tukey test, $P<0.05$). **(b)** Regression curve of the response of water potential to increasing PEG concentrations. Values were fitted using a quadratic model. Dots represent single values.

Polymerization of MSR medium at increasing concentrations of PEG

The addition of PEG interfered with the polymerization of Gelrite in the MSR medium, particularly at the highest concentrations. Gel consistency was indeed too soft at 250 and 400 g L^{-1} of PEG and was breaking easily. The opacity of the gel also increased proportionally with the concentration of PEG. Observation through the gel using a compound microscope was not possible above 50 g L^{-1} of PEG in the MSR medium.

Spores germination of AM fungal strains MUCL 41833, 49410, 43204 and 46238 in response to increasing concentration of PEG in the gelled MSR medium

Effect of PEG on the percentages of spore germination of AM fungal strains

The impact of 25 or 50 g L^{-1} PEG in the MSR medium on the percentage of spore germination percentage of AM fungal strains is described in Figure 25. After 15 days, PEG significantly affected the percentage of spore germination of the four AM fungal strains ($P<0.0001$). The percentage of spore germination of MUCL 43204 was significantly lower for PEG^{25} and PEG^{50} treatments when compared with the Control treatment. It was also significantly lower for the PEG^{50} treatment when compared with PEG^{25} treatment. For MUCL 41833, the percentage of spore germination was significantly lower in the PEG^{50} treatment when compared with the Control and PEG^{25} treatments, while no difference was noticed between these last two treatments. For MUCL 46238, the percentage of spore germination was significantly lower for PEG^{25} and PEG^{50} treatments when compared with the Control treatment and no significant difference was found between these last two treatments. For MUCL 49410, no significant difference was found between the Control and PEG^{50} treatments but PEG^{25} treatment was significantly lower when compared with these two treatments. After 30 days, PEG significantly affected the percentage of spore germination of MUCL 41833 and 43204 ($P<0.0001$), MUCL 46238 ($P= 0.0026$) and MUCL 49410 ($P=0.0154$). The percentage of spore germination of MUCL 41833 and MUCL 43204 were significantly decreased under PEG^{50} treatment as compared to the Control and PEG^{25} treatments. The percentage of spore germination of these last two treatments were similar. For MUCL 46238, the

percentage of spore germination was significantly lower in the PEG^{50} treatment when compared to the Control treatment while no difference was found between PEG^{25} treatment and the other treatments. Finally, no difference was noticed for MUCL 49410. After 50 days, PEG significantly affected the percentage of spore germination of MUCL 41833 and 43204 ($P<0.0001$), MUCL 46238 ($P=0.0321$) and MUCL 49410 ($P=0.0097$). The percentage of spore germination for MUCL 41833 was significantly decreased under PEG^{50} treatment as compared to the Control treatment. No significant difference was found between PEG^{25} treatment and the other treatments. For MUCL 43204, the percentage of spore germination was significantly decreased in the PEG^{50} treatment as compared to the Control and PEG^{25} treatments. No difference was noticed between the Control and PEG^{25} treatments. Finally, PEG treatments did not affect significantly the percentage of spore germination for MUCL 46238 and MUCL 49410.

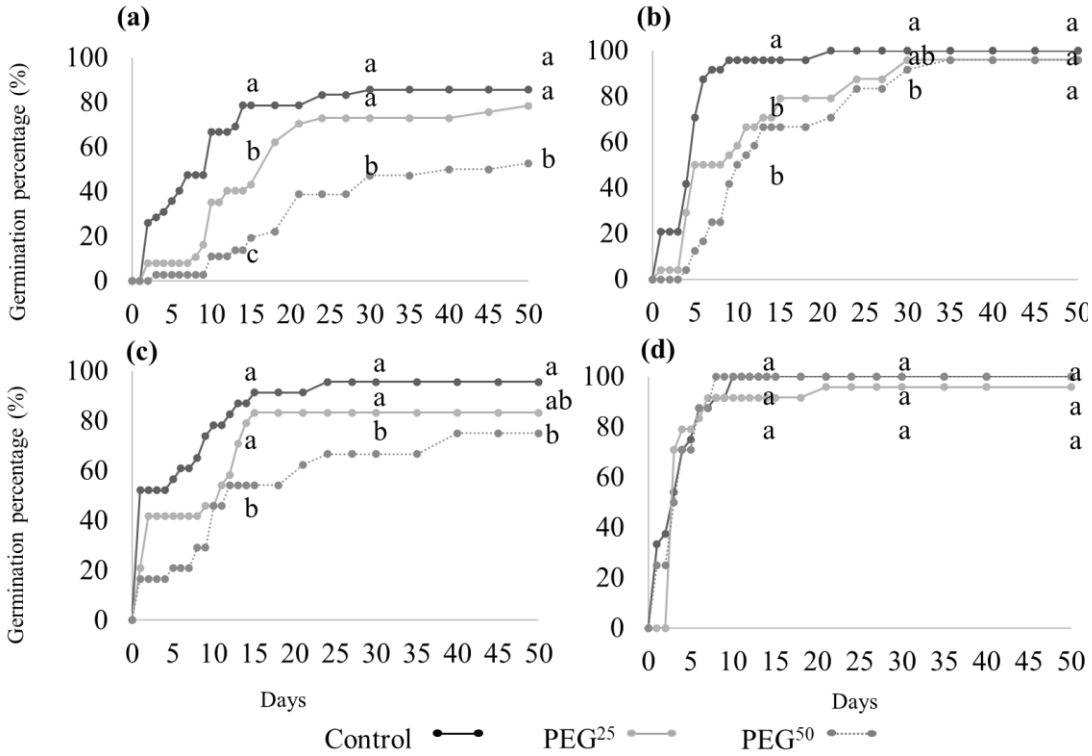

Figure 25 (a-d) Percentages of spore germination of four strains of *Rhizophagus* monitored during 50 days. **(a)** *R. sp* MUCL 43204, **(b)** *R. clarus* MUCL 46238, **(c)** *R. irregularis* MUCL 41833, **(d)** *R. intraradices* MUCL 49410. Spores were germinated on MSR medium without sucrose in absence of PEG (Control) or in presence of 25 or 50 g L^{-1} of PEG (PEG^{25} and PEG^{50} treatments, respectively). Different letters stand for significant differences between treatments using pairwise comparisons and Bonferroni's correction (Chi-square test, $P<0.017$, n=23 - 42)

Comparison of percentages of spore germination between AM fungal strains

In the absence of PEG (Control treatment), significant differences in the percentage of spore germination were found among the AM fungal strains on days 15, 30 and 50 ($P<0.0001$) (Table 4). After 15 days, the percentage of spore germination was significantly lower for MUCL 43204 when compared with MUCL 49410 and MUCL 46238 but did not differ with MUCL 41833. It was significantly decreased in MUCL 41833 when compared with MUCL 49410 but not with MUCL 46238. No significant difference was found between MUCL 49410 and MUCL 46238. After 30 days, the percentage of spore germination of MUCL 49410 and MUCL 46238 was significantly higher when compared to MUCL 43204 and MUCL 41833. No significant differences were found between MUCL 49410 and MUCL 46238 or between MUCL 43204 and MUCL 41833. After 50 days, the percentage of spore germination of MUCL 49410 and MUCL 46238 was significantly higher when compared to MUCL 43204 but not with MUCL 41833. No significant differences were found among MUCL 49410, MUCL 46238 and MUCL 41833.

The addition of PEG to the MSR medium impacted the percentage of spore germination of the strains and this effect differed with time and strain (Figure 26).

On day 15, the percentage of spore germination was significantly different among strains in the PEG^{25} and PEG^{50} treatments ($P<0.0001$). In the PEG^{25} treatment, the percentage of spore germination of MUCL 49410 and MUCL 41833 were significantly higher when compared with MUCL 43204 and did not significantly differ with MUCL 46238. No significant difference was found between MUCL 49410 and MUCL 41833 or between MUCL 43204 and MUCL 46238. In the PEG^{50} treatment, the percentage of spore germination was significantly higher for MUCL 49410 when compared to the other strains. The percentage of spore germination was also significantly higher for MUCL 41833 and MUCL 46238 when compared with MUCL 43204 and did not significantly differ among them.

On day 30, the percentage of spore germination was significantly different among strains in the PEG^{25} and PEG^{50} treatments ($P<0.0001$). In the PEG^{25} treatment, the percentage of spore germination was significantly higher

for MUCL 49410 and MUCL 46238 when compared with MUCL 43204 and MUCL 41833. No significant difference was found between MUCL 49410 and MUCL 46238 or between MUCL 43204 and MUCL 41833. In the PEG^{50} treatment, the percentage of spore germination was significantly higher for MUCL 49410 when compared with MUCL 46238, in MUCL 46238 when compared with MUCL 41833 and significantly higher in MUCL 41833 when compared with MUCL 43204.

On day 50, the percentage of spore germination was significantly different among strains in the PEG^{25} and PEG^{50} treatments ($P<0.0001$). In the PEG^{25} treatment, the percentage of spore germination was significantly higher for MUCL 49410 and MUCL 46238 when compared with MUCL 43204 and MUCL 41833. No significant difference was found between MUCL 49410 and MUCL 46238 or between MUCL 43204 and MUCL 41833. In the PEG^{50} treatment the percentage of spore germination was significantly higher for MUCL 49410 and MUCL 46238 when compared with MUCL 43204 and MUCL 41833 while no significant difference was found between MUCL 49410 and MUCL 46238. The percentage of spore germination was significantly higher for MUCL 41833 when compared with MUCL 43204.

Table 4 Percentage of spore germination of the AM fungal strains *R. irregularis* MUCL 41833, *R. sp* MUCL 43204, *R. clarus* MUCL 46238, and *R. intraradices* MUCL 49410 in the absence of PEG (Control treatment), measured on days 15, 30 and 50 of the experiment.

Strain		Percentage spore germination (%)		
		15 days	**30 days**	**50 days**
R. irregularis	MUCL 41833	91.30 ^{bc}	95.65 ^{B}	95.65 ^{ab}
R. sp	MUCL 43204	78.57 ^{c}	85.71 ^{B}	85.71 ^{b}
R. clarus	MUCL 46238	95.83 ^{ab}	100 ^{A}	100 ^{a}
R. intraradices	MUCL 49410	100 ^{a}	100 ^{A}	100 ^{a}

Different letters stand for significant differences between treatments using pairwise comparisons (Wilcoxon test, $P<0.0082$).

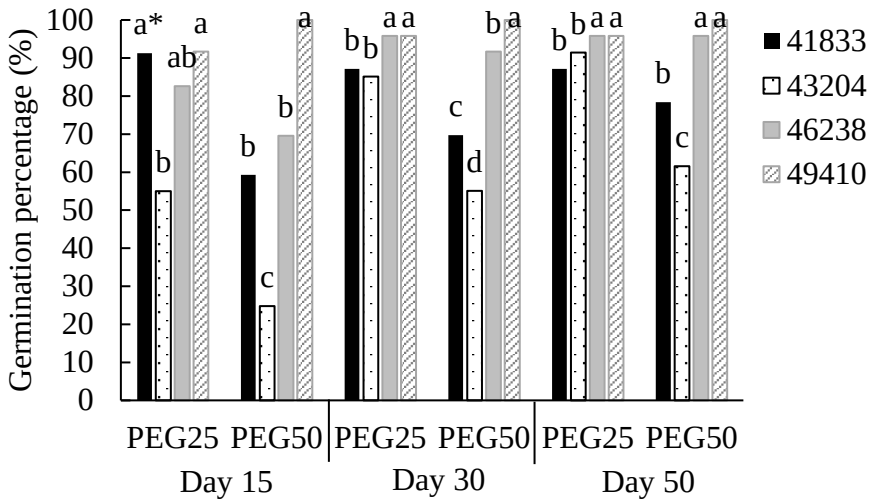

Figure 26 Percentage spore germination under increasing concentration of PEG (25 and 50 gL^{-1}, PEG^{25} and PEG^{50} treatments, respectively) relative to the Control treatment. Germination of *R. irregularis* MUCL 41833, *R. sp* MUCL 43204, *R. intraradices* MUCL 49410 and *R. clarus* MUCL 46238 was assessed 15, 30 and 50 days after the beginning of the experiment. ***** Significant differences between strains were tested independently for each PEG treatment and day. Different upper-case letters stand for significant differences between the different strains, using pairwise comparisons (Wilcoxon test, $P<0.0082$, $n\geq14$).

Germ tube length of AM fungal strains MUCL 41833, 49410, 43204 and 46238 in response to PEG addition in the gelled MSR medium

Effect of the PEG treatment on the spore germ tube length of the AM fungal strains

The Figure 27 presents the impact of PEG on the mean germ tube length of the spores of the four strains on days 30 and 50. On day 30, germ tube length of spores was impacted by the PEG treatment for MUCL 46238, MUCL 41833 and MUCL 43204 ($P<0.0001$) and MUCL 49410 ($P=0.0164$). The germ tube length of spores of MUCL 41833 was significantly decreased in the PEG^{25} and PEG^{50} treatments when compared with the Control treatment.

No difference was found between the two PEG treatments. For MUCL 43204 and MUCL 46238, germ tube length of spores was significantly decreased in the PEG^{50} treatment when compared with the Control and PEG^{25} treatments and significantly decreased in the PEG^{25} treatment when compared with the Control treatment. Finally, for MUCL 49410, germ tube length of spores was significantly increased in the PEG^{25} treatment when compared with the PEG^{50} treatment but did not differ with the Control treatment. No significant differences were found between then PEG^{50} and Control treatments.

On day 50, germ tube length of spores was also impacted by the PEG treatments for MUCL 41833 and MUCL 43204 ($P<0.0001$), MUCL 46238 ($P=0.0011$) and MUCL 49410 ($P=0.0113$). The germ tube length of spores of MUCL 41833 and MUCL 46238 were significantly decreased in the PEG^{50} and PEG^{25} treatments as compared with the Control treatment while germ tube length of spores in the two PEG treatments were similar. For MUCL 43204, germ tube length of spores was significantly decreased in the PEG^{50} treatment when compared with the Control and PEG^{25} treatments, and significantly decreased in the PEG^{25} treatment when compared with the Control treatment. Finally, germ tube length of spores was significantly decreased in the PEG^{50} treatment when compared with the Control treatment but not when compared with the PEG^{25} treatment. No significant differences were found between the Control and PEG^{25} treatments.

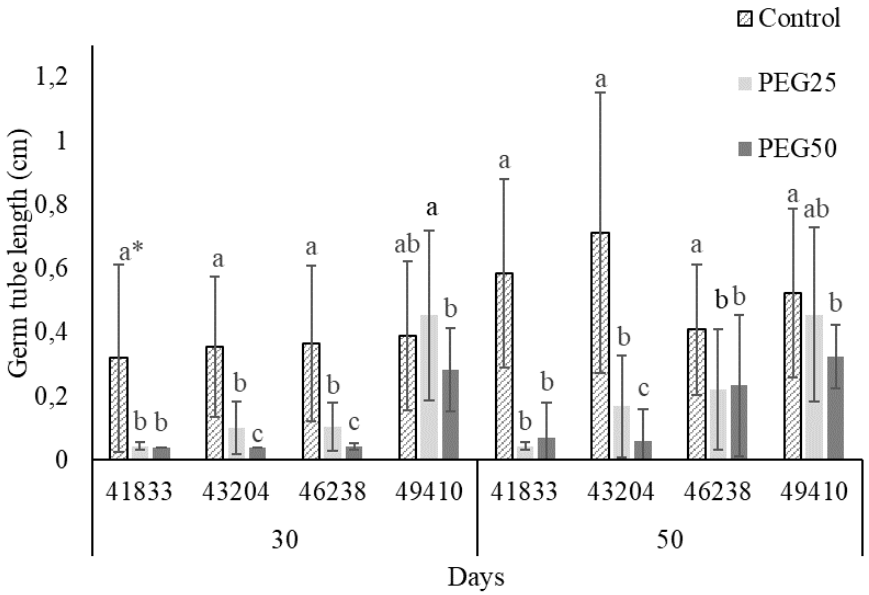

Figure 27 Germ tube length of spores of the different AM fungal strains in the absence of PEG (Control treatment) or under increasing concentration (25 and 50 gL^{-1}) of PEG (PEG^{25} and PEG^{50} treatments, respectively). Germ tube length of spores of *R. irregularis* MUCL 41833, *R. sp* MUCL 43204, *R. intraradices* MUCL 49410 and *R. clarus* MUCL 46238 was assessed 30 and 50 days after the beginning of the experiment. ***** Significant differences between treatments were tested independently for each strain. Different lower-case letters stand for significant differences between the Control, PEG^{25} and PEG^{50} treatments using pairwise comparisons (Wilcoxon test, $P<0.0166$, $n\geq14$).

Comparison of the germ tube length of spores between the AM fungal strains

In absence of PEG (Control treatment), significant differences in germ tube length of spores were found among the AM fungal strains on day 30 ($P<0.0001$) and day 50 ($P=0.0085$) (Table 5). On day 30, germ tube length of spores was significantly decreased for MUCL 41833 when compared with MUCL 49410, MUCL 43204 and MUCL 46238, while no significant difference was found among these three strains. On day 50, the germ tube length of spores of MUCL 46238 was significantly lower as compared with MUCL 43204. Germ tube length of spores of MUCL 41833 and MUCL 49410

were not significantly different and didn't differ either with MUCL 41833 or MUCL 46238.

The Figure 28 presents the germ tube length of spores of the different strains in presence of PEG^{25} and PEG^{50} treatments expressed as a percentage relative to the Control treatment.

On day 30, in both the PEG^{25} and PEG^{50} treatments, the germ tube length of spores was significantly different among strains ($P<0.0001$). The germ tube length of spores of MUCL 49410 was significantly longer when compared with the three other strains that did not differ among them. In the PEG^{50} treatment, germ tube length of spores was significantly longer for MUCL 49410 when compared with other strains. The germ tube length of spores was also significantly longer for MUCL 41833 when compared with MUCL 43204 and MUCL 46238, and significantly longer for MUCL 43204 when compared with MUCL 46238.

On day 50, in both the PEG^{25} and PEG^{50} treatments, the germ tube length of spores was significantly different among strains ($P<0.0001$). The germ tube length of spores was significantly longer for MUCL 49410 and MUCL 46238 when compared with MUCL 43204 and MUCL 41833. No significant difference was found between MUCL 43204 and MUCL 41833. In the PEG^{50} treatment, germ tube length of spores was significantly longer for MUCL 49410 and MUCL 46238 when compared with MUCL 43204 and MUCL 41833.

Table 5 Mean germ tube length of spores of AM fungal strains MUCL 41833, 43204, 49410 and of MUCL 46238 in the Control treatment, measured on day 30 and 50 of the experiment.

Strain	Germ tube length (cm)	
	30 days	50 days
MUCL 41833	0.32 ± 0.29^{b}	0.59 ± 0.30^{AB}
MUCL 43204	0.35 ± 0.22^{a}	0.71 ± 0.44^{A}
MUCL 46238	0.36 ± 0.24^{a}	0.41 ± 0.21^{B}
MUCL 49410	0.39 ± 0.23^{a}	0.52 ± 0.26^{AB}

Different letters stand for significant differences between treatments using pairwise comparisons (Wilcoxon test, P<0.0082).

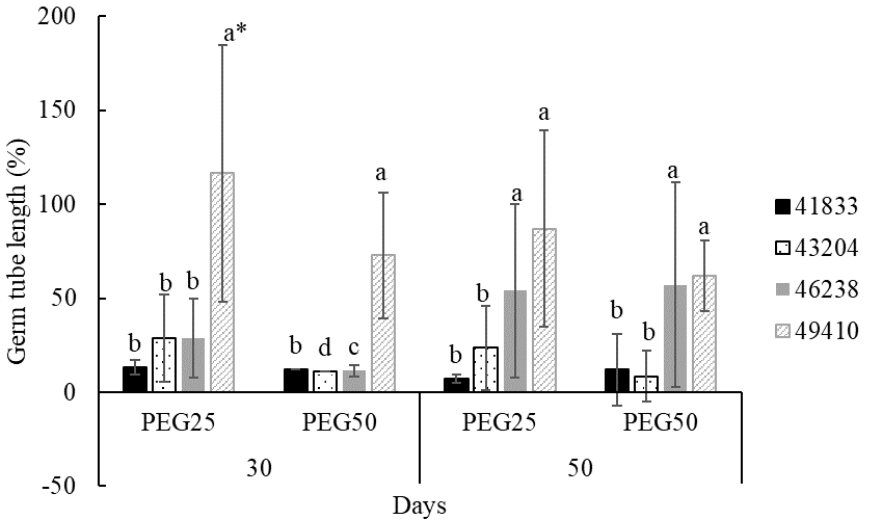

Figure 28 Germ tube length of spores under increasing concentration of PEG (25 and 50 gL^{-1}, PEG^{25} and PEG^{50} treatments, respectively) expressed as percentages relative to the Control treatment. Germ tube length of spores of *R. irregularis* MUCL 41833, *R. sp* MUCL 43204, *R. clarus* MUCL 46238 and *R. intraradices* MUCL 49410 was assessed 30 and 50 days after the beginning of the experiment. ***** Significant differences among strains were tested independently for each PEG treatment and day. Different lower-case letters stand for significant differences among the different strains, using pairwise comparisons (Wilcoxon test, P<0.0082, n≥14).

Discussion

The utilization of *in vitro* cultures of AM fungi allows studying the AM fungal life cycle (i.e. dynamics of spore germination, germ tube elongation, development and architecture of extraradical mycelium) under highly controlled conditions. Here, this system was used to investigate the impact of decreasing water availability induced by PEG on spore germination and germ tube length of four different AM fungal strains. Prior to this experiment, the impact of PEG on water potential of MSR medium as well as on its stiffness and opacity were assessed.

The addition of PEG to the MSR medium resulted in a significant decrease in water potential at 250 and 400 g L^{-1} of PEG and in a slight decrease, although not significant, at 50 and 100 g L^{-1} of PEG when compared with the Control treatment. The lack of significant difference in water potential between the Control treatment and the treatments with 50 and 100 g L^{-1} of PEG could be explained by a lack of precision in the measurement. Indeed, the precision in measurement of water potential using a vapor pressure osmometer could be limited at low osmolarities (below 100 mmol/kg) because the contamination of the thermocouple by solutions is more likely to affect the measurement (Sweeney and Beuchat, 1993). This hypothesis was supported by preliminary studies (data not shown) in which very close values of water potential were noticed in MSR solution in absence of PEG or supplemented with 50 and 100 g L^{-1} of PEG.

Although a very small decrease in water potential was measured in liquid MSR medium at 50 g L^{-1} of PEG (−0.13 MPa), it affected the germination and germ tube length of the AM fungal strains when compared with the Control treatment. PEG at 25 g L^{-1} also decreased spore germination and germ tube length. Our results seem in agreement with the study of Douds and Schenck (1991) in which the germination percentages of some AM fungal strains were decreased when the soil water potentials was slightly decreased (from −0.01 MPa to −0.05 MPa). However, it is not excluded that other indirect effects of PEG on water availability could impact the germination and germ tube length of spores. Indeed, it was shown that for a same water potential, growth medium supplemented with PEG as compared with medium supplemented with glycerol, did not change the germination response of *Fusarium graminearum* but had a higher impact on their germ tube elongation (Ramirez *et al.*, 2004). Gessner (1976) also found that the growth of the

ascomycete *Buergenerula spartinae* was more impacted by PEG when compared with mannitol. While Ramirez *et al*. (2004) attributed this effect to the matric component of the water potential, Gessner (1976) suggested that this effect could be due to the higher viscosity of PEG solution.

Similarly, numerous studies have reported that small increases in the concentration of gelling agents (which are biopolymer) affected the development of plant tissues cultured *in vitro* although it could not be related to a significant decrease in ψ of the medium as measured with a tensiometer (Spomer and Smith, 1996) or a vapor pressure osmometer (Klimazewska *et al*., 2000). By contrast, increasing gelling agent concentrations were found to impact the water availability as measured by the water absorbed by filter paper discs (Owens and Wosniak, 1991; Klimazewska *et al*., 2000; Huang *et al.*, 2009). It is therefore possible that PEG could affect other physiochemical properties of the medium.

PEG cannot be sterilized by autoclaving, because of the fusion temperature of the polymer (Majumdar *et al*., 2010). The exposition to UV light for certain time is neither recommended as it can degrade the backbone of the PEG molecule (Das and Gupta, 2005). PEG powder was thus mixed with the cold but autoclaved MSR medium without Gelrite to produce liquid MSR medium while it was mixed with hot MSR medium containing Gelrite (T° lower than 60°C) to produce the gelled medium. The maintenance of PEG powder in a clean place and its careful manipulation in a clean environment must have played an important role in preventing contaminations of the medium as only a few contaminations were observed. However, the direct addition of PEG at high concentrations (250 and 400 g L^{-1}) to the MSR medium interfered with the polymerization of the Gelrite as previously anticipated. Van der Weele *et al*. (2000) suggested the addition of PEG into agar medium by infusing the gel with a PEG overlay solution overnight. Unfortunately, this protocol was tested and did not show good results probably because the concentration of the gelling agent (Gelrite 3 g L^{-1}) in the MSR medium was much lower. At high concentrations of PEG, the MSR medium was opaque. These effects were more marked with increasing concentrations of PEG. Different hypothesis could be made on the interaction of Gelrite with PEG. This molecule could form complexes with cations *(*Balijepalli *et al*., 2013) which are necessary for the polymerization of polysaccharides. As PEG molecules are relatively large, they could also simply disturb the polysaccharide network.

A small decrease in water potential (from $\psi = -0.1$ MPa in the Control treatment to $\psi = -0.13$ MPa for the PEG^{50} treatment) was tested for the first time on spore germination and germ tube length of AM fungi. Whatever the AM fungal strain, concentrations of 25 and 50 g $L^{-1}$of PEG had an impact on both parameters evaluated on day 15 or 30. This effect seemed stronger at the highest concentration of PEG. The differences between AM fungal strains also suggested different resistances/tolerances to PEG concentrations. When comparing the percentages of spore germination of the different strains it seemed that MUCL 49410 and MUCL 46238 strains were more tolerant to PEG than MUCL 41833 and MUCL 43204. Indeed, for MUCL 49410 and MUCL 46238 strains no difference between the Control treatment and any of the PEG treatment was found after 50 days of germination, while the percentage of spore germination of the other strains was significantly decreased in the PEG^{50} treatment. The effect of PEG on the percentage of spore germination on days 15 and 30 was also less pronounced for the MUCL 49410 and MUCL 46238 when compared with MUCL 41833 and MUCL 43204 strains (Figure 25). Similar results were found for the germ tube length of spores. This result seems to corroborate the studies of Sylvia and Schenck b (1983) and Douds and Schenck (1991) who found different impacts of a decrease of the water potential on the percentage of spore germination depending on the AM fungal strain. However the determinants of spore germination sensitivity to a decrease of water potential are not clear. Spores could have a sensing mechanism that would inhibit spore germination according to their moisture requirements. Tommerup (1984) showed that the hydration level of spores determined their ability to germinate. Although all AM fungal strains tested here belonged to the *Rhizophagus* family it is likely that variations in spore wall thickness and structure exist. This physical differences could confer to spores different resistances to osmotic/matric pressure and/or influence the hydration levels required for germination. Finally, the differences among strains could globally result from a specific adaptation of the AM fungal strain to its environment. The MUCL 49410 and MUCL 46238 strains seemed to be closer to AM fungal strains that Chagnon *et al.* (2013) qualified as “stress tolerators”. These AM fungal strains have a low growth rate and allocate more resources to their descendants enabling them to survive under stressed environment. Interestingly, we observed that in the Control treatment the MUCL 43204 and MUCL 41833 had longer germ tube lengths of spores than the two other strains that seem more drought tolerant. It is possible that this characteristic would allow them to colonize plants more rapidly than the other strains under optimal conditions.

Conclusion

This study showed that PEG can significantly decrease the water potential of liquid MSR medium for PEG concentrations above 100 g L^{-1}. However using the protocol presented in this work, it was possible to prepare a gelled medium suitable for *in vitro* studies of AM fungi with PEG concentrations of 25 and 50 g L^{-1}. Although these low concentrations didn't significantly decrease the water potential of the medium, they were, however, sufficient to impact the spore germination and germ tube length of the AM fungal strains, without excluding the possible effect of PEG on other characteristics of the growth medium (e.g. viscosity) impacting fungal strains. These strains showed also different responses to lowering water availability. This experiment lays the foundations for future works on the implementations of PEG in *in vitro* study of AM fungi and could open an interesting avenue to study the functional ecology of different strains in response to water stress. Further investigations on the water potential of the medium at low PEG concentrations are required to better understand the response of the strains.

Supplementary material

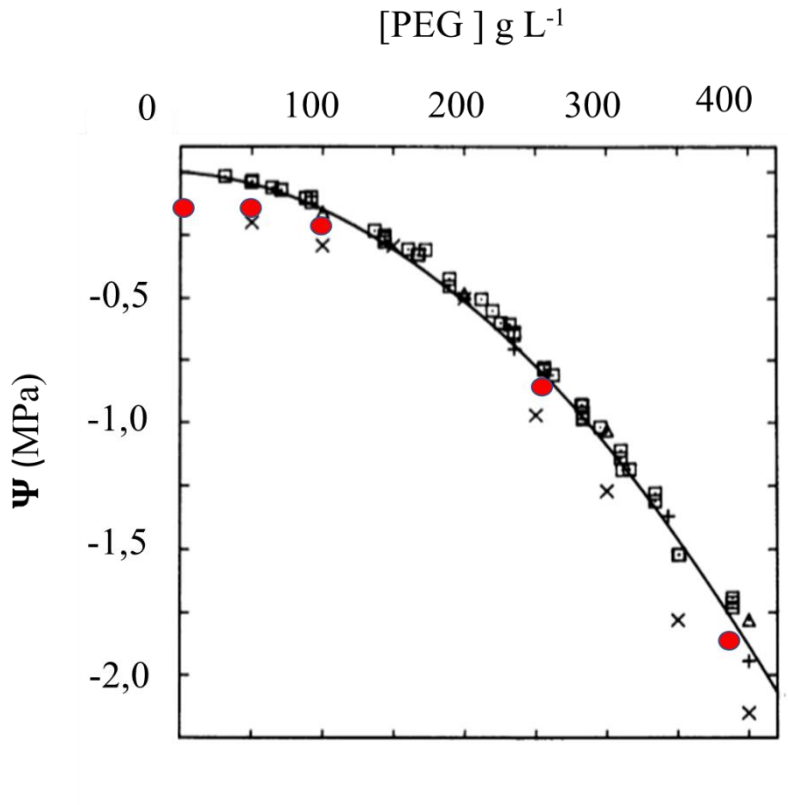

Figure S1 Water potentials (ψ) of different solutions added with increasing concentrations of PEG (8000) *(adapted from* Michel, 1983). *Water potentials are measured in water solutions with thermocouple hygrometer except those of McClendon: data are from Williams and Shaykewich , (1969) (□); from Michel and Kaufmann, (1973) (Δ) ; from Steuter et al., (1981) (×) ; and from McClendon, (1981) (+). Solid line is from Eq. 1 in Michel, (1983). Water potential measured in MSR medium without Gelrite with a vapor pressure osmometer (●) in our study.*

Chapter 2

Reducing water availability impacts the development of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* MUCL 41833 and its ability to take up and transport phosphorus under *in vitro* conditions

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Preface

In Chapter 1, the effects of PEG 8000 (hereafter cited as PEG) on water availability in the Modified Strullu-Romand (MSR) medium was estimated by measuring its impact on water potential. The water potential was decreased at 50, 100, 250 and 400 g L^{-1} of PEG in the MSR medium when compared with the Control (i.e. absence of PEG) but only the values obtained at 250 and 400 g L^{-1} were significantly lower as compared to the Control. The percentage of spore germination and germ tube length of spores were evaluated at 25 and 50 g L^{-1} of PEG in the MSR medium. The results differed between the AM fungal strains with higher values for *Rhizophagus intraradices* MUCL 49410 and *R. clarus* MUCL 46238 as compared with *R. irregularis* MUCL 41833 and *R. sp* MUCL 43204, suggesting different sensitivities of AM fungal strains to a decrease of water availability.

Although the utilization of PEG seemed adequate to study the effects of a decrease in water availability on spore germination and germ tube length, there was no clear evidence of an effect of PEG at concentrations below or equal to 50 g L^{-1}. Moreover, the effects of the PEG-induced decrease in water availability on extraradical mycelium development and uptake and transport of phosphorus (a major function of AM fungi) of the AM fungus were not investigated.

In Chapter 2, it was the objective, first, to test the impact of low concentrations of PEG (10, 25 and 50 g L^{-1}) on water potential and secondly to assess the effects of these PEG concentrations on the germination of spores and germ tube length as well as on the extraradical development of the fungus and its ability to take up Pi and transport it to the roots. The AM fungus used was *Rhizophagus irregularis* MUCL 41833 grown in association with *Medicago truncatula* in bi-compartmented Petri plates, separating a root compartment containing the plant and fungus from a hyphal compartment in which only the fungus was allowed to grow. Pi depletion in the hyphal compartment (indirectly meaning Pi uptake and transport by the extraradical mycelium of the AM fungus) was measured under increasing concentrations of PEG.

Abstract

Climate change scenarios predict higher variability in rainfall and an increased risk of water deficits during summers for the coming decades. For this reason, arbuscular mycorrhizal fungi (AM fungi) and their mitigating effects on drought stress in plants are increasingly considered in crop management. However, the impact of a decrease in water availability on the development of AM fungi and their ability to take up and transport inorganic phosphorus (Pi) to their hosts remain poorly explored. Here, *Medicago truncatula* plantlets were grown in association with *Rhizophagus irregularis* MUCL 41833 in bi-compartmented Petri plates. The system consisted in associating the plant and the AM fungus in a root compartment (RC), allowing only the hyphae to extend in a root-free hyphal compartment (HC). Water availability in the HC was then lowered by increasing the concentration of polyethylene glycol-8000 (PEG-8000) from 0 to 10, 25, and 50 g L^{-1} (corresponding to a slight decrease in water potential of −0.024, −0.025, −0.030, and −0.056 MPa, respectively). Hyphal growth, spore production and germination were severely impaired at the lowest water availability. The dynamics of Pi uptake by the AM fungus was also impacted, although total Pi uptake evaluated after 24 h stayed unchanged. The percentage of metabolically active extraradical hyphae remained above 70%. Finally, at the lowest water availability, a higher P concentration was observed in the shoots of *M. truncatula.* At reduced water availability, the extraradical mycelium (ERM) development was impacted, potentially limiting its capacity to explore a higher volume of soil. Pi uptake was slowed down but not prevented. The sensitivity of *R. irregularis* MUCL 41833 to a, even small, decrease in water availability contrasted with several studies reporting tolerance of AM fungi to drought. This suggests a species or strain dependent effect and support the necessity to compare the impact of water availability on morpho-anatomy, nutrient uptake and transport capacities of other, potentially more drought tolerant (e.g. isolated from dry environments) AM fungi.

Keywords: arbuscular mycorrhizal fungi, polyethylene glycol (PEG), drought stress, hyphal development, phosphorus uptake, phosphorus transport**.**

Introduction

Drought associated with extreme heat has been responsible for a significant drop in yield of crops and cultivable surfaces over the last 40 years (Lesk *et al*., 2016). Projected climate changes indicate a higher variability in rainfall in the coming decades, with an increasing risk of water deficits during summers, while water resources for irrigation will be at best maintained. Enhancing crops resilience to drought is thus a priority to secure nowadays and future food production (Wheeler and von Braun, 2013). There are various soils, water and crop management practices which, together with improved genotype, have the potential to increase water use efficiency of crops. For instance, irrigation is a practical solution to increase crop yield, although an intensification of this method is not viable on the long-term as freshwater resources are limited (Elliott *et al*., 2014). Irrigation methods that avoid salinization of soil, soil leaching, and improve water use efficiency are alternatives, but are not always affordable for smallholders (Wezel *et al*., 2014; Xie *et al*., 2014). Soil microorganisms living in close relations with plant roots might also play a role in plant water stress mitigation via various mechanisms (see review by Kim *et al*., 2012). Among these below-ground inhabitants are the beneficial and naturally occurring arbuscular mycorrhizal fungi (AM fungi) (Cardoso and Kuyper, 2006; Rodriguez and Sanders, 2014). Arbuscular mycorrhizal fungi are obligate root symbionts that have co-evolved with plants for an estimate of 400 million years (Parniske, 2008). They are nowadays associated with an approximate of 72% of Angiosperms (Brundrett,2009) in almost all ecosystems (Smith and Read, 2008). Their beneficial effects on plant nutrition and growth as well as on biotic and abiotic stresses alleviation have been amply described (Miransari, 2014; Latef *et al*., 2016; Plouznikoff *et al*., 2016) so that they are today considered as essential components of agro-ecosystems. In recent years, many studies have focused on the role of AM fungi in plant water stress mitigation (Aroca and Ruiz-Lozano, 2009; Jayne and Quigley, 2014; Fernández-Lizarazo and Moreno-Fonseca, 2016; Farooq *et al*., 2017). Under dry environments, AM fungi have been reported to regulate plant physiology and metabolism (Wu and Zou, 2017) by changing abscisic acid/cytokinin hormone balance (Goicoechea *et al*., 1997) and by protecting the host plants from oxidative damages under osmotic stress conditions (Porcel and Ruiz-Lozano, 2004). They have been shown to improve osmotic adjustment (Wu and Xia, 2006) and water lift from roots to shoots via higher water apoplastic flow (Bárzana *et al*., 2012).They have also been reported to increase cell to cell water transport through

increased expression of aquaporin membrane proteins (Aroca *et al*., 2007; Ruiz-Lozano *et al*., 2009). Increased drought tolerance of AM fungi-colonized plants has also been associated to an improved nutrition, especially inorganic phosphorus (Pi) that is transported by the extraradical hyphae of the fungus to the root cells (Nelsen and Safir, 1982; Bethlenfalvay *et al*., 1988; Fitter and Nichols, 1988). The extraradical hyphae can increase the access of plants to soil water (Sánchez-Díaz and Honrubia, 1994; Ahmad Khan *et al*., 2003) and increase soil water retention through glomalin excretion (Augé, 2001; Wu *et al*., 2008). Despite the quite large number of studies reporting effects of AM fungi on plant physiology and growth under drought stress conditions, little is known on the impact of decreased water availability on the development of the fungus and its capacity to take up and transport Pi to plants, an essential attribute of these fungi. Any change with respect to their growth and development or capacity to provide the host plant with nutrients would indeed partly compromise their agro-ecological value. Today, most studies have been conducted in pots and a few in bi-compartmented systems making it possible to differentiate Pi uptake by the plant from that of the fungus (Thingstrup *et al*., 2000; Jansa *et al*., 2003; Merrild *et al*., 2013). However, these systems do not allow the non-destructive observation of AM fungal development. Moreover, the dynamics of Pi uptake by the sole extraradical mycelium (ERM) of the fungus is nearly impossible to assess due, among others, to the presence of unwanted microbial contaminants. *In vitro* cultivation systems associating excised roots (Declerck *et al*., 2005) or whole plants (Voets *et al*., 2005) in mono or bi-compartmented Petri plates (St-Arnaud *et al*., 1996) are more indicated to overcome some of these issues. They have been used to study the development (Bago *et al*., 1996, 1998; St-Arnaud *et al*., 1996; Declerck *et al*., 2005) and architecture (Bago *et al*., 1998; de la Providencia *et al*., 2005; Voets *et al*., 2006) of the ERM as well as the intimate plant-AM fungal association under abiotic stress conditions (e.g., heavy metals – Pawlowska and Charvat, 2004; potentially toxic elements such as chromium – Gil-Cardeza *et al*., 2017; salt – Jahromi *et al*., 2008; Aroca and Ruiz-Lozano, 2009 ; fungicides– Zocco *et al*., 2011; polyaromatic hydrocarbons (PAHs) – Calonne *et al*., 2012). In particular, the utilization of bi-compartmented Petri plates (with the fungus developing in a root-free compartment) has allowed to monitor Pi uptake and transport in presence of fungicides (Zocco *et al*., 2011) or PAHs (Calonne *et al*., 2014). A few *in vitro* studies have also been focused on the effects of water stress on AM fungi. Indeed, mannitol has been reported to decrease germination and germ tube growth of *Funneliformis mosseae* (Estaun, 1989) on gelled medium. Glycerol has also been reported to decrease

mycelium biomass, spore production and spore size of *R. irregularis* DAOM 197198 (Hammer and Rillig, 2011). More recently, osmotic stress induced by the application of polyethylene glycol (PEG-8000) in liquid Minimum (M) medium upregulated the expression of the fungal aquaporins GintAQP1 and GintAQP2 of *Rhizophagus irregularis* DAOM 197198 associated to transformed *Daucus carota* roots in bicompartmented Petri plates (Li *et al*., 2013). PEG is an inert and low toxic polymer (Lawlor, 1970; Mexal and Reid, 1973), thus representing an adequate option to create water shortage in gelled medium. Over the last decades, it has become the principal compound used to study physiological response of plants cultivated *in vitro* to water stress (Rai *et al*., 2011). Therefore, it appears to be a good candidate to investigate the role of water deficit on the development and architecture of AM fungi and on the uptake and transport of Pi. In the present work, we first conducted a preliminary study to assess the water potential achieved by increasing concentrations of PEG in liquid medium. We then evaluated and compared the effects of PEG-induced decreased water availability on the development (i.e., spore germination and ERM development) and on the Pi uptake and transport of *R. irregularis* MUCL41833 associated to *Medicago truncatula*. Albeit water potential achieved under our experimental conditions are much higher than encountered under dry environments, we hypothesize that slight changes in water availability can trigger differential response of the fungus.

Material and methods

Biological material

Seeds of *Medicago truncatula* Gaertn.cv.Jemalong A17 (SARDI, Australia) were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 10 min and rinsed in sterilized (121°C for 15 min) deionized water. Seeds were germinated in Petri plates (90 mm diameter) containing 35 ml of modified Strullu–Romand (MSR) medium (Declerck *et al*., 1998) without vitamins and supplemented with 10 g L^{-1} sucrose and 3 g L^{-1} Gelrite (Merck & Co., Kenilworth, NJ, United States). The MSR medium was adjusted to pH 5.5 before sterilization (121°C for 15 min). The Petri plates were incubated at 27°C in the dark. The seedlings were ready to use four days following seed germination. The AM fungus *R. irregularis* (Błaszk., Wubet., Renker and Buscot) C. Walker and A. Schüßler comb. nov. MUCL41833 was

supplied by the Glomeromycota *in vitro* collection (GINCO[6]). The fungus was grown in bi-compartmented Petri plates (90 mm diameter) in association with *M. truncatula* plantlets following the method developed by Voets *et al*. (2009).

Preliminary experiment: water potential of MSR medium supplemented with increasing concentrations of polyethylene glycol

Polyethylene glycol 8000 (PEG-8000; Applichem, Darmstadt, Germany) was used to induce a decrease in water availability. The efficiency of PEG in decreasing the water potential and thus water availability of the MSR medium was determined as follows: liquid MSR medium without PEG or supplemented with 10, 25, or 50 g L^{-1} PEG was prepared. The dew point at each concentration was determined using a vapor pressure osmometer (VAPRO® 5520, Wescor Inc., Logan, UT, United States). The osmolarities obtained from the different solutions were then converted into water potentials (ψ). Response curve of water potential to PEG concentrations was finally built using a quadratic model in the JMP statistic software (JMP R Pro 12.1.0) from SAS. Quality of fit between the model and the data was evaluated by computation of the R^2. For the various assays described below, PEG at the various concentrations was mixed with sterilized (121°C for 15 min) liquid or solid (with 3% of Gelrite) MSR medium without sucrose and vitamins. Solubilization of PEG in the MSR medium was done with a sterilized magnetic rod and at a temperature of 45°C to prevent medium solidification before pouring in the Petri plates.

Preparation of the mycelium donor plants to study the hyphal development, spore production, and Pi uptake by the AM fungus

Spores (±100) of *R. irregularis* MUCL 41833 were associated to a four-days-old *M. truncatula* plantlet in the root compartment (RC) of a 90 mm diameter bi-compartmented Petri plate (named Mycelium Donor Plant (MDP) *in vitro* culture system – see for details Voets *et al*., 2009). The roots were plated on the MSR medium with the shoot extending outside the MDP *in vitro* culture system via a hole cautiously plastered with silicon grease (VWR, Radnor, PA, United States) to avoid contaminations. The RC and adjacent hyphal compartment (HC) were filled with 25 ml MSR medium without

[6] www.mycorrhiza.be/ginco.bel

sucrose and vitamins and solidified with 3 g L^{-1} gelrite. The MDP *in vitro* culture systems were then transferred to a growth chamber at 22/18°C (day/night), 70% relative humidity (RH), a photoperiod of 16 h day^{-1} and a photosynthetic photon flux (PPF) of 225 µmol m^{-2} s^{-1}. The systems were covered with dark plastic sheets to protect *M. truncatula* roots and the AM fungus from the light, while the shoots developed under light conditions. To nourish the plantlet, RC was supplied every week with approximately 10 ml of fresh MSR medium using a 25 ml sterile pipette (VWR). To allow the passage of the pipette, a hole in the lid above the RC was made using a hot cork-borer and was closed after pipetting using a double layer of sterilized plaster (Micropore TM, 3M TM, Maplewood, USA). Hyphae started to cross the partition wall separating the RC from the HC within eight to 12 weeks. Two four-days-old *M. truncatula* plantlets were subsequently transferred in the HC of each system to allow pre-mycorrhization of new plantlets. Roots were plated on the MSR medium without sucrose and vitamins and shoots extended outside the system via two holes, similarly plastered with silicon grease to avoid contaminations. The new plantlets were homogeneously colonized within 12 days.

Impact of decreasing water availability on hyphal development and spore production of *R. irregularis* MUCL 41833

Single 12-days-old pre-mycorrhized *M. truncatula* plantlets were plated in the RC of new bi-compartmented Petri plates using the same technique as for the preparation of MDP systems (see above). Using a sterile pipette, the RC was supplied with 15 ml of solid MSR medium without sucrose and vitamins every two weeks during the whole experiment. After nine weeks, 24 systems showing profuse ERM development in the HC were selected. To allow homogenous growth of the AM fungus in this compartment, the MSR medium was cut 2 mm away from the partition wall and removed. The HC was then supplemented with 25 ml MSR medium lacking sucrose and vitamins and solidified with 3 g L^{-1} of gelrite in absence ($Control^{-PEG}$ treatment) or supplemented with 10, 25, and 50 g L^{-1} PEG (PEG^{10}, PEG^{25}, and PEG^{50} treatments, respectively). Hyphae re-colonized the MSR medium within four weeks. Hyphae and spore densities were homogenous along the partition wall, although their respective length and number gradually decreased along the growing front. Hyphal length and spores number were counted under binocular microscope (Olympus SZ, Olympus Optical,

Germany – 40× magnification) in three 10 mm^2 squares located in the middle of the HC, perpendicular to the partition wall and parallel to the growing axis. Hyphal length (L) was estimated using the Newmann's formula modified by Marsh (1971): L(cm) = N × (11/14) × grid unit (cm) where N is the number of hyphal intersections with the grid, 11/14 is the ideal size of the grid sides that gives direct estimation of length in cm and grid unit = 1 cm. Spores number was summed over the three squares (Declerck *et al*., 2003). Six replicates were considered per treatment.

Impact of decreasing water availability on spore germination and germ tube length of *R. irregularis* MUCL 41833

Spores of a two-month-old MDP *in vitro* culture of *R. irregularis* MUCL 41833 were isolated from the MSR medium following solubilization of the gelled medium with 10 mM filter-sterilized sodium citrate buffer (Doner and Becard, 1991). Spores were separated with forceps and placed single with a micropipette in each well of two 24-well tissue culture plates (VWR, Radnor, PA, United States) containing 2 ml of solid MSR medium without sucrose and vitamins and without PEG ($Control^{-PEG}$ treatment) or supplemented with 10, 25, and 50 g L^{-1} PEG (PEG^{10}, PEG^{25}, and PEG^{50} treatments, respectively). The 24-well tissue culture plates were then sealed with cellophane (Toppits R, Melitta Group, Germany) and incubated in the dark at 27°C. Spore germination was estimated at regular intervals for 110 days and hyphal length of germinated spores was similarly evaluated on day 110 under binocular microscope (30× magnification). For $Control^{-PEG}$, PEG^{10} and PEG^{25} but not PEG^{50} treatment, Gompertz model was fitted to the germination counts by nonlinear (NLIN) regression using Gauss–Newton minimization algorithm in SAS software. Effect of the treatment was evaluated by testing a significant reduction of the residual sum of square when parameterizing with and without an effect of the treatment using the Fisher–Snedecor F-test with 1 and t−4 df (Seber and Wild, 1989; Declerck *et al*., 2001): Fr=(SSEr−SSEf)/q MSEf where SSEr is the residual sum of squares in reduced model and SSEf in the full model, MSEf = residual mean square in the full model. Parameter estimates of the model in the different treatments were further compared to assess an effect on the dynamics of germination in the HC. Confidence intervals were built around the following estimated parameters: coefficient "a" referred to the cumulated number of germinated spores when the plateau phase was reached, "b" referred to the maximum spore germination rate (tangent of the curve at the inflection point), and "c"

referred to the lag time (time to the intersection between the tangent at the inflection point and the time axis).

Impact of decreasing water availability on Pi uptake by *R. irregularis* MUCL 41833, SDH activity of hyphae, plant biomass, and phosphorus concentration

Phosphorus uptake by *R. irregularis* MUCL 41833 was measured indirectly by monitoring Pi concentration remaining in the medium of the HC of the MDP *in vitro* culture systems. This approach cannot exclude that some of the Pi is adsorbed on the hyphae, although most studies conducted *in vitro* (e.g., Nielsen *et al*., 2002; Zocco *et al*., 2011; Calonne *et al*., 2014; Gil-Cardeza *et al*., 2017) clearly show that the vast majority of Pi is taken up and transported by the mycelium. Single 12-days-old pre-mycorrhized *M. truncatula* plantlets were plated in the RC of bi-compartmented Petri plates using the same technique as for the preparation of MDP systems (see above). The RC was supplied with 15 ml of solid MSR medium without sucrose and vitamins every two weeks during the whole experiment. A profuse ERM was produced in the RC within nine weeks and started to cross the partition wall separating the RC from the HC (Figure 29). At that time, the gelled MSR medium in the HC was entirely removed and replaced by 15 ml of liquid MSR medium without sucrose and vitamins (Figure 29). A hole in the lid of the system was made above the HC, similarly to the procedure described above in *Preparation of the mycelium donor plants to study the hyphal development, spore production, and Pi uptake by the AM fungus*. Using a 25 ml sterile pipette, the HC was then supplied weekly with approximately 15 ml of liquid MSR medium. Profuse hyphal regrowth in the HC was obtained within seven weeks. The MDP *in vitro* culture systems were then separated in four homogenous groups based on the surface covered by the hyphae in the HC. Hyphal surface was estimated following the method described by Voets *et al*. (2009) by tracing on a transparent sheet the growing front constituted by the hyphal network. This measure confirmed no significant differences ($P = 0.6395$) between treatments. Four treatments, each with six replicates, were considered: MDP *in vitro* culture systems without PEG in the HC ($Control^{-PEG}$ treatment) or supplemented with 10, 25, and 50 g L^{-1} PEG (PEG^{10}, PEG^{25}, PEG^{50} treatments, respectively). A Control (six replicates) without mycelium in the HC ($Control^{-AMF}$ treatment) was also considered by cutting and removing the ERM from the HC. Liquid MSR medium was retrieved from the HC and the compartment was rinsed two times with sterilized deionized water

(Figure 29). Fifteen ml of fresh liquid MSR medium was added to the Control^{-AMF} and Control^{-PEG} treatments, whereas liquid MSR medium containing 10, 25, and 50 g L^{-1} PEG was added to the other treatments (Figure 29). The Pi concentration in the HC at the start of experiment was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES; ICP 6500, Thermo-Scientific). The value was 0.59 mg L^{-1} and total P content 0.09 mg.

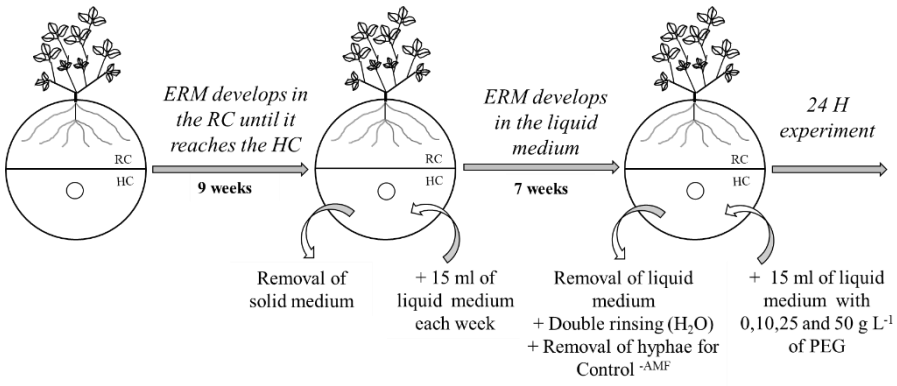


Figure 29 Schematic representation of the preparation of bi-compartmented Petri plates to assess the impact of decreasing water availability on the inorganic phosphorus uptake by *R. irregularis* MUCL 41833. The fungus is associated with *M. truncatula* roots in the root compartment (RC) and extraradical mycelium (ERM) extends in the hyphal compartment (HC). Small circles represent the opening made in the lid of the Petri plate used for adding modified Strullu Romand (MSR) medium. Addition of solid MSR medium in the RC is not represented in the scheme.

For Pi concentrations analysis in the HC, 0.5 ml of MSR medium was sampled every 2 h during 24 h from the HC of each system. The samples were diluted (1/10) and digested with 20 µl of nitric acid (65%, Suprapur®, Merck, Germany) and finally analyzed by ICP-AES. Data of Pi concentration obtained in ppm were subsequently converted into mg L^{-1} and standardized as in Garcés-Ruiz *et al*. (2017). Data were finally transformed into % of Pi remaining in the medium which is the percentage of the Pi concentration remaining in the medium of the HC at time 2, 4, 6, 8, 12, 18, 24 h relative to the Pi concentration in the medium of the HC at time T0:

$$\%\ of\ Pix = ([Pix]t + [Pic]t - [Pic]t0)/([Pix]\ t0 \times 100$$

where:

[Pix] = Pi concentration in the medium of the HC for the sample x considered

t = time considered (2, 4, 6, 8, 12, 18 or 24 h after the start of the experiment)

t0 = 0h before the start of the experiment

[Pic] = Mean Pi concentration measured over 6 replicates in the Control^{-AMF} treatment

At the end of the sampling phase, the ERM in the HC was harvested from the liquid MSR medium and stained for succinate dehydrogenase (SDH) detection. The SDH staining method enabled to evaluate the proportion of metabolically active hyphae (Saito, 1995; Vierheilig *et al*., 2005). Hyphae were harvested and incubated overnight at room temperature in the dark in a freshly prepared staining solution containing Tris–HCl (50 mM), $MgCl_2$ (0.5 mM), sodium succinate (25.1 mM) and Nitroblue tetrazolium (1.22 mM). The samples were then washed with deionized water, counterstained in 0.1% acid fuchsin in lactic acid for 15 min and transferred to lactoglycerol for de-staining. Hyphae samples were mounted on microscope slides with lactoglycerol. The precipitation of substrate after enzymatic reaction was assessed under a bright field microscope (Olympus BH2-RFCA, Japan) at 400× magnification. For each sample, an approximate of 100 hyphal intersections was observed. According to Van Aarle *et al*. (2002), intersections of hyphae were classified as active or non-active following substrate precipitation or not.

Following harvest, shoots and roots were separated and dry weights evaluated after 48 h at 45°C in an oven. The dried shoots and roots material were then grinded and subsamples heated overnight at a temperature of 60°C with 4 ml of nitric acid 65% followed by an evaporation step at 120°C. Subsamples were then dissolved in 2 ml solution of aqua regia (3/4 HCL 38% for 1/4 HNO_3 65%). The solutions were filtered and diluted in 25 ml of purified MilliQ (Millipore™) water for P concentration analysis via ICP-AES.

Statistical analysis

Data analysis was performed with JMP or SAS statistic softwares (SAS Institute Inc., Cary, NC, USA) with a 5% α-threshold. Pairwise comparisons following chi-square tests were analyzed using Bonferroni's correction. Significant differences between treatments for water potential measurements, hyphal lengths, shoots and roots dry biomass, shoots and roots P concentrations and content as well as for hyphal surfaces and relative water contents were tested using analysis of variance with one factor (ANOVA1) followed by a post-hoc Tuckey (α=5%) test. Levene's test was run prior to the ANOVA analysis to confirm homogeneity of variance in the distribution. Sporulation counts were tested using a likelihood ratio *test* for *negative binomial* model. Data corresponding to the % of Pi remaining in the medium were submitted to an $\arcsin^{-1}$ transformation and submitted to a repeated-measures ANOVA with REML estimation where "Treatment" was regarded as a fixed factor and "Time" as random factor. Additionally, % of Pi remaining in the medium was compared for each time using ANOVA1 test as described above. Finally, SDH activity and spore germination rates were submitted to a chi-squared test.

Results

Water potential of MSR medium supplemented with increasing concentrations of polyethylene glycol

Water potential of the MSR medium was significantly lower in the PEG^{50} treatment as compared to the $Control^{-PEG}$, PEG^{10} and PEG^{25} treatments (Figure 30). No significant differences were noticed between the $Control^{-PEG}$ treatment and the PEG^{10}, PEG^{25} treatments. Modelling of the response by linear regression (data not shown) using a quadratic model showed high statistic fitting (R^2= 0.93501).

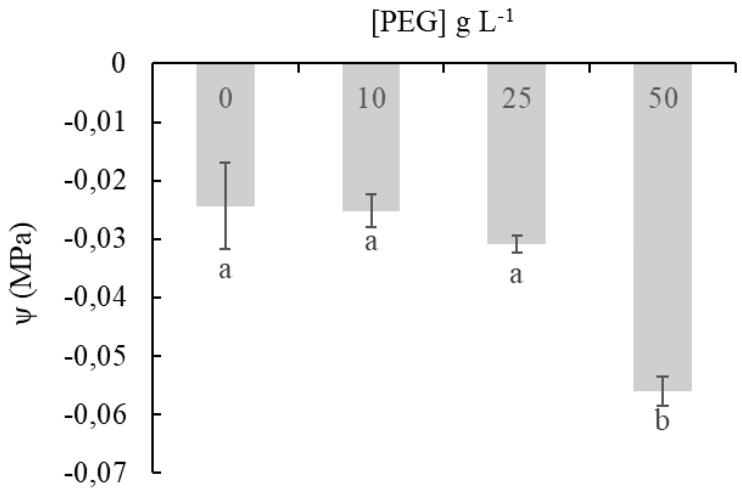


Figure 30 Water potential (Ψ) response of the modified Strullu Romand medium supplemented with increasing concentrations of polyethylene glycol (0, 10, 25 and 50 g L^{-1}). Data are represented as means ± SD (n=3). Different letters below bars indicate significant differences between treatments using pair-wised comparison (Tuckey's test, $P<0.05$).

Impact of decreasing water availability on hyphal development and spore production of *R. irregularis* MUCL 41833

The hyphal length and number of newly produced spores in presence of increasing concentrations of PEG are presented in Table 6. After four weeks of growth in the HC, the hyphal length was significantly higher in the $Control^{-PEG}$ treatment as compared to the PEG^{50} treatment but did not differ from the PEG^{10} and PEG^{25} treatments (Table 6). No differences were found between the PEG^{10}, PEG^{25} and PEG^{50} treatments (Table 6).

The number of newly produced spores was significantly higher in the $Control^{-PEG}$ treatment as compared to the PEG^{25} and PEG^{50} treatments but did not differ from the PEG^{10} treatment (Table 6). Similarly, the number of spores in the PEG^{10} treatment was significantly higher as compared to the PEG^{50} treatment but did not differ from the PEG^{25} treatment (Table 6). No significant

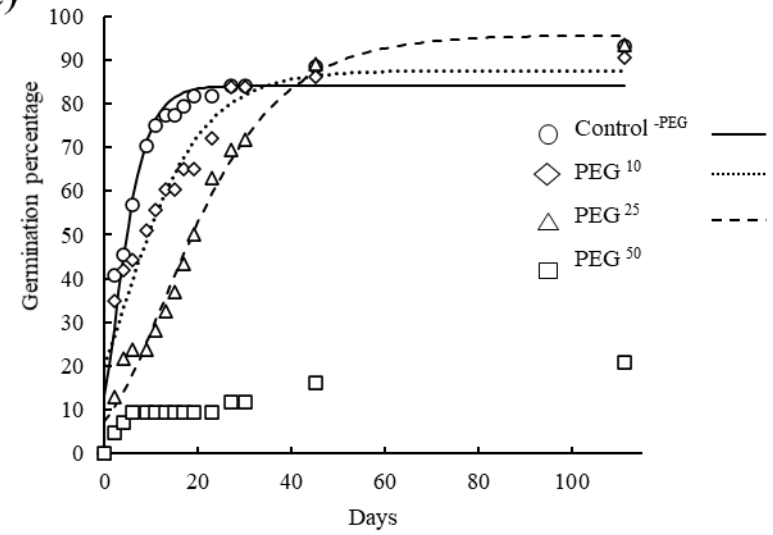
difference in number of spores was noticed between the PEG^{25} and PEG^{50} treatments (Table 6).

Impact of decreasing water availability on spore germination and germ tube length of *R. irregularis* MUCL 41833

The percentage of germinated spores at the end of the experiment (i.e. on day 110) was significantly lower in the PEG^{50} treatment (i.e. 20.9%) as compared to the $Control^{-PEG}$ (i.e. 93.2%), PEG^{10} (i.e. 90.7%) and PEG^{25} (i.e. 93.6%) treatments. No significant differences were noticed between the $Control^{-PEG}$, PEG^{10} and PEG^{25} treatments.

Comparison of modelled germination curves between $Control^{-PEG}$, PEG^{10} and PEG^{25} treatments (Figure 31 a) demonstrated a significant impact of the treatments ($F= 29.99$, $P<0.001$). Parameter "a" (referring to the cumulated number of germinated spores when the plateau phase was reached) did not differ between the $Control^{-PEG}$ and the PEG^{10} and PEG^{25} treatments (Figure 31b). Parameter "b" (corresponding to the maximum spore germination rate) was significantly higher in the $Control^{-PEG}$ treatment as compared to the two other treatments that did not differ amongst them (Figure 31b). Parameter "c" (referring to the lag time) was significantly higher in the PEG^{25} treatment as compared to the $Control^{-PEG}$ and PEG^{10} treatments (Figure 31b). No differences were found between $Control^{-PEG}$ and PEG^{10} treatments (Figure 31b). The germ tube length of germinated spores under decreasing water availability, is presented in Table 6. The germ tube length, estimated at 110 days, was significantly lower in the PEG^{10}, PEG^{25} and PEG^{50} treatments as compared to the $Control^{-PEG}$ treatment (Table 6). No significant differences were found between the three PEG treatments (Table 6).

(a)



(b)

	Treatment		
Curve parameter	Control $^{-PEG}$	PEG ^{10}	PEG ^{25}
Coefficient a	0.841 a	0.876 a	0.957 a
Coefficient b	0.271 a	0.104 b	0.073 b
Coefficient c	2.295 a	3.772 a	12.85 b

Figure 31 Germination percentage of spores of *R. irregularis* MUCL 41833. **(a)** in response to decreasing water availability. Treatments consisted in concentrations of 10, 25 and 50 g L^{-1} of polyethylene glycol (PEG) added to the medium (respectively, PEG^{10}, PEG^{25} and PEG^{50}) as well as a Control without PEG ($Control^{-PEG}$). Germination percentages were modeled using a non-linear regression and a 3-parameter Gompertz model ($y = aexp\left(-e^{-b(x-c)}\right)$). Observed percentages are represented by symbols whereas lines represent modelized curves under $Control^{-PEG}$, PEG^{10} and PEG^{25} treatments. **(b)** Coefficients of the models are estimated in each treatment. Coefficient "a" is the cumulated number of germinated spores when the plateau phase was reached, "b" the maximum spore germination rate and "c" the lag time. Different letters indicate significant differences between treatments using comparison of confidence intervals

Impact of decreasing water availability on Pi uptake by *R. irregularis* MUCL 41833, SDH activity of hyphae and plant parameters

Short-term dynamics of Pi uptake (throughout the text expressed as the % of Pi remaining in the medium, see definition in Materials and method section) under decreasing water availability is reported in Figure 32. Whatever the treatment ($Control^{-PEG}$, PEG^{10}, PEG^{25}, PEG^{50}), the % of Pi remaining in the medium decreased over time suggesting an efficient uptake of Pi by the extraradical hyphae (Figure 32).

No significant differences in the % of Pi remaining in the medium at T_{24} was found between treatments. This result is consistent with total SDH activity measured at the end of the experiment (comprised between 72 and 89%) that revealed no significant differences between the treatments (data not shown, P=0.9127). Analysis of the % of Pi remaining in the medium with repeated measures over the time showed, however, a significant effect of the factor "treatment" (P<0.0001) (Figure 32). Further analyses were thus conducted at each time point to compare % of Pi remaining in the medium. At T_4 and T_6, the % of Pi remaining in the medium in the $Control^{-PEG}$ treatment was significantly lower as compared to the PEG^{50} treatment but did not differ with PEG^{10} and PEG^{25} treatments (Figure 32). No significant differences were found between PEG^{10}, PEG^{25} and PEG^{50} treatments (Figure 32). Finally, at T_2, T_8, T_{12}, T_{18} and T_{24}, no significant differences were noticed in the % of Pi remaining in the medium between treatments (respectively P= 0.1302, P=0.0853, P=0.5294, P=0.4609 and P=0.4699).

At the end of the experiment, no significant differences were noticed in shoot and roots dry weights (P=0.1557 and P= 0.1224, respectively) and water content (P= 0.1281 and P= 0.119 respectively) (Table 7). P concentration in shoots differed significantly between treatments (P=0.0049) (Table 7). P concentration in shoots was significantly higher in PEG^{50} as compared to $Control^{-AMF}$, $Control^{-PEG}$ and PEG^{10} treatments but did not differ from PEG^{25} treatment. No differences were found between $Control^{-AMF}$, $Control^{-PEG}$, PEG^{10} and PEG^{25} treatments. No significant differences were found in roots P concentrations (P= 0.2944) and in shoots and root P contents (P=0.5672 and P=0.0681 respectively).

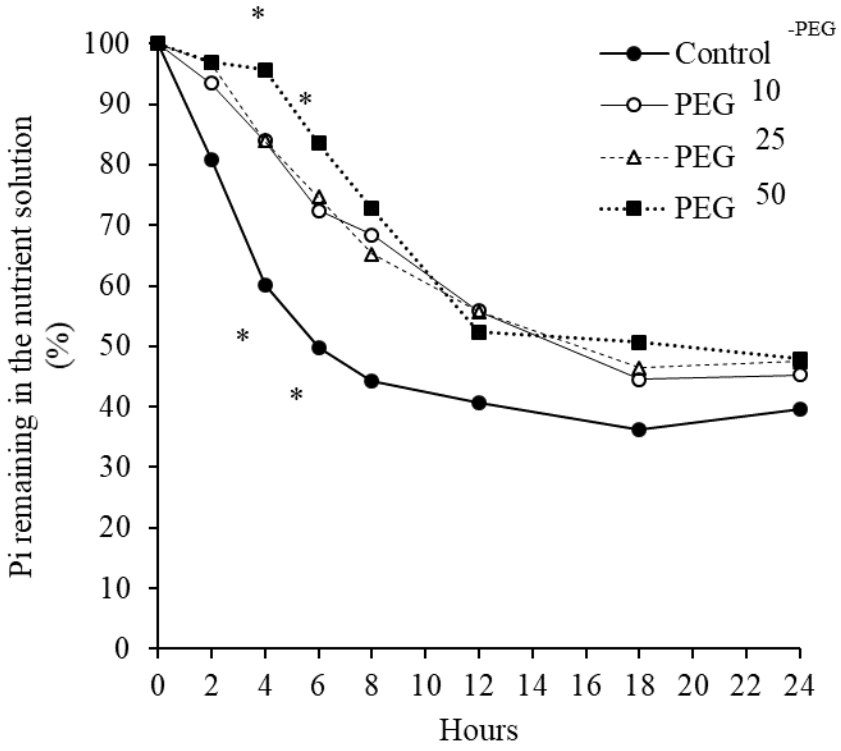


Figure 32 Percentage of inorganic phosphorus (Pi) remaining in the medium in presence of *R. irregularis* MUCL 41833 developing in the hyphal compartment under decreasing water availability during 24 hours. Treatments consisted in concentrations of 10, 25 and 50 g L^{-1} of polyethylene glycol (PEG) added to the medium (respectively, PEG^{10}, PEG^{25} and PEG^{50}) as well as a Control without PEG ($Control^{-PEG}$). Pi concentrations at each sampling time were standardized after Pi concentrations in the $Control^{-AMF}$ treatment and expressed in percentages relative to their T_0 values (see Material and Methods for further details). Data are presented as means. At T_4 and T_6, stars above marks represent significant differences between treatments after ANOVA1 analysis followed by pair-wised comparisons (Tuckey's test, $P<0.05$, n=6).

Table 6 Effect of decreasing water availability on (i) hyphal length and spore productions of *R. irregularis* MUCL 41833 developing in the hyphal compartment (HC) of a mycorrhizal donor plant *in vitro* culture system and on (ii) % of germination and germ tube length of isolated spores. Treatments consisted in concentrations of 10, 25 and 50 g L^{-1} of PEG added in the modified Strullu Romand medium (respectively, PEG^{10}, PEG^{25} and PEG^{50}) as well as a Control without PEG ($Control^{-PEG}$). Hyphal length and number of newly produced spores were estimated four weeks after the beginning of the experiment. Percentage of germination and germ tube length of spores were measured on day 110.

Parameters	Treatments			
	$Control^{-PEG}$	PEG^{10}	PEG^{25}	PEG^{50}
Hyphal length (cm)	1482.6 ± 741.0 [a]	1093.7 ± 368.0 [ab]	782.8 ± 211.2 [ab]	711.1 ± 246.3 [b]
Spore number	927.0 ± 180.0 [a]	662.7 ± 191.8 [ab]	504.8 ± 65.9 [bc]	411.5 ± 155.1 [c]
Germ tube length (cm)	1.010 ± 0.399 [a]	0.584 ± 0.314 [b]	0.522 ± 0.289 [b]	0.405 ± 0.239 [b]
Final germination (%)	93.18 [a]	90.69 [a]	93.48 [a]	20.93 [b]

Data are presented as means ± SD (n=6). Different letters indicate significant differences between treatments using pair-wised comparison (Tuckey's test, $P<0.05$).

Table 7 Shoot and root dry weights, water content and phosphorus content and concentration [P] of *Medicago truncatula* plantlets associated to *R. irregularis* MUCL 41833 developing in a hyphal compartment under decreased water availability during 24 hours. Treatments consisted in concentrations of 10, 25 and 50 g L^{-1} of PEG added to the medium (respectively, PEG^{10}, PEG^{25} and PEG^{50}) as well as a Control without PEG ($Control^{-PEG}$) and without mycelium in the hyphal compartment ($Control^{-AMF}$).

		Treatments				
	Plant part	$Control^{-AMF}$	$Control^{-PEG}$	PEG^{10}	PEG^{25}	PEG^{50}
Dry weight (mg)	Shoot	367 ± 61 [a]	410 ± 42 [a]	342 ± 87 [a]	335 ± 39 [a]	327 ± 41 [a]
	Root	353 ± 43 [a]	420 ± 52 [a]	358 ± 75 [a]	368 ± 49 [a]	410 ± 39 [a]
Water content (%)	Shoot	74.8 ± 1 [a]	73.9 ± 0.9 [a]	75.3 ± 1.1 [a]	75.7 ± 1.6 [a]	76 ± 1.7 [a]
	Root	90.2 ± 0.6 [a]	90 ± 0.9 [a]	89.3 ± 0.8 [a]	89.6 ± 1.2 [a]	88.8 ± 1.3 [a]
P content (mg)	Shoot	0.22 ± 0.05 [a]	0.22 ± 0.02 [a]	0.21 ± 0.07 [a]	0.20 ± 0.05 [a]	0.25 ± 0.03 [a]
	Root	0.27 ± 0.02 [a]	0.29 ± 0.04 [a]	0.25 ± 0.03 [a]	0.26 ± 0.02 [a]	0.27 ± 0.02 [a]
[P] (mg Kg^{-1})	Shoot	545 ± 63 [b]	522 ± 51 [b]	566 ± 104 [b]	571 ± 143 [ab]	714 ± 49 [a]
	Root	749 ± 76 [a]	671 ± 112 [a]	679 ± 113 [a]	663 ± 65 [a]	642 ± 48 [a]

Data are presented as means ± SD (n=6). Different letters indicate significant differences between treatments using pair-wised comparison (Tuckey's test, $P<0.05$).

Discussion

Drought stress is an increasing problem in agriculture and methods to alleviate or at least decrease its impact on crops are highly demanded. Plant roots are intimately associated with microorganisms. Some of them (e.g. AM fungi) have been reported to increase the resistance of plants to environmental stresses such as water deficit (Fernández-Lizarazo and Moreno-Fonseca, 2016; Jayne and Quigley, 2014). A few studies conducted within field also reported significant increase of plant biomass under dry environment (Caravaca *et al*., 2003; Pellegrino *et al*., 2011; Querejeta *et al*., 2006). This suggested that the utilization of AM fungi may potentially represent an innovative approach to address the issue of decreasing crop production under drought stress conditions. If the beneficial effects of AM fungi on plants under water stress have been reported (Fernández-Lizarazo and Moreno-Fonseca, 2016; Jayne and Quigley, 2014), limited knowledge is available on the impact of decreasing water availability on the development of AM fungi and their capacity to take up and transport Pi to plants. Here we reported on the impact of decreasing water availability on the development of *R. irregularis* MUCL 41833 and its capacity to take up and transport Pi under *in vitro* culture conditions. Water deficit was established via the application of increasing amounts (0, 10, 25 and 50 mg L^{-1}) of PEG to the medium (corresponding to a decrease in water potential in a range from −0,024 to −0,056 MPa). At the highest concentration of PEG and thus lowest availability of water, hyphal development and spore production, as well as spore germination were decreased. Similarly, the dynamics of Pi depletion in the root-free hyphal compartment was impacted at the highest concentration of PEG, even if total depletion was similar at the end of the experiment (i.e. after 24 h) suggesting an effective but slower dynamics of Pi uptake by the ERM.

Water potential measured in the MSR medium decreased inversely to PEG concentration and accordingly to previous results followed a quadratic model (Michel and Kaufmann, 1973). PEG significantly decreased the water potential of the MSR medium at the concentration of 50 g L^{-1}. Conversely, 10 and 25 g L^{-1} did not significantly decrease the water potential as compared to the $Control^{-PEG}$ treatment. Water potentials considered in our experiment (from −0,024 to −0,056 MPa) were above the limit of what is considered a drought stress environment (< −1MPa). However, it was sufficient to establish a gradient of water availability to the fungus impacting at the highest concentration development and to a lesser extend Pi uptake by the AM fungi.

Higher concentrations of PEG in the MSR medium were also tested and decreased further the water potential (see Figure 24 in Research Results − Chapter 1). However, under these conditions, the MSR medium did not solidify completely, probably due to a decreased polymerization of the gelling agent (i.e. Gelrite) as earlier reported by van der Weele *et al*. (2000). An increased opacity of the medium was also noticed, preventing the easy and rigorous observation of the AM fungi.

Hyphal length was significantly decreased in the PEG^{50} treatment and spore production in the PEG^{25} and PEG^{50} treatment as compared to the $Control^{-PEG}$ treatment. These results were consistent with the observations of Khalvati *et al*. (2005) in a pot experiment conducted in the greenhouse. These authors noticed that the extraradical hyphae density of *G. intraradices* was decreased under drought conditions. Conversely, Bethlenfalvay *et al*. (1988) and Davies *et al*. (1992) noticed an increase in hyphal length of *Glomus mosseae* and *Glomus deserticola*, respectively under greenhouse pot experiments. Similar contrasting results were reported for spore production. Drought drastically decreased their number in *Gigaspora albida* and *Scutellospora heterogama* (Silva *et al*., 2015), *Entrophospora columbiana* and *Acaulospora longula*, (Sieverding and Toro, 1988), while no effects were noticed on *Gigaspora margarita, Glomus clarum* and *G. mosseae* (Sylvia and Schenck, 1983) under greenhouse pot experiments. Spore germination was also significantly decreased in the PEG^{50} treatment as compared to the other treatments and germ tube length was significantly decreased at all PEG concentrations as compared to the $Control^{-PEG}$ treatment. This corroborates the results of Douds and Schenck (1991) and Sylvia and Schenck (1983). These authors, respectively, reported a decrease in germination of *G. intraradices, G. mosseae* and *A. longula* spores placed in soil substrate of water potentials equal or lower than −0.5 MPa and a decrease in germination of *G. clarum, G. etunicatum and G. macrocarpum* spores placed in soil substrate of water potentials below −0.01 MPa. Under *in vitro* conditions, Estaun (1989) observed a negative effect of glycerol–induced osmotic stress on germination and germ tube length of *G. mosseae* starting at a water potential of −0.21 MPa.

Noticeably, these results clearly demonstrated that the impact of decreasing water availability can drastically differ between AM fungal species. Although water potentials in the above-mentioned studies were much lower than in our experiment, *R. irregularis* MUCL 41833 seemed to be very

sensitive to water availability as noticed by the decreasing hyphal growth, spore production and spore germination obtained in the PEG^{50} treatment (corresponding to −0,056 MPa). In previous greenhouse pot experiments studies, the effects of drought on the decrease in spore production and extraradical development could not be strictly attributed to a direct impact on the fungus. Indeed, it was not possible to separate fungus from plant and thus direct from indirect effect on the fungus. Nevertheless, the authors suggested that the effects were due to a decrease of the carbon sink constituted by the plants (Augé, 2001; Manoharan *et al*., 2010; Silva *et al*., 2015). In our study conducted strictly *in vitro*, thus avoiding any confounding effects, only the ERM of the fungus was exposed to a decrease in water availability. The results clearly demonstrated that *R. irregularis* was directly impacted by a decrease in water availability. The effects observed were thus only attributable to the water potential and the resulting osmotic stress.

One of the most noticeable attributes of AM fungi is the ability of the fungus to take up and transport Pi to its host. The *in vitro* culture system used in this study was particularly adequate to investigate Pi uptake by the AM fungus as earlier demonstrated (Calonne *et al*., 2014; Nielsen *et al*., 2002; Zocco *et al*., 2011) but was never applied under conditions of decreased water availability. Under PEG-induced decrease in water availability, Pi uptake by the fungus was significantly impacted at the lowest water availability (PEG^{50}), while no impact was noticed for PEG^{10} and PEG^{25}. Indeed, within the first 6 h, the % of Pi remaining in the medium was lower in the HC of the $Control^{-PEG}$ treatment as compared to the PEG^{50} treatment indicating a probable effect of lower water availability on the Pi uptake and possibly on the Pi translocation to the plant. Interestingly, at the end of the experiment, no differences were noticed in % of Pi remaining in the medium whatever the water availability. Since SDH activity of the mycelium was similar between the different treatments, the decrease in Pi uptake by the fungus within the first hours cannot be clearly attributed to the death of a substantial proportion of hyphae. We hypothesized that the decreased rate of Pi uptake by the fungus could be related to a lower diffusion of Pi due to the viscosity of PEG solution or to an impact on the Pi transporters in the hyphae. It was, indeed, reported that under lower phosphate concentrations in the environment, the expression of the fungal Pi transporter *GintPT* was decreased (Maldonado-Mendoza *et al*., 2001). PEG could decrease water mass-flow and Pi diffusion to the hyphae causing *GintPT* to decrease its expression. The lower Pi uptake may be

accompanied by a slower translocation within the hyphae, although this needs to be demonstrated.

No significant difference in shoots and roots P content was found in the absence (Control^{-AMF}) or in the presence (Control^{-PEG}, PEG^{10}, PEG^{25}and PEG^{50} treatment) of mycelium in the HC. These results were expected as no differences in the Pi uptake by the mycelium could be detected between treatments after 24 h. Furthermore, it is unlikely that Pi uptake by the hyphae could directly impact P content in plants. Indeed, Pi quantities taken up by the hyphae are very small compared to the whole plant P content. Moreover, the short time of the experiment might not be sufficient to observe a complete translocation of Pi to the plant. Indeed, it has been demonstrated that Pi accumulated in symbiotic structures in the RC only after 37 h (Nielsen *et al*., 2002).

Surprisingly, we found that P concentrations in shoots were significantly increased under PEG^{50} treatment compared to the Control^{-AMF} (× 1.32), Control^{-PEG} (× 1.37), and PEG^{10} (× 1.26), treatments. Shoot P content was expected to follow the same trend given that shoot biomass was not significantly different between treatments. However, shoot P content did not differ significantly between treatments, probably because of the impact of high standard deviations. As no difference was found in root P concentration between treatments, increased P concentration in shoots under the PEG^{50} treatment might be due to a higher root to shoot P translocation compared to other treatments. In a similar experiment (Gil-Cardeza *et al*., 2017), an increased translocation of P from root to the shoot was also observed. Pi uptake by the mycelium was monitored in response to formaldehyde applied in the HC. After 24 h, P concentration in shoots of *M. truncatula* was significantly increased (×1.9) as compared to the treatment without stress. This effect was explained by a plant response to the high stress applied on the mycelium in the HC. Although the role of AM symbiosis in the plant P translocation is still barely known, some studies point the interaction of AM symbiosis long distance signals with the plant P homeostasis regulation. Analysis of miRNA expression pattern in mycorrhized *M. truncatula* plants under low phosphate conditions showed that AM fungi could increase the expression of miR399 (Branscheid *et al*., 2010), a P starvation signal involved in the regulation of root-shoot P compartmentation in the *Arabidospis thaliana* model (Doerner *et al*., 2008). Moreover, AM symbiosis can up or down

regulate the expression of numerous Pht1-type P transporter in plants and are believed to play a possible role in P translocation (Javot *et al*., 2007).

It was demonstrated that extraradical hyphae could directly transfer soil water to the plant and improve plant water status (Khalvati *et al*., 2005). However, the presence of ERM in the hyphal compartment seemed not to improve water status of the plant as no significant increase in shoots and roots water content of *M. truncatula* were found. Similarly, no effect was found by adding PEG while Li *et al*. (2013) demonstrated that PEG addition in the hyphal compartment enhanced expression of the fungal aquaporin genes *GintAQPF1* and *GintAQPF2* of *R. irregularis* and may increase water absorption by the hyphae. The absence of effect of the treatments on water content, in shoots and roots of *M. truncatula* in our experiment was expected because of the relative short time application of stress (24 h). Another explanation is that PEG concentration tested were not as severe as water potential level found under a drought environment.

Conclusion

The present study cannot be compared *per se* with drought conditions in natural environments as the water potentials tested in the *in vitro* system were not low enough to induce drought stress in soil. However, the *in vitro* culture system was adequate to investigate the impact of a gradient of water availability on various fungal parameters and function (i.e. Pi uptake and transport) under strict controlled conditions. PEG-induced decrease in water availability impacted hyphal development and spore production, spore germination and Pi uptake dynamics at the highest concentration suggesting a probable higher effect under even more limiting water conditions. The *in vitro* system used appeared adequate to investigate various parameters of AM fungi. It opens the door for comparative studies including AM fungal strains isolated from drought environments as well as for molecular studies involving expression of aquaporin genes or genes involved in stress resistance.

Supplementary material

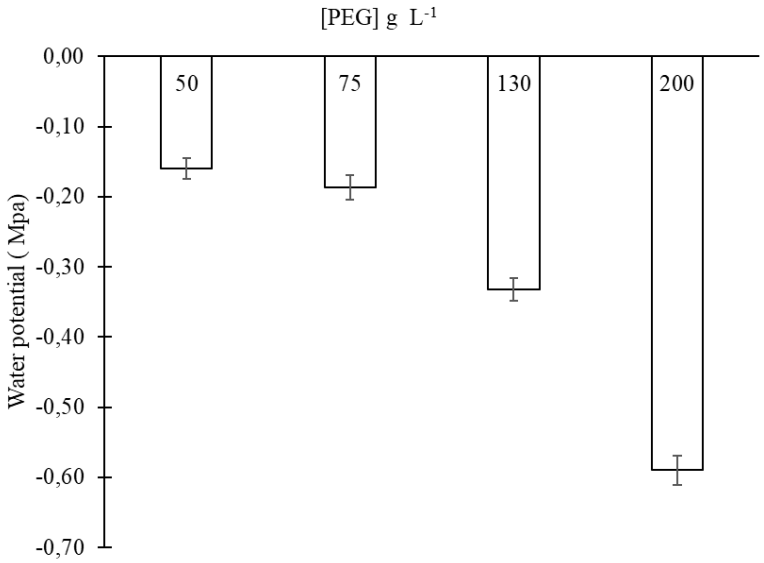


Figure S2 Water potential (Ψ) response of the Modified Strullu Romand medium supplemented with sugar (3g L^{-1}) and increasing concentrations of Polyethylene glycol 8000 (0, 50, 75, 130 and 200 g L^{-1}). Data are represented as means ± SD (n=3).

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Contribution of the authors

OLP contributed to the development of experiments, data collection, analysis, and interpretation of the data, drafted the work, and gave the final approval and agreed with all aspects of the work. SD made substantial contributions to the conception and design of the experiments, interpretation of the data, draft corrections, and gave the final approval and agreed with all aspects of the work.

Chapter 3

Rhizophagus irregularis MUCL 41833 improves phosphorus uptake and water use efficiency in maize plants during recovery from drought stress

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Preface

In chapter 2, we demonstrated that the life cycle of *Rhizophagus irregularis* MUCL 41833 grown under *in vitro* culture conditions was impacted by a slight decrease in water availability induced by the addition of PEG to the Modified Strullu-Romand (MSR) medium. The capacity of the fungus to take up Pi from the liquid MSR medium was slowed down as compared to the Control without PEG, even though the uptake at the end of the experiment was similar to the Control. This indicated that *R. irregularis* MUCL 41833 is able to grow and transport Pi to its host under reduced water availability, potentially sustaining plant growth under 'moderate' drought stress conditions. A number of studies supported these results (under *in vitro* or greenhouse conditions) but whether the AM fungus can also help plants recovering from drought stress remains almost unknown.

There is a growing interest in understanding the capacity of plants to recover from water stress as an adaptation strategy to drought. However, only a few studies have evoked the role of AM fungi in this process. These studies showed that when plants had an unlimited access to water and nutrients in the soil solution following water stress (during recovery), AM fungi could still enhance water and Pi contents in plants. So far, the mechanisms responsible for these beneficial effects have not been elucidated.

In chapter 3, it was the objective to better understand how AM fungi help plants to recover from drought by studying their effects on the dynamics of Pi uptake and leaf gas exchange parameters. In this experiment, maize, a model plant for drought stress studies, was associated or not with the AM fungus *R. irregularis* MUCL 41833. Maize plants were grown in a semi-hydroponic cultivation system and submitted to three water regimes (no drought stress, moderate and severe droughts). After the stress period, plants were watered at field capacity and the dynamics of Pi depletion during recovery was assessed by monitoring the Pi depletion (corresponding to Pi uptake/immobilization by the maize-AMF associates) in an impoverished Hoagland nutrient solution circulating in a closed loop through the plants.

Abstract

Irregular precipitations are likely to affect maize production in the future. Arbuscular mycorrhizal fungi (AM fungi) have been reported to increase maize resistance to drought, but their role on the short-term inorganic phosphorus (Pi) uptake, leaf gas exchange parameters and water content during recovery after drought remains poorly understood. Here, we investigated these parameters in maize plants colonized or not by *Rhizophagus irregularis* MUCL 41833. The mycorrhizal (M) and non-mycorrhizal (NM) plants were grown for a 3-weeks period in a circulatory semi-hydroponic cultivation system and were submitted to well-, moderately- or poorly-watered conditions (WW, MW and PW, respectively), the two latter conditions corresponding to moderate and severe droughts. The plants were then watered at field capacity for 42 h with a Pi impoverished Hoagland nutrient solution and the dynamics of Pi depletion in the nutrient solution, corresponding to Pi uptake/immobilization by the maize-AM fungus associates, was evaluated at 0, 9, 21 and 42 h. The CO_2 assimilation rate (A), stomatal conductance (g_s), transpiration (E) and instantaneous water use efficiency (WUE_i) were also assessed at 0 and 42 h of circulation. Plant biomass, plant water content, phosphorus concentration and content, and leaf relative water content were evaluated at harvest. During recovery, Pi uptake was significantly higher in M versus NM plants whatever the water regime (WR) applied before recovery. AM fungal colonization did not affect leaf gas exchange parameters before recovery but modulated g_s and E, and improved WUE_i after 42 h of recovery. At harvest, no significant difference in dry biomass was found between M and NM plants but shoot fresh weight was significantly higher in M plants. This resulted in an increased shoot water content in M plants grown in the MW and PW treatments. Surprisingly, leaf relative water content was significantly lower in M plants when compared with NM plants. Finally, P content and concentration were significantly higher in roots but not in shoots of M plants. Our results suggested that AM fungi can play a role in drought resistance of maize plants by increasing the Pi uptake and WUE_i during recovery after drought stress.

Keywords: maize, arbuscular mycorrhizal fungi, drought recovery, phosphorus uptake, leaf gas exchange, water use efficiency.

Introduction

Maize (*Zea mays* L.) is the third most consumed cereal worldwide, after rice and wheat (FAO, 2013). It is considered a main staple food in Southern and Eastern Africa, Central America and Mexico, playing an important role in food security (Ranum *et al*., 2014). The trend for more erratic rainfall in the next 50 years poses, however, a severe threat to maize production (Challinor *et al*., 2014; Li *et al*., 2009; Rosenzweig *et al*., 2014). Indeed, as a C_4 plant, maize is known to have a higher water use efficiency than C_3 plants such as wheat and barley (Akita and Moss, 1972). Yet, maize tend to have higher grain yield reduction under water shortage conditions and a higher drought sensitivity when compared to wheat (Daryanto *et al*., 2016). This could be explained partly by the drought avoidance strategy of maize plants that show decreased stomatal conductance, transpiration and eventually lower photosynthesis rate under lower soil water content (Sinclair *et al*., 1975).

The mechanisms involved in plant resistance to drought have been widely documented (see review by Fang and Xiong, 2015), while those involved during drought recovery have been less explored. A recent study conducted on maize indicated, however, that plant physiological responses differed during drought and recovery and that the recovery phase could play a more important role than previously believed (Chen *et al*., 2016). This corroborated the study of Hayano-Kanashiro *et al*. (2009) who observed that gene expression patterns between drought tolerant and drought sensitive maize genotype differed more during recovery from drought than during drought. Similar findings were reported in drought-tolerant cowpea genotype (Anyia and Herzog, 2004; Rivas *et al*., 2016) pointing at the importance of studying plant recovery to improve adaptability of plants to water paucity.

A number of studies have demonstrated the role of soil microorganisms in increasing drought resistance to drought stresses (Coleman-Derr and Tringe, 2014; Kim *et al*., 2012). Among these are the arbuscular mycorrhizal fungi (AM fungi). These soil microorganisms form symbiotic associations with an estimate of 72% of land plant species (Brundrett and Tedersoo, 2018) and have been suggested as promising root-associates for drought mitigation in plants (Boomsma and Vyn, 2008) via several mechanisms. For instance, improved cell turgor via osmotic adjustment in shoots (Wu and Xia, 2006), neutralization of reactive oxygen species in tissues via the synthesis of enzymes (e.g. superoxide dismutase) (Ruiz-Lozano *et al*., 1996), the increased

access to soil pores inaccessible to roots or root hairs (Smith and Read, 2008) and transport of water via the extraradical mycelium to the root cells (Ahmad Khan *et al*., 2003; Sánchez-Díaz and Honrubia, 1994), the enhanced water uptake in plants by stimulating the growth of root hairs (Zou *et al*., 2017) and by increasing root hydraulic conductivity through modulation of root aquaporin expression (Bárzana *et al*., 2012; Quiroga *et al*., 2018) have been reported (for details see reviews by Augé, 2001; Plouznikoff *et al*., 2016; Latef *et al*., 2016). Remarkably, in inorganic P (Pi) limiting soils, AM fungi can ameliorate P nutrition of plants by accessing to remote locations of Pi through their extraradical hyphae (Smith and Read, 2008) and it has been demonstrated that this improved Pi nutrition can maintain optimal growth and water relations and therefore increase plant resistance to drought (Augé, 2001; Fitter, 1988; Nelsen and Safir, 1982).

In contrast to the many studies conducted on the role of AM fungi in plant drought resistance (see above), the beneficial effects of these microorganisms during recovery from drought is much less investigated, especially in maize. An improved Pi nutrition was reported in AM fungi-colonized maize (Subramanian and Charest, 1997) and wheat (Bryla and Duniway, 1997) plants following drought recovery. However, it was not clear which from the Pi uptake during recovery or during drought contributed the most to the improved P nutrition. Studies conducted by Levy and Krikun (1980) in citrus plants and by Goicoecha *et al*. (2004) in *Anthyllis cytisoides* revealed an improved water status in AM fungi-colonized plants together with an improved photosynthesis, leaf stomatal conductance and transpiration, during a five and 20 days recovery period, respectively. In maize, Subramanian and Charest (1997) indicated an accelerated recovery of leaf-turgidity in AM fungi-colonized plants. To understand how AM fungi may help plants to better recover from drought it could be important to examine and compare the short-term dynamics of Pi uptake and to analyze leaf gas exchanges in presence/absence of these root symbionts. Recently, a semi-hydroponic cultivation system developed by Colpaert *et al*. (1999) for pine associated to ectomycorrhizal fungi, was adapted by Garcés *et al*. (2017) to study the short-term dynamics of Pi uptake/mobilization by maize plants associated with AM fungi. The mycorrhizal maize plants were grown in containers on perlite and irrigated with a Hoagland Pi-impoverished nutrient solution circulating in a closed loop. The Pi uptake dynamics by the plant/AM fungus associates was deduced from the Pi depletion in the circulating nutrient solution.

In the present study, the semi-hydroponic cultivation system was used to assess the effect of AM fungi on the dynamics of Pi uptake and on leaf gas exchanges of maize plants during the first 42 h of recovery from drought. The plants inoculated with *Rhizophagus irregularis* MUCL 41833 and their non-colonized counterparts were grown under well-, moderately- or poorly-watered conditions for three weeks and then watered at field capacity for 42 h with a Pi-impoverished nutrient solution circulating in a close loop. The dynamics of Pi uptake was monitored at 0, 9, 21 and 42 h, and leaf gas exchanges measured at times 0 and 42 h after setup of the circulatory system. Finally, the plant biomass, P content and concentration, water content and leaf relative water content were assessed at the end of the experiment.

Materials and Methods

Biological material

The arbuscular mycorrhizal fungus (AM fungus) *Rhizophagus irregularis* (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as [‘irregulare'] MUCL 41833 was supplied by the Glomeromycota *in vitro* collection (GINCO – http://www.mycorrhiza.be/ginco-bel) and grown *in vitro* on excised Ri T-DNA transformed roots of carrot (*Daucus carota* L.) as described in Cranenbrouck *et al*. (2005).

Seeds of maize (*Zea mays* L. cv. ES Ballade) were supplied by the Centre Indépendant de Promotion Fourragère (CIPF – http://www.cipf.be/). They were surface-disinfected as described in Garcés-Ruiz *et al*. (2017) and placed on wet paper (N°1 Whatman, United Kingdom) in closed plastic containers (Aarts Plastics, Netherlands) in the dark at room temperature (~20°C) for germination during four days.

Preparation of the maize plantlets

Three 5 L pots containing a sterilized (121°C for 15 min) substrate composed of vermiculite (PullRhenen, Netherlands), sand (quartz N°4, Euroquartz, Belg) and lava stone (DCM, Belgium) (w:w:w, 1:1:1) were prepared to produce mycorrhizal (M) plants. In each pot, six maize seedlings were placed with their roots in contact with an approximate of 100 spores of an *in vitro* culture of *R. irregularis*. Three other pots containing a similar substrate but without AM fungal inoculum were used to produce non-

mycorrhizal (NM) plants. All the pots were maintained 4 months under greenhouse conditions (21°C/18°C (day/night), a relative humidity (RH) varying from 40 to 70%, a photoperiod of 16 h day^{-1} and a photosynthetic photon flux (PPF) of 120 µmol m^{-2} s^{-1}) to produce maize plants with strong root systems whether colonized or not with the AM fungus. The shoots of the plants were then cut, and six new three-days-old disinfected maize seedlings were planted in the pots containing or not the AM fungus extending from the roots of the cut maize plants. The plants were watered twice a week with 1400 ml of deionized water and received additionally 500 ml of Pi-impoverished modified Hoagland solution ($Hoagland^{lowPi}$ – see Garcés-Ruiz *et al*. (2017) for chemical composition). The plants were kept in a grow chamber set at 21/21°C (day/night), a RH of 75%, a photoperiod of 16 h day^{-1} and a PPF of 300 µmol m^{-2} s^{-1}.

Experimental set up

After three weeks, the maize plants were gently removed from the pots and roots carefully rinsed from the substrate with deionized water. Three plants from the M and NM treatments were randomly selected and root colonization evaluated (see below). The other plants were transferred to the greenhouse into individual containers that consisted of 500 ml wash bottles filled with 25 g of dry perlite (see Garcés-Ruiz *et al*. (2017) for details). The bottles were inverted (i.e. with the neck below closed by a nylon mesh of 100 µm size pore (Prosep B.V.B.A., Belgium) to avoid substrate loss) and disposed in holes made in flex foam supports disposed on tables. Nine containers without plants were also included as non-vegetated (NV) Controls. Every two days, all the plants and the NV Control containers received the same volume (i.e. between 60 and 100 ml) of $Hoagland^{lowPi}$ solution. Deionized water was further added until saturation of the substrate (estimated until the first drops of water were released from the opening at the bottom of the containers). After 24 days of acclimatization in the perlite, the M and NM maize plants had the same height ($P = 0.923$). They were separated in three homogenous groups and grown under three water regimes (WR): well-watered (WW^{M} and WW^{NM}), moderately-watered (MW^{M} and MW^{NM}) or poorly-watered (PW^{M} and PW^{NM}), the two later corresponding to moderate and severe drought conditions, respectively (see below for details). Six replicates were considered per treatment and three NV Control containers were assigned to each WR. Treatments and Controls were randomly distributed among 5 tables, with each treatment represented at least once on

each table. Position of the containers were finally randomized on each table. The plants were subsequently grown under the three WR for a three weeks-period. Every two days, the plants in the WW treatment were supplied with deionized water to maintain saturation of the perlite (see details above), while in the MW and PW treatments, the plants received 60 and 30 ml of deionized water corresponding to 50% and 25% of the volume of water necessary to saturate the substrate (estimated to 120 ml deionized water, data not shown). After one week, the circulatory system was started a first time with Hoagland^{lowPi} solution for 42 h without any analysis. In the two following weeks, plants were watered as described above before setting the circulatory system a second time. During this second period, the MW and PW treatments induced drought stresses in the maize plants as noticed by the severe decrease in CO_2 assimilation rate and the wilting of the leaves (data not shown). To summarize, the maize seeds were germinated for three days, then associated or not with the AM fungus in 5 L pots for 21 days. The M and NM plants were then transferred in the semi-hydroponic cultivation system and acclimatized for 24 days. Three water regimes (WW, MW and PW) were then applied for one and two weeks respectively, both periods being separated by 42 h of recovery. At harvest, the plants were grown in the semi-hydroponic cultivation system for 49 days and were 73 days old.

Measurement of short-term Pi depletion in the Hoagland^{lowPi} solution circulating through the containers containing the M and NM maize plants and non-vegetated Controls

The short-term depletion of Pi in the Hoagland^{lowPi} solution was monitored using a closed semi-hydroponic cultivation system as described by Garcés-Ruiz *et al*. (2017). Briefly, 1 L of Hoagland^{lowPi} solution contained in glass bottles circulated via peristaltic pumps through the containers with M or NM maize plants and returned to the bottles in a closed loop (see protocol in Material and Methods - section 4.8.). After a flushing step to equally saturate perlite in Hoagland^{lowPi} solution (Garcés-Ruiz *et al*., 2017), the Pi uptake by the plants and AM fungus was deduced from the Pi depletion in the circulating solution during 42 h. Initial Pi concentration in the nutrient solution was determined by sampling 15 ml of the solution from each bottle (considered as Time 0 hour (T_0)) before the start of the circulatory system. The circulatory system was then initiated at a rate of 7.4 ml min^{-1} and 15 ml of nutrient solution was collected at 9, 21 and 42 h. The samples were then stored at 4°C in the dark and Pi concentration subsequently measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) as described in Garcés-Ruiz *et al*. (2017). Data were then normalized according to the NV Controls (Garcés-Ruiz *et al*., 2017). Finally, the data expressed in mg kg^{-1} were converted into % of Pi concentrations remaining in the nutrient solution relative to the initial concentration at T_0 using the following formula:

$$\% \text{ of } Px = ([Px]T + [P_{NV}]T_0 - [P_{NV}]T) / [Px]T_0 \times 100$$

where:

[P] = Pi concentration in the solution

x = sample

NV = non-vegetated containers (i.e. perlite Control) respective to the sample analyzed

$[P_{NV}]$ = mean Pi concentration (3 replicates) for the non-vegetated containers

T= time considered (9, 21, or 42 h after the start of the circulatory system)

T_0 = 0 h before the start of the circulatory system

Leaf gas exchange parameters in maize leaves

Net CO_2 assimilation rate (A), stomatal conductance (g_s) and transpiration rates (E) were measured in the morning (from 8 to 12 AM) before starting the circulatory system (considered as time = 0 h), and at the end of the circulation (at time = 42 h), using an InfraRed Gaz Analyzer (IRGA-LCi Photosynthesis system, ADC BioScientific Ltd). For each plant, an average of three measures recorded from a 5.8 cm^2 area on the 4 or 5^{th} leaf was considered for the analysis. Instantaneous water use efficiency was calculated using the following equation (Bacelar *et al*., 2012): WUE_i= A/E.

Plant harvest

At the end of the experiment, i.e. 49 days after transfer into the systems, the plants were harvested. The shoot and roots fresh weights (SFW and RFW, respectively) of each plant were evaluated and a leaf sample (approx.1x7cm) was cut from the 5^{th} leaf to evaluate leaf relative water contents (see below). Shoots and roots were oven-dried at 50°C for seven days for evaluation of shoot and roots dry weights (SDW and RDW, respectively) and leaf samples were further used for the determination of the leaf relative water contents (RWC_L− see below).

The root systems were subsequently separated in two parts to evaluate AM fungal root colonization and P concentration in tissues. After evaluation of dry weight, 500 mg of shoot and roots were ground separately for P analysis. Samples were digested following the procedure described in Garcés-Ruiz *et al*. (2017). Phosphorus concentration was quantified using ICP-AES.

RWC_L was determined at harvest from a sample of maize leaf (see above). After determination of the FW, the leaf sample was placed in a Petri dish containing water at 4°C in the dark for one night and turgid weight (TW) was estimated. Leaf samples were then oven-dried at 50°C for 4 hours and dry weight (DW) was estimated. The RWC_L was calculated using the equation of Barrs and Weatherley (1962): RWC_L (%) = (FW–DW)/(TW–DW) ×100

AM fungal root colonization

Root colonization was estimated on 3 replicates from the M and NM plants at the time of transfer to the systems (i.e. 24 days old plants) and on each replicate of the six treatments at harvest (i.e. 49 days after transfer in to the systems – 73 days old plants). Roots were sampled, washed from the substrate, cut into small pieces and placed into 50 ml Falcon tubes (Sarstedt, Germany). Twenty-five ml of KOH 10% was added to the roots before incubation at 70°C in a water bath for 1 h. The KOH solution was removed and roots were washed with HCl 1%. The roots were further stained with ink 2% (Parker blue ink, USA) in HCl 1% (Walker, 2005) by placing the tubes at 70°C in a water bath for 1 h. The roots were rinsed and stored in deionized water before observation (Walker, 2005). Percentages of total (%TC), arbuscular (%AC) and vesicular (%VC) root colonization were estimated under a compound microscope (Olympus BH2–RFCA, Japan) at 100× magnification (McGonigle *et al*., 1990). At each intersection, the presence or absence of fungal structures was noted. Around 100 intersections were observed per plant.

Statistical analysis

Data analysis was performed with JMP 13.2 and SAS 9.4 statistical software (SAS Institute Inc., Cary, NC, USA) with a 5% α-threshold. Levene and Shapiro-Wilk tests were run prior to the statistical analysis to confirm homogeneity of variance and normality in the distribution, respectively. Phosphorus depletion in the Hoagland^{lowPi} nutrient solution was analyzed with a mixed model for repeated measurements, where "time" of sampling (i.e. 9, 21, 42 h), "plant number" (from 1 to 36) and "table" (5 different tables) were considered as random factors while "AMF"(AM fungal colonization) and "WR" (water regime) (i.e. six conditions) were regarded as fixed factors. Plant biomass, tissue P content and concentration, and RWC_L were analyzed using a mixed model analysis with REML estimation where "AMF" and "WR" were regarded as fixed factors and "table" as random factor. Following significance of the factors, contrast analysis ($p \leq 0.05$) was conducted to determine differences between groups. Data of g_s at time 0 h and data of g_s and E at time 42 h were submitted to a square root transformation to meet the assumption of the homogeneity of variance. Before and after circulation, A, g_s and WUE_i were analyzed using a mixed model with REML estimation followed by contrast analysis as described above. When a significant interaction between

factors was found, a one-way ANOVA was run for each level of each factor "AMF" and "WR", separately. Water contents was analyzed using Wilcoxon's non-parametric comparison for each level of each factor "AMF" and "WR", separately. Results of multiple pairwise comparisons were taking in account Bonferroni's correction of p-values. Finally, root mycorrhizal colonization percentages were arcsine transformed and analyzed using a one-way ANOVA followed by a pairwise comparison using Tukey-HSD test. The raw data are provided as supplementary material (Appendix 4).

Results

AM fungal root colonization

The root colonization parameters were measured at transfer of the maize plants into the containers and at harvest in the WW, MW and PW treatments (Table 8). The %TC in the plants at transfer did not differ from the %TC in the plants of the WW treatment at harvest, while it was significantly higher as compared to the plants in the MW^M and PW^M treatments. Whatever the water regime, no significant differences were observed in %AC between the plants at transfer and those at harvest. The %VC was significantly higher in the plants of the WW^M treatment as compared to the plants in the PW^M treatment but did not change significantly from the plants in the MW^M treatment and from the plants at transfer. No significant difference in %VC was noted between the plants in the MW^M and PW^M treatments as compared to the plants at transfer.

Table 8 Percentages of arbuscular- (%AC), vesicular- (%VC) and total colonization (%TC) of maize roots associated with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* MUCL 41833 at transfer into the semi-hydroponic container system (after 21 days of pre-mycorrhization) and at harvest (in 73-days-old plants that were grown for a three-weeks period under well- (WW), moderately (MW) or poorly-watered (PW) condition).

	AC (%)	**VC (%)**	**TC (%)**
At plant transfer	68 ± 6.4 [a]	42 ± 6.3 [ab]	97 ± 1.8 [a]
Harvest time			
WW	69 ± 13.5 [a]	47 ± 12 [a]	77 ± 15 [ab]
MW	51 ± 8.7 [a]	37 ± 15 [ab]	69 ± 18 [b]
PW	54 ± 13 [a]	25 ± 11 [b]	63 ± 19 [b]

Data were analyzed with a one-way ANOVA followed by pairwise comparison using Tukey-HSD test. Data in a column followed by similar letter do not differ significantly ($p \leq 0.05$). Three replicates were considered at plant transfer into the containers and six per water regime (WW, MW, PW) at harvest. Data are presented as mean ± SD.

Phosphorus depletion in the nutrient solution

The short-term dynamics of Pi depletion in the nutrient solution was assessed on maize plants that were grown for a three-weeks period under three contrasting WR (WW, MW, PW) before watering them at field capacity for 42 h. Pi depletion (throughout the text expressed as a relative percentage of Pi concentration in the medium or %Pi) was measured 0, 9, 21 and 42 h after the setup of the circulatory system. Whatever the WR preceding circulation of the nutrient solution, a decrease was noticed in the %Pi in the nutrient solution between times 0 and 42 h (Figure 33), suggesting a concomitant Pi-uptake by the plant and AM fungus associates. Using the mixed model for repeated measures, the %Pi was affected by the factors AMF, WR, Time, and the interaction WR*Time ($P<0.0001$), this later indicating that the impact of the WR significantly differed with time. Indeed, the %Pi was lower for the plants in the WW treatment when compared with those in the MW or PW treatments at any time but this difference was less significant at time 42 h than at times 9 and 21 h (At times 9 and 21 h, $P<0.0001$ for plants in both MW and PW

treatments when compared with plants in the WW treatment while at time 42 h, P= 0.007 and P=0.004 for the plants in the MW and PW treatments respectively when compared with those in the WW treatment). No difference was found between the plants in the MW or PW treatments at time 9, 21 and 42 h. (P=0.425, P=0.883 and P=0.967, respectively). Conversely, the %Pi depletion was not affected by AMF*WR, AMF*Time and AMF*WR*Time interactions (P>0.05). This suggested that the effect of the AM fungus on the %Pi depletion in the nutrient solution was effective whatever the WR or time considered. Interestingly, the plants in the MW^{M} and PW^{M} treatments took up proportionally more Pi (as compared to their respective NM Controls) as compared to the plants in the WW^{M} treatment. Indeed, for the plants in the MW^{M} treatment, the decrease in % Pi at 9 and 21 h was two times higher than for those in the MW^{NM} treatment (i.e. 18 and 9% at 9 h and 43% and 23% at 21 h for the M and NM plants, respectively). For the plants in the PW^{M} treatment the decrease in %Pi as compared to the plants in the PW^{NM} treatment was 50% and 18% at times 9 and 21 h respectively, while for the plants in the WW^{M} treatment, it was only 10 and 13% at times 9 and 21 h, respectively as compared to those in the WW^{NM} treatment.

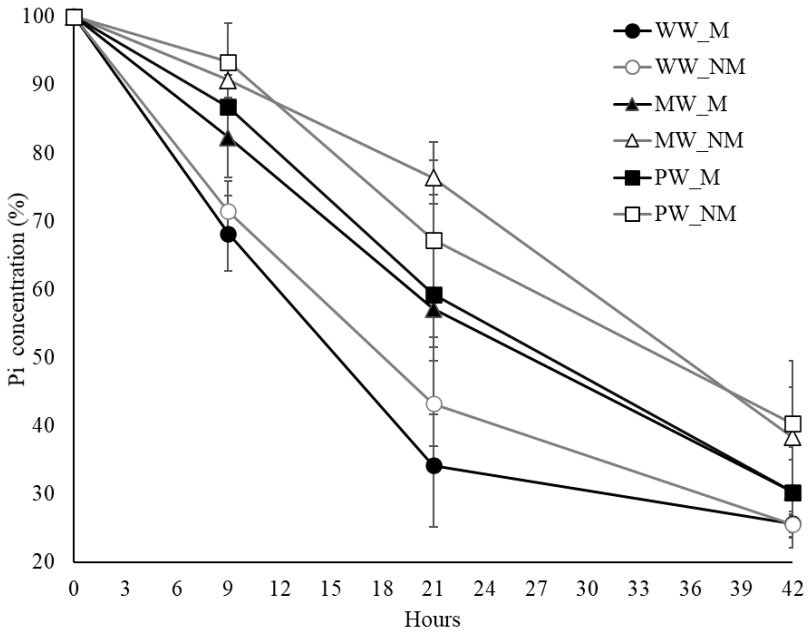

Figure 33 Dynamics of Pi depletion in the Hoagland^{lowPi} nutrient solution circulating in containers with mycorrhizal (M) and non-mycorrhizal (NM) maize plants grown under well- (WW), moderately- (MW) or poorly-watered (PW) conditions for a three-week period before the start of the circulatory system. Percentages of Pi concentration remaining in the nutrient solution were expressed from Pi concentrations measured at times 9, 21 and 42 h relative to Pi concentrations measured at time 0 h. The AM fungus was *Rhizophagus irregularis* MUCL 41833. Data are presented as mean ± SD of six replicates per treatment.

Physiological parameters

The values for A, g_s, E and WUE_i were analyzed in leaves before (i.e. Time 0) and after circulation of the nutrient solution (i.e. Time 42 h) (Table 9). At time 0 h, no significant effects of the factor AMF or the interaction AMF*WR were noticed for any of the parameters evaluated in the leaves (Table 9). A significant effect of the factor WR was noticed on A, g_s and WUE_i, but not on E. When compared with the plants in the WW treatment, the A, g_s and WUE_i significantly decreased in leaves by 58, 83 and 48%

respectively, in the plants of the MW treatment, and decreased by 25, 60 and 34% respectively, in the plants of the PW treatment.

At 42 h following circulation, the WUE_i measured in leaves was significantly higher in the M plants as compared to the NM ones (Table 9). The WR had an impact on A. Indeed, A significantly increased for the plants on the MW and PW treatment when compared to those in the WW treatment (Table 9). The interaction AMF*WR significantly influenced g_s and E, suggesting that the impact of WR was dependent of the root colonization by the AM fungus and *vice-versa*. Indeed, whereas the parameters E and g_s remained similar in leaves of NM plants whatever the WR, their value increased in the leaves of the plants in the PW^M treatment as compared to those in the WW^M treatment. In addition, the plants in the WW^M treatment had a significant lower g_s than those in the WW^{NM} treatment, while under MW and PW conditions, no significant differences were noticed between M and NM plants (Table 9). Indeed, the g_s measured in the leaves of the plants in the WW^M treatment was 72.3% lower as compared to those in the WW^{NM} treatment. Similarly, E significantly decreased by 57 and 42.9% in the leaves of the M plants as compared to NM leaves, under WW and MW WR, respectively.

Table 9 Impact of different water regimes (WR) and AM fungal colonization (AMF) by *Rhizophagus irregularis* MUCL 41833 on the net CO_2 assimilation rate (A), stomatal conductance (g_s), transpiration rate (E) and instantaneous water use efficiency (WUE_i) measured in maize leaves at the start (0 h) and after 42 h of circulation of the nutrient solution. Mycorrhizal (M) and non-mycorrhizal (NM) maize plants were grown under well- (WW), moderately- (MW) or poorly-watered (PW) conditions for three weeks.

Time = 0 h	A ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	g_s ($mmol\ m^{-2}\ s^{-1}$)	E ($mmol\ H_2O\ m^{-2}\ s^{-1}$)	WUE_i ($\mu mol\ CO_2\ mol^{-1}\ H_2O$)
Groups				
M	0.8 ± 0.5	2.8 ± 4	0.2 ± 0.1	4.1 ± 2.4
NM	0.9 ± 0.4	2.2 ± 3.4	0.2 ± 0.1	5.4 ± 3.4
WW	1.2 ± 0.3 α	4.7 ± 4.4 α	0.2 ± 0.1	6.5 ± 3.7 α
MW	0.5 ± 0.4 γ	0.8 ± 2.1 β	0.2 ± 0.1	3.4 ± 2.6 β
PW	0.9 ± 0.2 β	1.9 ± 3.3 β	0.2 ± 0.1	4.3 ± 1.5 β
WW^{M}	1.2 ± 0.5	5 ± 4.6	0.2 ± 0.1	6.1 ± 2.6
WW^{NM}	1.2 ± 0.2	4.4 ± 4.6	0.2 ± 0.1	7 ± 4.7
MW^{M}	0.4 ± 0.3	1.1 ± 2.7	0.2 ± 0.1	2.1 ± 0.9
MW^{NM}	0.7 ± 0.5	0.6 ± 1.4	0.2 ± 0	4.7 ± 3.2
PW^{M}	0.8 ± 0.2	2.2 ± 4.0	0.2 ± 0.1	4.1 ± 1.8
PW^{NM}	0.9 ± 0.3	1.7 ± 2.8	0.2 ± 0.1	4.5 ± 1.3
Effects				
AMF	ns	ns	ns	ns
WR	***	*	ns	*
AMF × WR	ns	ns	ns	ns
Table (% of the total effect)	0	18.2	4.45	16.6

To be continued→

Table 9 (continued)

Time = 42 h	A ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	g_s ($mmol\ m^{-2}\ s^{-1}$)	E ($mmol\ H_2O\ m^{-2}\ s^{-1}$)	WUE_i ($\mu mol\ CO_2\ mol^{-1}\ H_2O$)
Groups				
M	1.4 ± 1.1	17 ± 22	0.5 ± 0.5 b	3.0 ± 1.6 a
NM	1.1 ± 1	23 ± 15	0.7 ± 0.4 a	1.8 ± 1.2 b
WW	0.8 ± 0.6 γ	14 ± 15	0.5 ± 0.4	2.4 ± 1.9
MW	1.3 ± 0.6 β	17 ± 13	0.5 ± 0.3	2.7 ± 1.4
PW	1.6 ± 1.5 α	28 ± 25	0.8 ± 0.5	2.1 ±1.3
WW^{M}	0.8 ± 0.6	6.1 ± 8* B	0.3 ± 0.2* B	3.5 ± 1.9
WW^{NM}	0.8 ± 0.8	22 ± 16* *A*	0.7 ± 0.4* *A*	1.2 ± 0.8
MW^{M}	1.4 ± 0.8	12 ± 6.9 AB	0.4 ± 0.2* AB	3.1 ± 1.4
MW^{NM}	1.3 ± 0.5	22 ± 15 *A*	0.7 ± 0.4* *A*	2.4 ± 1.4
PW^{M}	1.9 ± 1.6	32 ± 32 A	0.8 ± 0.7 A	2.5 ± 1.4
PW^{NM}	1.3 ± 1.5	24 ± 11 *A*	0.7 ± 0.4 *A*	1.8 ± 1.2
Effects				
AMF	ns	ns	*	**
WR	**	ns	ns	ns
AMF × WR	ns	*	*	ns
Table (% of the total effect)	60.62	32.96	45.3	21.16

The effect of the fixed factors AM fungal colonization (AMF) and water regime (WR) as well as their interactions on plant physiological parameters were tested using the mixed model with REML analysis ($p \leq 0.05$). Percentages of the effect due to the random factor Table are reported. When no significant interactions between fixed factors were found, significant differences among WR and between M and NM plants are indicated using contrast analysis. In the case of a significant interaction, the significant differences between the different levels of each treatment were tested separately using a one-way ANOVA followed by Tukey-HSD test ($p \leq 0.05$).

- Different lower-case letters indicate significant differences between M and NM plants according to the contrast analysis.

- Different Greek letters indicate significant differences between WR according to the contrast analysis

- Different upper-case letters and different upper-case letters in italics indicate significant, differences between WR inside each M and NM level, respectively.

- * indicate significant differences between M and NM plants inside each WR level.

Data are represented as mean ± SD of six replicates per treatment. For the P-values, ns =no significant difference, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Plant growth parameters

At the end of the experiment, plants were harvested and the FW and DW, the P concentration and content, as well as the WC and RWC_L were evaluated (Table 10). Whatever the WR, the SDW, P concentration and total P content in shoots did not differ significantly between the M and NM plants, while, the SFW was significantly higher in the M plants as compared to the NM ones (Table 10). Conversely, the RWC_L was significantly higher in the NM plants as compared to the M plants. No significant difference was noticed in the shoot WC between the plants in the WW^M and WW^{NM} treatments, while under MW and PW conditions, a significant increase of shoot WC (by 2.9 and 2.6%, respectively) was noticed in the M plants as compared to the NM ones. Whatever the presence or absence of the AM fungus within roots, the WR significantly impacted the SFW, SDW, and P concentration in shoots (Table 10), but not the total P contents and RWC_L. Indeed, the SFW and SDW significantly decreased in the plants of the MW and PW treatments. A significant lower P concentration was measured in the shoots of the plants in the WW treatment as compared to those in the MW or PW treatments. Nonetheless, when reported to P contents in shoots, no significant impact of WR was noticed. No significant interaction of WR*AMF was observed for any of the shoot parameters, demonstrating the absence of interactions between WR and presence/absence of the AM fungus (Table 10).

In roots, no significant effect of the factor AMF was observed on the RFW and RDW whatever the WR, while P concentration and content were significantly higher in the M plants as compared to their respective NM Controls (Table 10). These parameters were also significantly affected by the factor WR but not by the interaction AMF*WR. Indeed, the RFW and RDW as well as the P contents in roots decreased in the plants of the MW and PW treatments whatever the presence/absence of the AM fungus. To the contrary, the P concentration in roots increased in the plants of the MW and PW treatments as compared to those in the WW treatment (Table 10). No significant impact of the AM fungus or WR could be observed on root WC.

Table 10 Impact of different water regimes (WW, MW, PW) and AM fungal colonization (AMF) by *Rhizophagus irregularis* MUCL 41833 on fresh weight (FW), dry weight (DW), water contents (WC), P concentration ([P]) and P contents of shoots and roots and on leaf relative water contents (RWC_L) of maize at harvest time, after 68 days of growth. Mycorrhizal (M) and non-mycorrhizal (NM) maize plants were grown under well- (WW), moderately- (MW) or poorly-watered (PW) conditions for three weeks.

Shoots		FW (g)	DW (g)	WC (%)	RWC_L (%)	[P] (mg P g^{-1} of DW)	P contents (mg $plant^{-1}$)
Groups	M	52 ± 7.7 a	6.4 ± 2.3	88 ± 3.1 a	75 ± 18 b	1.5 ± 0.6	9 ± 2.6
	NM	46 ± 12 b	6 ± 1.8	87 ± 2.4 b	90 ± 25 a	1.5 ± 0.5	8.4 ± 2.6
	WW	59 ± 7.3 α	8.1 ± 2.4 α	86 ± 3.8	78 ± 23	1 ± 0.5 γ	8 ± 3.3
	MW	49 ± 7.3 β	5.7 ± 1 β	88 ± 2.3	84 ± 20	1.5 ± 0.3 β	8.8 ± 2.4
	PW	40 ± 6.4 γ	4.8 ± 0.6 β	88 ± 1.6	88 ± 26	2 ± 0.4 α	9.4 ± 2.0
	WW^{M}	60 ± 5.3	8.9 ± 2.3	85 ± 3.7 B	71 ± 21	0.9 ± 0.2	7.6 ± 2.5
	WW^{NM}	59 ± 9.4	7.4 ± 2.5	87 ± 3.9 *A*	83 ± 24	1.2 ± 0.6	8.4 ± 4.2
	MW^{M}	52 ± 4	5.5 ± 1.1	89 ± 2.1* AB	77 ± 14	1.7 ± 0.2	9.2 ± 2.5
	MW^{NM}	46 ± 8.7	6 ± 0.9	86 ± 1.5* *A*	92 ± 22	1.4 ± 0.3	8.3 ± 2.4
	PW^{M}	44 ± 4.5	4.9 ± 0.7	89 ± 0.7* A	79 ± 21	2.1 ± 0.5	10 ± 2.5
	PW^{NM}	35 ± 3.7	4.7 ± 0.4	86 ± 1.1* *A*	96 ± 30	1.8 ± 0.3	8.4 ± 0.9
Effects	AMF	*	ns	*	*	ns	ns
	WR	***	***	ns	ns	***	ns
	Table (% of the total effect)	0	0	na	8.7	7.6	0

To be continued →

Table 10 (Continued)

Roots		FW (g)	DW (g)	WC (%)	[P] (mg P g^{-1} of DW)	P contents (mg $plant^{-1}$)
Groups	M	23 ± 8.6	2.3 ± 1.0	89 ± 4	1.3 ± 0.2 a	3 ± 1.3 a
	NM	23.4 ± 7.1	2.0 ± 0.6	91 ± 2	1 ± 0.2 b	2 ± 0.5 b
	WW	30 ± 6.6 α	2.8 ± 0.8 α	90 ± 4	1 ± 0.6 γ	2.9 ± 1.2 α
	MW	23 ± 5.4 β	2.1 ± 0.6 β	91 ± 3	1.1 ± 0.3 β	2.4 ± 1.1 β
	PW	17 ± 4.5 γ	1.5 ± 0.6 β	91 ± 3	1.3 ± 0.3 α	1.9 ± 0.7 γ
	WW^{M}	30 ± 8.5	3.2 ± 0.9	89 ± 5	1.1 ± 0.1	3.7 ± 1.3
	WW^{NM}	30 ± 4.8	2.4 ± 0.6	91 ± 2	0.9 ± 0.1	2.2 ± 0.4
	MW^{M}	22 ± 6.3	2.0 ± 0.8	90 ± 4	1.4 ± 0.3	2.8 ± 1.5
	MW^{NM}	23 ± 4.8	2.2 ± 0.4	91 ± 1	0.9 ± 0	2 ± 0.4
	PW^{M}	16 ± 4.4	1.6 ± 0.6	90 ± 2	1.4 ± 0.1	2.2 ± 0.8
	PW^{NM}	17 ± 5.1	1.5 ± 0.6	91± 4	1.1 ± 0.3	1.5 ± 0.5
Effects	AMF	ns	ns	ns	***	**
	WR	***	***	ns	***	**
	Table (% of the total effect)	3	0	na	6.5	5.6

The effect of the fixed factors AM fungal colonization (AMF) and water regime (WR) on plant growth parameters was tested using mixed model with REML analysis ($p \leq 0.05$). Percentages of the effect due to the random factor Table are reported. Water contents (WC) were analyzed by running Wilcoxon's test on each factor separately and using Bonferroni's correction for pairwise comparisons, without interaction analysis. For the [P], data in ppm were converted in mg g^{-1} of dry matter.

- Different lower-case letters indicate significant differences between M and NM plants according to the contrast analyses.
- Different Greek letters indicate significant differences between WR according to the contrast
- Different upper-case letters and lower-case letters in italic indicate significant differences between WR inside each M and NM level, respectively.
- * indicate significant differences between M and NM treatments inside each WR level.
- No interaction between factors was found.

Data are represented as mean ± SD of six replicates per treatment. For the P-values, ns = no significant difference, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, na = test not applicable.

Discussion

Drought is a major threat to maize production and many strategies (e.g. irrigation, breeding) have been put in place to limit its impact. In maize, recovery after drought is poorly studied and the role played by AM fungi in this process is almost unknown. Here, we reported on the impact of *R. irregularis* MUCL 41833 on the dynamics of Pi uptake and on the modifications in leaf gas exchanges parameters (CO_2 assimilation rate (A), stomatal conductance (g_s), transpiration (E) and instantaneous water use efficiency (WUE_i) in maize plants that were grown under three contrasting water regimes (i.e. well-, moderately- and poorly-watered, WW, MW and PW, respectively) and subsequently watered at field capacity for 42 h. Plants were grown in a semi-hydroponic cultivation system to follow-up the depletion of Pi in the circulating nutrient solution. During the circulation, Pi uptake was increased in the mycorrhizal plants, whatever the WR. At initiation of circulation (time 0 h), A, g_s, and WUE_i were impacted by the WR but not by AMF, except for E which stayed unchanged whatever the condition. A, g_s, and WUE_i were indeed lower in plants grown in the MW and PW treatment when compared with those in the WW treatment. After 42 h of recovery, A recorded in the plants in the MW and PW treatments exceeded those in the WW treatment, a significant interaction between AMF and WR was found for g_s and E, and WUE_i was improved in M plants when compared with NM plants whatever the WR. At harvest, the plants in the MW^M and PW^M treatments had higher shoot WC but also lower leaf RWC_L when compared to their non-mycorrhizal counterparts.

Total and arbuscular root colonization parameters evaluated at harvest were not impacted by the WR. This result is consistent with previous studies conducted on maize (Bárzana *et al*., 2014; Quiroga *et al*., 2017) and wheat under moderate and severe drought stress (Beltrano and Ronco, 2008) and suggested that the fungus was able to develop in the root system of plants under different water regimes, even the most severe, as in our study. However, a significant decrease in %VC was found in the plants grown in the PW^M treatment when compared to those grown in the WW^M treatment. This corroborated the study of Martínez-García *et al*. (2012) that observed a decrease in vesicles but not in arbuscules in shrubs during seasonal drought. They suggested that under drought conditions, AM fungi would rather allocate energy to the production of arbuscules than to storage structures (i.e. vesicles) to maintain transfer of water and nutrients to the plant. Our results on Pi uptake

during recovery (see below) tend to support this hypothesis with a proportionally higher uptake of Pi in the plants grown in the PW^{M} treatment versus those grown in the PW^{NM} treatment as compared to the plants grown in the WW^{M} treatment versus those grown in the WW^{NM} treatment, even if the data showed no significant differences.

The dynamics of Pi uptake/immobilization by the maize-AM fungus associates was measured indirectly by monitoring the % of Pi concentration remaining in the nutrient solution circulating through the plants. Whatever the presence/absence of the AM fungus, the decrease in %Pi in the circulating solution in the plants of the WW treatment was fast from 0 to 21 h and slowed down onwards 21 until 42 h. This dynamics is similar to the one encountered in studies using the same semi-hydroponic cultivation system with maize (Garcés-Ruiz *et al*. 2017) and medic (Calonne-Salmon *et al*. 2018) plants. A comparable, but less pronounced, decrease in %Pi was noticed in the M and NM plants grown under MW and PW treatments, although no slowdown was noticed after 21 h of circulation. Indeed, the Pi uptake in the plants of the MW and PW treatments was significantly lower as compared to those in the WW treatment whatever the time considered even if this difference was less significant at time 42 h. The lower Pi uptake could be explained by the significant lower root biomass of the plants in the MW and PW treatments as compared to those in the WW treatment, probably combined with a negative effect of drought on the plant and/or AM fungus physiology.

Whatever the WR, the Pi uptake by the plant/AM fungus associates during the 42 h circulation, increased significantly in the M versus NM plants. The absence of differences in root biomass between M and NM plants under similar WR, combined with the high levels of root colonization, suggested that the increased rate of Pi uptake in the M plants could be attributed to an increased acquisition of Pi by the extraradical mycelium extending in the substrate. However, the relative small volume of the container but also the continuous percolation of the nutrient solution through the substrate, might prevent the formation of Pi-depletion zones around roots and hyphae. Therefore, the increased Pi uptake in the M plants could probably result from an increased expression of fungal Pi transporters (Colpaert *et al*., 1999) rather than to an increased foraging by the extraradical hyphae. It is also not excluded that AM fungi could also improve direct Pi uptake by the maize roots. This result was also associated with an improved Pi content in maize roots. However, shoot and root biomass as well as shoot P content and concentration

at harvest were not improved in the M plants as compared to the NM plants under similar WR, as already reported in the study of Subramanian and Charest (1997) and Zhu *et al*. (2012) with maize. As suggested by Garcés-Ruiz *et al*. (2017), it is not excluded that Pi accumulated in the intraradical structures of the AM fungus (i.e. hyphae and vesicles) in the form of polyphosphate granules. Interestingly, similar shoot P contents under all WR were associated with significantly lower shoot dry weight in the plants of the MW and PW treatments as compared with those in the WW treatment. This suggested that water was the principal factor limiting plant growth. Pi accumulation in the intraradical structures of the fungus could thus been explained by the lower demand of Pi by the plant.

Leaf gas exchange parameters measured at the initiation of circulation (time 0 h) drastically differed after 42 h of circulation. A high increase of g_s and E was noticed between time 0 h and 42 h of circulation. As environmental conditions did not differ between both times (i.e. vapor pressure deficit, temperature, photosynthetically active radiation, not presented data), we hypothesized that these differences were related to the 42 h circulation. Interestingly, an increase of g_s and E values in plants of the WW treatment was also noted between both times suggesting that perlite moisture at time 0 h was not optimal. It is indeed not excluded that a decrease in perlite moisture in the WW treatments had happened within two days, impacting gas exchange parameters event if no leaf rolling was observed in the plants in contrast to the MW and PW treatments.

At time 0 h, i.e. before plants received the nutrient solution, A, g_s and WUE_i in maize leaves were decreased in the plants of the MW and PW treatments as compared to those in the WW treatment. Conversely, after 42 h of circulation, g_s and WUE_i were not impacted anymore by the WR indicating that rehydration occurring through the circulation of the nutrient solution was sufficient to eliminate the differences between plants in the WW treatment and drought-stressed plants as already observed (de Carvalho *et al*., 2011; Grzesiak *et al*., 2006; Hayano-Kanashiro *et al*., 2009). In addition, the plants in the MW and PW treatments had significantly higher A values than those in the WW treatment at time 42 h and significantly increased shoot water contents at harvest. Increased CO_2 assimilation has been considered a plant strategy to alleviate drought stress (Gale and Zeroni, 1985). This increased metabolism and water demand compared to the plants in the WW treatment

suggest that plants in the MW and PW treatments tried to compensate the lack of growth and water supply during recovery.

At time 0 h, the AM fungus did not improve leaf gas exchange parameters in plants that were grown under WW, MW or PW conditions. Although various studies showed a beneficial effect of AM fungi on these parameters in well-watered or drought-stressed maize plants (Subramanian *et al*., 1995; Zhu *et al*., 2012; Augé *et al*., 2015), a few others reported no impact of AM fungi on g_s in drought-stressed maize plants (Quiroga *et al*., 2017) and on E parameters in well-watered maize plants (Subramanian and Charest, 1999). As suggested by Quiroga *et al*. (2017), the absence of effect could be due to the variability of response in different maize genotype. After 42 h of circulation, the AM fungus did not improve A but modulated E and g_s in function of the WR. Indeed, E and g_s were significantly lower in the plants of the WW^M treatment when compared to those in the MW^M and PW^M treatments. As photosynthesis was also significantly lower in the plants of the WW treatment when compared with those in the MW and PW treatments, it mechanically resulted in an improved WUE_i in the M plants comparatively to the NM plants.

An increased WC was observed in the plants of the MW^M and PW^M treatments during drought recovery which could be partly explained by the improvement of the WUE_i as observed in the plants at harvest. Other mechanisms such as improved root hydraulic properties during drought (Bárzana *et al*., 2012) or following recovery (Levy and Krikun, 1980) have also been proposed as possible reasons for increased WC in M plants under drought stressed conditions. Curiously, the higher WC in the plants of the MW^M and PW^M treatments was not associated with higher RWC_L measured from leaves. Indeed, RWC_L was significantly decreased by 15% in M plants when compared to NM plants. However, WC and RWC_L cannot strictly be compared as WC measurement is based on whole plant and takes into account water in leaves, stems, grains and flowers (if there are any), whereas RWC_L is measured from a small sample taken from the 4^{th} to 5^{th} leaf. This result seems also contradictory with previous studies which observed a higher RWC_L (Subramanian *et al*., 1997) and leaf moisture percentages (Zhao *et al*., 2015) in M maize plants during recovery. It has been shown that structure and physiology of maize leaves differed between mature and juvenile leaves (Bongard-Pierce *et al*., 1996). This difference in RWC_L could thus potentially be explained by the different maturity of the leaf measured in our study.

Meanwhile, the values of RWC_L measured in the plants of the WW treatment also tended to be lower than those in the plants in the MW and PW treatments. Thus, it seems that lower RWC_L values measured in plants of WW treatment as well as in M plants could reflect higher water binding properties of the plant tissues rather than a difference in the turgidity of the leaves. Plants tissues can indeed display different water binding properties depending on their structural and macromolecular composition. In wheat for example, it has been shown that leaves of drought tolerant cultivars had a higher affinity for strongly bound water when compared with drought sensitive ones (Rascio *et al*., 1999). This hypothesis is reinforced as leave water contents (calculated without the contribution of the turgid weight) shows no significant differences between the treatments (not presented data).

In this study we demonstrated a beneficial effect of AM fungi on the recovery of maize plants after a drought stress. An improved Pi uptake as well as an increased WUE_i and WC in shoots was noticed in the plant/AM fungus associates during the 42 h of recovery. Under a drought environment, where Pi is less available (Gahoonia *et al*., 1994) and precipitations are erratic, a more efficient Pi uptake by the fungus can help plants to mitigate the impact of drought stress and maintain growth (Singh *et al*., 1999) by improving net photosynthesis and water contents for instance (Garg *et al*., 2004). The ability of AM fungi to increase plant WUE_i during recovery from drought is of high agronomical importance as limited transpiration in maize plants is a major driver of yield performance in drought-susceptible environments (Messina *et al*., 2015). Therefore, a future challenge could be to develop drought-adapted inbred lines highly responsive to AM fungi for faster recovery after drought stress.

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Author contributions

OLP contributed to the data collection, analysis, and interpretation, drafting of the manuscript, commentary corrections, and final approval and agreement with all aspects of the work. MG contributed to the data collection, analysis, and interpretation, drafting of the results section and final approval and agreement with all aspects of the work. MC-S and SD contributed to the conception and design of the experiments, interpretation of the data, draft corrections, and final approval and agreement with all aspects of the work. FBD contributed to the data collection, and final approval and agreement with all aspects of the work.

Supplementary Material

The Supplementary Material for this article can be found online at : https://www.frontiersin.org/articles/10.3389/fpls.2019.00897/full#supplementary-material

General Discussion

Drought is a major constraint for crop production (Li *et al*., 2009; Lesk, 2016). For instance, in Angola, Botswana, the USA and Australia, it has been reported to impact cereal production by 4 to 6% within the period 1961-2014 (Mehrabi and Ramankutty, 2017). With the increase in global temperature, estimated at 2 °C by 2050, it is expected that the frequency and severity of drought periods will increase, especially in tropical regions (Li *et al*., 2009). This will require the application at large scale of agricultural practices such as irrigation or the wide recourse to drought-resistant genotype or crops. Unfortunately, these practices are often costly or even inaccessible to less fortunate farmers.

A potential alternative or complementary option to mitigate the effects of drought on crops is the use of beneficial soil microorganisms (Grover *et al*., 2011; Dodd and Ruiz-Lozano, 2012; Lau and Lennon, 2012; Nadeem *et al*., 2014), among which are the AM fungi (Jayne and Quigley, 2014). These soil inhabitants establish symbiotic associations with most agricultural and horticultural crops, supplying them with nutrients (mostly P) in exchange for carbohydrates (Bago *et al*., 2003) and lipids (Keymer *et al*., 2017). They have been reported to mitigate the impact of drought on yield of various crops and plant species (Augé, 2001; Jayne and Quigley, 2014). Therefore the combination of adequate AM fungi and plant species may represent an option complementary to other practices to reduce food insecurity problems under water stress periods (Cardoso and Kuyper 2006; Rodriguez and Sanders, 2014).

The optimization of AM fungal/plant associations can be implemented by the application of fungal inoculants into the field (Douds *et al.*, 2004; Englander *et al.*, 2016) and/or by promoting local AM fungal population densities and communities diversity through adapted agricultural practices (Maiti, 2010). However, to be efficient, agricultural practices must rely on a deep knowledge of the function and biology of AM fungi in the context of drought. Unfortunately, there is a lack of research concerning the impact of drought on AM fungi (Augé *et al*., 2003), in particular on their life cycle (e.g. spore germination, extraradical mycelium development) and functions (e.g. nutrient uptake and transport to plants). This lack of knowledge can be partly

explained by the difficulties to study fungal structures because hyphae are intermingled in soil particles.

The response of AM fungi to drought conditions is often not sufficient to predict a beneficial effect once they are associated with plants in pots or fields. Therefore, their role on plant physiology must be investigated under conditions of drought but also during recovery periods.

Plant recovery from drought can be defined as "*A plant's capability to resume growth and gain yield after exposure to severe drought stress which causes a complete loss of turgor pressure and leaf* (Luo, 2010)". Until now, the majority of studies have mainly focused on the physiological response of plants to drought and very rarely on their response during recovery from drought. Several studies have shown that physiological responses and gene expression patterns in resistant plant genotypes of maize and cowpea during drought recovery differed with those during drought and were more positively correlated with final crop yields (Anyia and Herzog, 2004; Rivas *et al*., 2016; Chen *et al*., 2016). Similarly, the role of AM fungi during this phase of plant growth has been barely evaluated.

In order to better understand the impact of decreasing water availability on AM fungi and their roles in plant resistance to drought several experiments were conducted. In the first two chapters we investigated under strict *in vitro* culture conditions the impact of a decreased water availability on spore germination of four different AM fungal strains and on the extraradical mycelium (ERM) development, sporulation and Pi uptake and transport capacity of the ERM of *R. irregularis* MUCL 41833. Water availability in the growth medium was decreased by using polyethylene glycol (PEG). In the third chapter we inverstigated the Pi nutrition and leaf gas exchange parameters of maize plants during recovery from drought. To that end, mycorrhizal and non-mycorrhizal maize plants were grown in a semi-hydroponic cultivation system, submitted to drought stress for three weeks. The dynamics of Pi uptake of the AM fungal/plant association was monitored in a nutritive solution circulating in a closed loop through the pots. The leaf gas exchanges parameters were assessed from maize leaves before and after circulating the nutrient solution.

Impact of polyethylene glycol on the water availability in MSR medium

Using a novel protocol, PEG was added into the MSR medium in order to decrease the water availability in the gelled medium used for the *in vitro* study of AM fungi. It was first necessary to test the effects of PEG on water potential (ψ) and polymerization of the MSR medium. The ψ was tested under increasing concentrations of PEG (i.e. 50, 100, 250 and 400 g L^{-1} in Chapter 1 and 10, 25 and 50 g L^{-1} in Chapter 2 of Research Results section). In both experiments, the response of ψ was not linear but fitted (R^2>99%) a regression curve following a quadratic equation which confirms earlier works of Michel (1983) and Money (1989). As stated by Money (1989) the non-linearity of the response and the fact that osmolarity cannot be predicted by Van't Hoffs formula indicate that PEG did not behave as an ideal solute. Moreover, the response of ψ to increasing PEG concentrations can also result from the interactions between PEG and other solutes present in the solution (Michel, 1983; Aujla and Paulitz, 2017). Thus, it happens that the measure of the decrease of osmotic/water potential induced by PEG must be reconducted whenever a new growth medium is considered.

In the first experiment, the ψ of MSR medium was significantly decreased at 250 and 400 g L^{-1} of PEG in the MSR medium but not at 50 and 100 g L^{-1} when compared with the Control (i.e. without PEG, hereafter). Conversely, in the second experiment, water potential gradually decreased when PEG was added at concentrations of 10, 25 and 50 g L^{-1} and there was a significant decrease in ψ at 50 g L^{-1} of PEG in the MSR medium as compared to the Control. The ψ measured in the Control and at 50 g L^{-1} of PEG were also slightly higher (ψ = −0.024 and −0.056 MPa, respectively) when compared with the first experiment (ψ = −0.10 and −0.13 MPa for the Control and PEG^{50}, respectively). According to Money (1989) who found a gradual decrease of ψ for a low range of PEG concentrations (20 to 60 g L^{-1} of PEG), it seems that the second experiment has given more accurate results and reflected better the impact of PEG on the water potential of MSR solutions. To explain these differences, it is important to note that the accuracy of the measurements by a vapor pressure osmometer (VPO) for solutions with low osmolarity (below 100 mmol kg^{-1} which corresponds to ψ between 0 and −0.25 MPa) can be limited by the contamination of the chamber and the thermocouple by residues from solutions (Sweeney and Beuchat, 1993). Therefore, it is not excluded that the measurements of the first experiment

were less precise and gave more negative ψ values because they were impacted by contamination in the chamber.

Impact of decreasing water availability on the germination response of AM fungi

Although the ψ values PEG concentrations of 10, 25 and 50 g L^{-1} in MSR medium were slightly decreased when compared with the Control (e.g. ψ= −0.024 for the Control, and ψ= −0.056 MPa at 50 g L^{-1} of PEG) it was sufficient to impact the germination of AM fungal strains, in particular, of *R. irregularis* MUCL 41833, under *in vitro* culture conditions.

The impact of decreased water availability on germination of spores and germ tube length of the AM fungus *R. irregularis* MUCL 41833 was tested at 10, 25 and 50 g L^{-1} of PEG in two independent experiments (see Research results - Chapter 1 and 2) conducted under similar conditions. Both experiments yielded contrasting results. The percentage of germinated spores at the end of the experiment varied from 20% to 65% in experiments 2 and 1, respectively and germ tube length varied from 0.29 cm to 0.590 cm, respectively. Similar differences were observed for the percentages of spore germination and germ tube length in the Control treatment of both experiments. It is not excluded that those differences could be related to the age of the *in vitro* cultures used to produce the spores (a two-months-old culture in experiment 2 versus a five-month-old culture in experiment 1). Indeed, newly formed spores have been reported to germinate more slowly than older ones (Tommerup, 1983). Spore age/maturation may play a crucial role in germination ability, a phenomenon that may be exacerbated under decreasing water availability. Comparing spores of different ages within the same experiment would help to comprehend the role of maturity on germination potential under no stress to stressed conditions.

The impact of decreasing water availability induced by PEG affected differently the germination of four AM fungal strains. Interestingly, the spores were sampled from cultures of the same age, and were thus presumably of the same maturity, although this needs to be demonstrated. If this were the case it would mean that some strains may be more resistant/tolerant to drought stresses than *R. irregularis* MUCL 41833 and potentially have higher abilities to colonize roots. However, these differences neither predict how the extraradical hyphae will develop nor how Pi uptake and transport to the host

will be affected under these conditions. Indeed, despite similar or higher root colonization rates, AM fungal strains might differ in life history strategies i.e. in their investment of energy towards their different structures. Once the plant is colonized, AM fungal strains behaving as "K strategist" will therefore mainly invest energy in extraradical structures and enhance the exchanges of nutrients with plants while the "r strategist" will rather invest in their offsprings (propagules) which could result in lower benefit for the plants (Ijdo *et al*., 2010).

Impact of decreasing water availability on R. irregularis MUCL 41833 and consequences for Pi uptake and transfer to the plant

The effects of decreasing water availability on the development (total hyphal length and the number of new spores produced) and Pi uptake of the ERM was thus investigated with *R. irregularis* MUCL 41833 associated to *Medicago truncatula* in bi-compartmented Petri plates.

As for spore germination, decreasing water availability impacted spore production, hyphal length and delayed the capacity of ERM to take up Pi, even if no effect was visible on the physiology of the fungus (as measured by the succinate dehydrogenase activity). Therefore, it was presumed that the AM fungus could still benefit the plant. This hypothesis was corroborated by the greenhouse assay with maize plants associated or not with *R. irregularis* MUCL 41833 in a semi-hydroponic cultivation system. In this study, AM fungal structures (i.e. the total and vesicules/spores AM colonization) were impacted by periods of drought before recovery. Similarly, the capacity of the AM fungus to take up Pi and transfer it to the plant was decreased as witnessed by the lower Pi contents and concentrations in roots at harvest and the delayed Pi uptake during recovery in AM colonized maize plants. However, this didn't prevent the fungus to benefit the plants as Pi uptake, root P contents and concentrations were still significantly increased in AM colonized plants when compared to non-mycorrhizal plants.

As previously shown under *in vitro* culture conditions it is probable that the growth of the extraradical hyphae in the pots and/or the Pi uptake by the mycelium was impacted under drought conditions reducing its capacity to take up Pi and transfer it to the maize plants. It was also hypothesized that the lower Pi uptake could have resulted from an impact of a lower water availability on the number and efficiency of Pi transporters in the membranes of the

extraradical hyphae in contact with the environment (medium/soil). Indeed, the lower water availability could decrease the diffusion of Pi in the medium or in the case of soil, in the solution, thereby decreasing its availability and reducing the expression of fungal Pi transporters as reported by Maldonado-Mendoza *et al*. (2001). Another study also reported that the translocation of Pi in fungal hyphae could vary positively with the level of water movement along the fungal-plant continuum which was modulated by the transpiration in the associated plant or by the expression of fungal aquaporins (Kikuchi *et al.*, 2016). It is thus hypothesized that decreasing water availability in the growth medium or a dry soil could decrease the water uptake and translocation into the hyphae and could therefore lead to a lower Pi uptake and translocation.

Impact of decreasing water availability on R. irregularis MUCL 41833 and consequences for water relations in plants

The role of AM fungi in maintaining/improving water homeostasis in plant submitted to drought stress is mentioned in countless studies (see reviews by Augé, 2000, 2001). Among the various mechanisms hypothesized some studies have shown that AM fungi could improve the soils water retention (Allen, 2007) and could take up water through extraradical hyphae and transfer it directly to the plant (Egerton-Warburton *et al*., 2007; Ruth *et al*., 2011). Therefore it could be hypothesized that the contribution of the AM fungus to the plant water uptake would drastically decrease when the total hyphal length of the ERM is impacted by drought. However, some studies suggested that AM fungi could adapt their physiology and increase water uptake in response to water shortage. Indeed, it was shown that the water absorption rate of AM fungi was enhanced during drought when compared to well-watered conditions (F. Zhang *et al*., 2018) while the expression of two aquaporins (*GintAQPF1-2*) in extraradical hyphae was found increased under 25% PEG osmotic stress (Li *et al*., 2013). Interestingly, the study on maize showed that mycorrhizal plants that had been moderately or poorly watered before watering at field capacity (during the circulation of the nutritive solution) had higher shoot water contents at harvest than non-mycorrhizal ones. This suggested that despite a possible impact of drought on the hyphal length of the ERM, the water uptake capacity of these hyphae could have been enhanced.

Role of R. irregularis MUCL 41833 in improving plant adaptation to drought

Improving drought adaptation strategies in plants is a main challenge for plant breeders. In particular, plants that are able to resist drought can continue to grow and develop under moderate drought conditions, giving better yields at harvest. To achieve this resistance, they can avoid tissue dehydration by improving their water supply and/or decrease water loss, or increase their tolerance to water shortage by adapting their physiology. Both physiological adaptations often coexist and are implemented to cope with drought stress (Volaire, 2018). In the literature, AM fungi have been found to improve both mechanisms of plant resistance. In our studies, we showed that the AM fungus could enhance drought avoidance strategy (or more precisely dehydration avoidance) in different ways. We found that despite the ERM of *R. irregularis MUCL 41833* was affected by drought it was still metabolically active and could potentially supply water to the plant. Another way the AM fungus can enhance dehydration avoidance in plants is by decreasing water loss, for instance through decreased E and g_s as reported in leaves of mycorrhizal maize plants. However, this decreased water loss was observed during recovery of plants. Therefore plants were optimizing tissue rehydration rather than avoiding tissue dehydration which pleads in favor for a specific physiological adaptation strategy during recovery.

Some studies linked an improved resistance to drought with the improvement of Pi nutrition in AM-colonized plants (Nelsen *et al*., 1982; Farahani *et al*., 2013). As Pi availability in dry soils is often decreased, an increased Pi supply by the AM fungus might help plants to better cope with drought stress. However, it was also previously shown by He and Dijkstra (2014) and confirmed in our experiment with maize, that Pi doesn't seem to be the limiting factor for plant growth under drought conditions. Nevertheless, studies have also reported that Pi spraying on plant leaves improved numerous physiological parameters under drought such as leaf chlorophyll contents, leaf gas exchanges or antioxidants activity (dos Santos *et al*., 2004; Ahmad *et al*., 2017) suggesting that Pi might play an important role in plant physiological adaptation to drought especially in shoots. In this context, the increased translocation of Pi from roots to shoots induced by the fungus under *in vitro* conditions could be interpreted as a strategy to increase resistance of plant tissues to drought.

Conclusion and Perspectives

In this thesis, we aimed at understanding the impact of decreasing water availability on AM fungi and the role of these root symbionts in plant resistance to drought. With this in view, two major objectives were addressed:

Evaluate the impact of decreasing water availability on life cycle and Pi uptake and transport capacity of AM fungi

The impact of a decrease in water availability on life cycle and Pi uptake and transport capacity of AM fungi was investigated under *in vitro* culture conditions. Water potential (reflecting water availability) of the MSR medium was decreased by using PEG and spore germination, germ tube lengths, spores number and hyphal length of the extraradical mycelium as well as its Pi uptake and transport capacity were evaluated.

A decrease in water availability affected the germination of different AM fungal strains as well as the overall AM fungal life cycle (spore germination and germ tube length, extraradical mycelium development and spores production) of *R. irregularis* MUCL 41833 under strict *in vitro* culture conditions. This result could lead to a reduced ability of AM fungi to colonize a suitable host and probably to a lower capacity to take up and transport minerals (e.g. phosphorus) and water from soil to plant. As the spore germination response to decreasing water availability differed between AM fungal strains it is also suggested that differences in resistance/tolerance to drought exist among strains. The capacity of the AM fungus *R. irregularis* MUCL 41833 to take up Pi under decreasing water availability was monitored under *in vitro* conditions. Decreasing water availability delayed the Pi uptake by the extraradical hyphae without impacting the hyphal metabollic activity and total Pi uptake at the end of the experiment. Proposed mechanisms include the impact of decreasing water availability on Pi concentration in the medium leading to a decreased expression of fungal Pi transporters (Maldonado-Mendoza *et al*., 2001) or an impact on water flow along the fungus to the plant, affecting the Pi translocation within the fungal hyphae (Kikuchi *et al*., 2016).

Perspectives:

- Differences in germination response to decreased water availability among four AM fungal strains were found suggesting differences in resistance/tolerance to drought. As this observation concerned only a limited number of strains, large-scale screening experiments comparing strains belonging to the same species isolated either from humid (temperate) or dry (deserts) environments or different species with contrasting life history strategies (r or K), should be considered to investigate the inherited or acquired resistance/tolerance of AM fungi to drought stress. However, differences in germination response to decreased water availability are not sufficient to confirm an improvement of plant resistance to drought. Further studies should thus be conducted to test if drought tolerant AM fungal strains have also a higher ability to develop extraradical networks and take up and transport Pi to the plant when placed under conditions of decreased water availability. Subsequent studies of the benefit of these strains on plant grown under water stress conditions could open the door to the selection of promising strains to be used in regions expected to be threatened with increasing frequencies and severities of drought periods.
- The experience conducted *in vitro* showed for the first time that decreased water availability impacted the Pi uptake by extraradical hyphae without impacting the number of metabolically active hyphae. To understand the mechanisms behind this observation, it could be necessary to reconduct the same experiment by exploring if the decreased Pi uptake is associated with a lower expression of fungal Pi transporters as well as water transporters (aquaporins) as these might be also involved in Pi uptake from the medium.
- Further studies using this *in vitro* system could also be conducted to better understand the role of the AM fungus in water uptake and transport to the plant. In particular, the time of exposition of the mycelium to a PEG-induced decrease in water availability should be extended over 24h to determine if the tendency of an increased water content in *Medicago truncatula* plants can be confirmed. As fungal aquaporins genes were found upregulated under 25% PEG-stress (Li *et al.*, 2013), their expression should be investigated at the same time to confirm a possible role in an increased plant water supply.

- The *in vitro* system would be also adequate to trace the water uptake by the hyphae and its subsequent transport to the plant in the root compartment using deuterium in the medium. This experiment could be coupled with an analysis of the expression of known fungal aquaporins from the extraradical mycelium to assess their possible role in water uptake.
- Finally, the *in vitro* system could be used to follow how mycorrhizal plants respond when decreased water availability is applied only to the extraradical hyphae. Indeed, application of decreased water availability in the hyphal compartment led to a higher shoot P concentration in *M. truncatula* plants suggesting a translocation of Pi from root to shoots. Analysis of gene expression of root and shoots phosphate and water transporter could easily be implemented to confirm this effect. Other genes more generally involved in the defense of plants against abiotic stress could also be investigated to highlight possible long distance signals from the fungus to the plant.

Evaluate the effects of AM fungi on plant recovery from drought via their impact on Pi mobilization and leaf gas exchange parameters

Dynamics of Pi uptake during recovery was followed by monitoring Pi depletion in a nutrient solution circulating on the roots of maize plants during 42 h following a period of three weeks of drought stress. During recovery, maize plants associated with *R. irregularis* MUCL 41833 took up Pi faster from the nutrient solution than non-mycorrhizal ones although both plants had similar root biomass. As the continuous percolation of the nutrient solution through the substrate might prevent the formation of Pi-depletion zones around roots and hyphae, the role of the foraging by extraradical hyphae could be minimized. It is suggested that the improved Pi uptake could also be due to an increased expression of phosphate transporters in mycorrhizal roots (Colpaert *et al.*, 1999). On a long term, this improved Pi uptake could possibly enhance plant growth, survival and reproduction after a period of drought.
After 42 hours of recovery, the AM fungus significantly affected leaf gas exchange parameters in the maize plants. Mycorrhizal plants had stable CO_2 assimilation rates but decreased transpiration and stomatal conductance (g_s) associated with increased instantaneous water use efficiencies (WUE_i) when compared to their non-mycorrhizal counterparts. The AM fungus also increased shoot water contents in maize plants that had been moderately and

poorly watered before recovery. This clearly suggested that the AM fungus *R. irregularis* MUCL 41833 was able to induce modifications in the metabolism of maize plants during recovery enabling them to save more water and rehydrate faster.

Perspectives:

- The mechanisms by which the fungus improved the Pi uptake in plants whose roots had unrestricted access to Pi in the substrate was not elucidated. As mentioned above, the foraging of the hyphae seems not to have a great influence on Pi uptake in this experiment as Pi depletion zones are unlikely to build around the roots. It is not excluded that the increased Pi uptake in mycorrhizal plants could also be due to an enhancement of direct Pi uptake capacity in mycorrhizal roots. Further studies should thus evaluate if these results are associated with an improvement of root architecture in mycorrhizal maize plants and with an overexpression of the plant phosphate transporters.
- However, it is not excluded that the presence of hyphae in the substrate still played a role by increasing the surface for Pi absorption. Interestingly, the ability of AM-colonized plants to take up Pi was impacted in AM colonized plants that were submitted to moderate to severe drought before recovery. This could be explained by an impact of water regime on the extraradical mycelium. This hypothesis could be confirmed by estimating the fungal biomass or hyphal length in the substrate at harvest.
- To determine the respective contributions of AM fungi and roots to Pi uptake in maize plants, *in vivo* systems using split-pot compartments and quantification of two different isotopes of P (^{32}P and ^{33}P) taken up by the mycorrhizal roots for one and the roots alone for the other can be used (Smith *et al*., 2004, 2011). However, it must be kept in mind that the Pi uptake can be affected by the experimental conditions of our study which would be difficult to recreate in split-pot systems.
- After 42 hours of recovery mycorrhizal plants that had been submitted to moderate to severe drought seemed to avoid water loss more than non-mycorrhizal plants as their water contents were enhanced and their stomatal conductance and transpiration were decreased. As this effect was not observed when plant were submitted to drought it seemed to be a specific physiological response to recovery. However, it is probable that this adaptative mechanisms also developed before

the recovery period, whenever drought-stressed plants were watered. To test this, leaf gas exchange parameters should be measured during the application of the water regimes right after watering the plants.

- As plant hydrate and resume their growth the physiological response of the plant as well the effect of the fungus on gas leaf exchange parameters might change. Repeated measurements of plant physiological parameters at different timings from time 0 of recovery could enable us to test whether the effect of the AM fungus on plant physiology remains or disappears with a longer recovery period.
- Finally, the recovery of maize varieties displaying varying resistance to drought and inoculated with the same AM fungal strain or other strains could be investigated to better understand the genetic and physiologic determinants of drought resistance and higher recovery potential. This could help select efficient combinations between microorganism and maize varieties to enhance crop yields in drought prone environments.

In conclusion, in this thesis we were able to assess the effects of decreasing water availability on the AM fungal life cycle and Pi uptake and transport, and highlighted the specific role played by AM fungi on plant physiology during recovery from drought stress. These information are crucial if we want to better understand the role and function of AM fungi and enhance the success of plant-fungi associations in dry environments but also in temperate areas subjected to variable periods of drought. The novel results brought in this thesis plead for higher investigation efforts in the comparison of different AM fungal strains and for a better consideration of the role of AM fungi in the recovery of plants.

Overview of the Scientific Achievements

1. Scientific papers published in peer-reviewed journals

Olivia Le Pioufle, Stéphane Declerck (2018) Reducing water availability impacts the development of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* MUCL 41833 and its ability to take up and transport phosphorus under *in vitro* conditions. Frontiers in Microbiology. 9:1254. doi: 10.3389/fmicb.2018.01254

Olivia Le Pioufle, Matike Ganoudi, Maryline Calonne-Salmon, Fatma Ben Dhaou, Stéphane Declerck (2019) *Rhizophagus irregularis* MUCL 41833 improves phosphorus uptake and water use efficiency in maize plants during recovery from drought stress. Frontiers in Plant science. 10: 897. doi: 10.3389/fpls.2019.00897

2. Research article in preparation

Olivia Le Pioufle, Stéphane Declerck. Polyethylene glycol decreases the water potential of the modified Strullu-Romand medium and impacts the growth of arbuscular mycorrhizal fungi

3. Conference Participation

Olivia Le Pioufle, Stéphane Declerck (2014) Poster. Impact of polyethylene glycol-induced drought stress on *in vitro* hyphal network development and germination of *Rhizophagus irregularis*. *International Congress on Mycorrhizae.* Mycorrhizal Symbiosis a key factor for improving plant productivity and ecosystems restoration, Marrakesh, Morocco, 15-17 October 2014.

Olivia Le Pioufle, Maryline Calonne-Salmon, Stéphane Declerck (2019) Poster. Germination of four arbuscular mycorrhizal fungal strains shows different responses to decreased water potential under *in vitro* conditions. *1st International symposium on Biodiversity Research*, Canakkale, Turkey, 2-4 May 2019.

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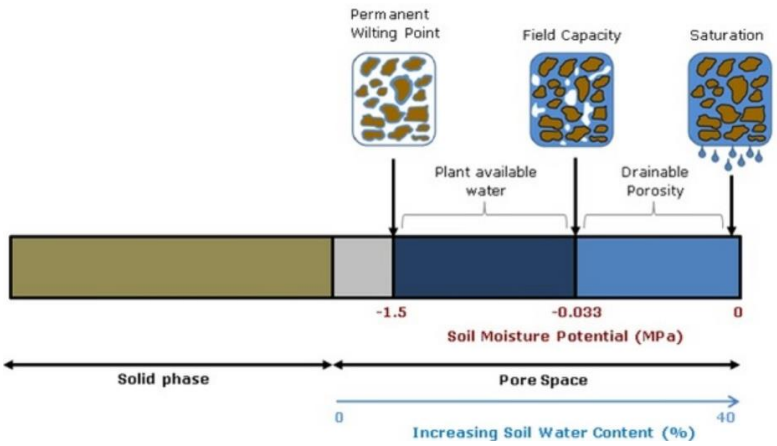
Appendix

Appendix 1 Water content and water potential at saturation, field capacity and permanent wilting point.



The difference in water content between field capacity and permanent wilting point is plant available water. Drainable porosity is the amount of water that drains from macropores by gravity between saturation to field capacity typically representing three days of drainage in the field. Figure adapted from Brady & Weil 2002 and McCauley *et al.* 2005. https://www.nature.com/scitable/knowledge/library/soil-water-dynamics-103089121/

Appendix 2 Composition of the Modified Strullu-Romand (MSR) medium

Elements	Concentration (μM)
$N(NO_3^-)$	3800
$N(NH_4^+)$	180
P	30
K	1650
Ca	1520
Mg	3000
S	3013
Cl	870
Na	20
Fe	20
Mn	11
Zn	1
B	30
I	-
Mo	0.22
Cu	0.96
*Vitamins (facultative *) :*	
Ca Panthotenate	1.88
Biotin	0.004
Pyridoxine	4.38
Thiamine	2.96
Cyanocobalamine	0.29
Nicotinic acid	8.10
pH 5.5	

*In root organ cultivation (ROC) vitamins must be added to the medium as well as sucrose at a concentration of 10 g L^{-1}**.**

Appendix 3 Preparation of a 90% Pi-impoverished Hoagland (Hoagland ^{lowPi}) solution

Hoagland ^{lowPi} solution is prepared right before use by adding 10 ml of each of the stock solutions (approx. concentrated 100 times, see table below) in 1 L of demineralized water. Finally, the pH is adjusted to 5.6.

Table … Composition of the stock solutions (**x100)** for preparation of Hoagland ^{lowPi}

Stock solutions	**Elements**	Cs (g L^{-1})	Cs (mol L^{-1})	Cf (g L^{-1})
Sol A	NH_4NO_3	8.00	0.1	0.0777
pH 5.3	$Ca(NO_3)_2 \cdot 4H_2O$	82.60	0.35	0.8019
	KNO_3	35.70	0.353	0.3466
	KCl.	4.51	0.061	0.0438
	K_2SO_4	10.54	0.061	0.1023
Sol B	KNO_3	5.00	0.049	0.0485
pH 4.4	KH_2PO_4	2.74	0.02	0.0266
	$MgSO_4$	12.04	0.1	0.1169
	$MnSO_4\ H_2O$	0.05	0.00031	0.0005
	H_3BO_3	0.14	0.0023	0.0014
	$CuSO_4 \cdot 5H_2O$	0.02	0.00006	0.0001
	$(NH_4)_6Mo7O_2 \cdot 4H_2O$	0.01	0.000007	0.0001
	$ZnSO_4 \cdot 7H_2O$	0.06	0.00021	0.0006
Sol C	Fe-EDTA	1.90	0.005200	0.0184

Cs= concentration of stock solution
Cf= final concentration

Appendix 4 Raw data of the greenhouse experiment (Chapter 3)

Maize root biomass and phosphorus parameters at harvest

Myco	Water regime	Table	Plant number	Root Water content (%)	Root fresh weight (g)	Root dry weight (g)	P mg kg^{-1}	P content (mg)
NM	WW	a	1	87.6	37.92	4.7	920.88	4.33
NM	MW	a	2	86.52	24.95	3.36	876.32	2.95
NM	PW	a	3	86.47	17.2	2.33	698.51	1.63
M	WW	a	4	87.24	28.46	3.63	1156.5	4.2
M	MW	a	5	85.6	24.68	3.55	892.73	3.17
M	PW	a	6	85.33	14.51	2.13	1356.14	2.89
M	WW	a	7	83.97	25.1	4.02	1202.87	4.84
NM	WW	b	10	85.32	26.13	3.83	1133.05	4.35
NM	MW	b	11	86.54	23.5	3.16	902.09	2.85
NM	PW	b	12	85.62	21.3	3.06	972.35	2.98
M	WW	b	13	80.74	27.19	5.24	1205.83	6.31
M	MW	b	14	86.39	32.59	4.44	1506.84	6.68
M	PW	b	15	84.79	23.97	3.65	1292.17	4.71
NM	MW	b	16	83.93	21.82	3.51	867.39	3.04
NM	WW	c	19	84.81	31.98	4.86	866.63	4.21
NM	MW	c	20	86.36	21.83	2.98	990.91	2.95
NM	PW	c	21	88.28	23.9	2.8	1176.78	3.3
M	WW	c	22	83.81	44.76	7.25	975.86	7.07
M	MW	c	23	85.71	20.4	2.91	1609.05	4.69
M	PW	c	24	81.19	10.59	1.99	1456.07	2.9
M	PW	c	25	83.95	16.48	2.64	1267.79	3.35
NM	WW	d	28	85.29	29.51	4.34	798.65	3.47
NM	MW	d	29	86	30.78	4.31	942.86	4.06
NM	PW	d	30	85.95	15.73	2.21	1092.98	2.42
M	WW	d	31	77.74	21.29	4.74	1271.58	6.03
M	MW	d	32	85.61	22.74	3.27	1186.21	3.88
M	PW	d	33	83.68	15.06	2.46	1554.28	3.82
NM	PW	d	34	84.36	14.65	2.29	1077.71	2.47
NM	WW	e	37	83.64	24.23	3.97	922.38	3.66
NM	MW	e	38	84.73	16.21	2.48	921.74	2.28
NM	PW	e	39	87.59	9.63	1.2	1589.97	1.9
M	WW	e	40	85.48	35.47	5.15	1080.88	5.57
M	MW	e	41	84.86	18.75	2.84	1462	4.15
M	PW	e	42	84.46	16.91	2.63	1485.32	3.9
NM	WW	e	43	83.24	30.5	5.11	882.22	4.51
M	MW	e	44	83.86	13.89	2.24	1457.2	3.27

Maize shoot biomass and phosphorus parameters at harvest

Myco	Water regime	Table	Plant number	Shoot fresh weight	Shoot dry weight	Water content (%)	P mg kg^{-1}	P content (mg)
NM	WW	a	1	62.19	6.05	90.27	2422.55	31.92
NM	MW	a	2	45.44	5.75	87.35	1826.6	11.82
NM	PW	a	3	32.65	4.17	87.21	1903.29	8.81
M	WW	a	4	62.08	8.27	86.67	890.98	9.69
M	MW	a	5	52.44	5.87	88.81	1665.18	11.96
M	PW	a	6	41.5	3.97	90.43	2376.6	11.59
M	WW	a	7	52.18	7.97	84.73	766.6	6.95
NM	WW	b	10	46.13	6.95	84.93	905.97	9.01
NM	MW	b	11	35.89	5.18	85.57	1187.77	7
NM	PW	b	12	36.62	5.34	85.43	1617.39	10.46
M	WW	b	13	65.2	10.58	83.78	936.16	11.02
M	MW	b	14	58.79	5.78	90.17	1583.83	13.65
M	PW	b	15	48.27	5.57	88.47	2116	13.46
NM	MW	b	16	36.72	5.05	86.26	1090.41	7.36
NM	WW	c	19	59.25	8.47	85.71	1103.5	10.89
NM	MW	c	20	39.41	6.35	83.88	1307.98	9.2
NM	PW	c	21	37.67	5.08	86.5	1420.87	8.75
M	WW	c	22	55.61	5.56	90	698.23	8.84
M	MW	c	23	48.53	3.74	92.29	1286.02	8.82
M	PW	c	24	37.15	4	89.24	2212.03	10.8
M	PW	c	25	45.33	5.23	88.46	2755.03	16.11
NM	WW	d	28	72.38	10.48	85.53	1044.67	12.18
NM	MW	d	29	60.78	7.48	87.69	1556.22	15.9
NM	PW	d	30	37.66	4.52	88.01	2182.77	12.02
M	WW	d	31	57.5	12.17	78.83	589.32	8.05
M	MW	d	32	47.23	4.78	89.88	1921.88	11.74
M	PW	d	33	45.02	5.11	88.64	1987.18	11.37
NM	PW	d	34	30.72	4.47	85.44	1845.8	11.01
NM	WW	e	37	52.66	8.81	83.27	706.01	8.14
NM	MW	e	38	47.67	5.91	87.61	1290.11	10.53
NM	PW	e	39	30.64	4.37	85.74	1985.49	10.25
M	WW	e	40	64.32	8.67	86.52	1253.55	13.69
M	MW	e	41	50.82	5.87	88.46	1715.26	12.57
M	PW	e	42	50.03	5.54	88.92	1294.47	8.56
NM	WW	e	43	54.7	3.47	93.66	827.39	10.18
M	MW	e	44	49.66	6.96	85.99	1797.85	13.39

Maize leaf water contents at harvest

Water regime	Myco	Table	Plant number	Leaf fresh weight	Leaf turgid weight	Leaf dry weight	Leaf relative water content	Leaf water content
WW	NM	a	1	0.32	0.32	0.06	1	0.81
MW	NM	a	2	0.25	0.24	0.03	1.05	0.88
PW	NM	a	3	0.13	0.15	0.02	0.85	0.85
WW	M	a	4	0.22	0.26	0.05	0.81	0.77
MW	M	a	5	0.09	0.12	0.02	0.7	0.78
PW	M	a	6	0.18	0.2	0.02	0.89	0.89
WW	M	a	7	0.12	0.21	0.04	0.47	0.67
WW	NM	b	10	0.15	0.14	0.03	1.09	0.8
MW	NM	b	11	0.16	0.21	0.03	0.72	0.81
PW	NM	b	12	0.17	0.2	0.03	0.82	0.82
WW	M	b	13	0.17	0.17	0.04	1	0.76
MW	M	b	14	0.27	0.31	0.05	0.85	0.81
PW	M	b	15	0.26	0.23	0.04	1.16	0.85
MW	NM	b	16	0.15	0.12	0.02	1.3	0.87
WW	NM	c	19	0.09	0.12	0.02	0.7	0.78
MW	NM	c	20	0.2	0.21	0.04	0.94	0.8
PW	NM	c	21	0.15	0.1	0.01	1.56	0.93
WW	M	c	22	0.23	0.29	0.06	0.74	0.74
MW	M	c	23	0.25	0.3	0.05	0.8	0.8
PW	M	c	24	0.18	0.28	0.03	0.6	0.83
PW	M	c	25	0.09	0.14	0.01	0.62	0.89
WW	NM	d	28	0.24	0.25	0.05	0.95	0.79
MW	NM	d	29	0.18	0.22	0.04	0.78	0.78
PW	NM	d	30	0.15	0.19	0.03	0.75	0.8
WW	M	d	31	0.18	0.33	0.06	0.44	0.67
MW	M	d	32	0.2	0.35	0.04	0.52	0.8
PW	M	d	33	0.17	0.22	0.03	0.74	0.82
PW	NM	d	34	0.09	0.11	0.01	0.8	0.89
WW	NM	e	37	0.08	0.16	0.02	0.43	0.75
MW	NM	e	38	0.19	0.24	0.04	0.75	0.79
PW	NM	e	39	0.12	0.12	0.02	1	0.83
WW	M	e	40	0.22	0.27	0.04	0.78	0.82
MW	M	e	41	0.18	0.2	0.02	0.89	0.89
PW	M	e	42	0.2	0.26	0.03	0.74	0.85
WW	NM	e	43	0.15	0.18	0.03	0.8	0.8
MW	M	e	44	0.2	0.23	0.04	0.84	0.8

Pi depletion in the Hoagland^{lowPi} solution

Myco	Water regime	Time	Plant number	Table	P (mg L^{-1})	%Pi
NM	WW	0	1	a	6.28	100
NM	MW	0	2	a	6.23	100
NM	PW	0	3	a	6.12	100
M	WW	0	4	a	6.22	100
M	MW	0	5	a	6.21	100
M	PW	0	6	a	6.33	100
M	WW	0	7	a	6.38	100
nv	WW	0	8	a	6.34	100
nv	PW	0	9	a	6.33	100
NM	WW	0	10	b	6.42	100
NM	MW	0	11	b	6.37	100
NM	PW	0	12	b	6.41	100
M	WW	0	13	b	6.41	100
M	MW	0	14	b	6.49	100
M	PW	0	15	b	6.47	100
NM	MW	0	16	b	6.46	100
nv	WW	0	17	b	6.51	100
nv	MW	0	18	b	6.53	100
NM	WW	0	19	c	6.48	100
NM	MW	0	20	c	6.57	100
NM	PW	0	21	c	6.05	100
M	WW	0	22	c	6.15	100
M	MW	0	23	c	6.14	100
M	PW	0	24	c	6.13	100
M	PW	0	25	c	6.11	100
nv	MW	0	26	c	6.13	100
nv	PW	0	27	c	6.19	100
NM	WW	0	28	d	6.19	100
NM	MW	0	29	d	6.24	100
NM	PW	0	30	d	6.29	100
M	WW	0	31	d	6.2	100
M	MW	0	32	d	6.36	100
M	PW	0	33	d	6.28	100
NM	PW	0	34	d	6.25	100
nv	WW	0	35	d	6.32	100
nv	MW	0	36	d	6.26	100
NM	WW	0	37	e	5.95	100
NM	MW	0	38	e	6.32	100
NM	PW	0	39	e	6.31	100
M	WW	0	40	e	6.27	100
M	MW	0	41	e	6.31	100

Myco	Water regime	Time	Plant number	Table	P (mg L^{-1})	%Pi
M	PW	0	42	e	6.29	100
NM	WW	0	43	e	6.33	100
M	MW	0	44	e	6.4	100
nv	PW	0	45	e	6.32	100
NM	WW	9	1	a	4.3	74
NM	MW	9	2	a	5.65	90.73
NM	PW	9	3	a	5.53	90.97
M	WW	9	4	a	4.29	74.56
M	MW	9	5	a	4.89	78.77
M	PW	9	6	a	5.29	84.21
M	WW	9	7	a	4.06	68.95
nv	WW	9	8	a	5.94	99.04
nv	PW	9	9	a	6.12	97.34
NM	WW	9	10	b	3.86	65.49
NM	MW	9	11	b	5.89	92.54
NM	PW	9	12	b	5.52	86.75
M	WW	9	13	b	3.44	59.06
M	MW	9	14	b	5.01	77.22
M	PW	9	15	b	5.24	81.64
NM	MW	9	16	b	5.89	91.35
nv	WW	9	17	b	6.05	98.21
nv	MW	9	18	b	6.26	96.01
NM	WW	9	19	c	4.25	70.77
NM	MW	9	20	c	5.93	90.41
NM	PW	9	21	c	6.06	100
M	WW	9	22	c	4.05	71.53
M	MW	9	23	c	5.67	92.42
M	PW	9	24	c	5.41	88.96
M	PW	9	25	c	5.78	95.22
nv	MW	9	26	c	6.32	100
nv	PW	9	27	c	6.1	99.23
NM	WW	9	28	d	3.87	68.09
NM	MW	9	29	d	5.86	93.96
NM	PW	9	30	d	6.18	98.88
M	WW	9	31	d	3.66	64.68
M	MW	9	32	d	5.27	82.93
M	PW	9	33	d	5.3	85.08
NM	PW	9	34	d	5.94	95.56
nv	WW	9	35	d	6.16	100
nv	MW	9	36	d	6.32	100
NM	WW	9	37	e	4.29	78
NM	MW	9	38	e	5.36	84.92

Myco	Water regime	Time	Plant number	Table	P (mg L^{-1})	%Pi
NM	PW	9	39	e	5.52	88.04
M	WW	9	40	e	4.08	70.47
M	MW	9	41	e	4.87	77.33
M	PW	9	42	e	5.33	85.26
NM	WW	9	43	e	4.23	72.27
M	MW	9	44	e	5.43	84.91
nv	PW	9	45	e	6.5	100
NM	WW	21	1	a	2.12	47.04
NM	MW	21	2	a	4.06	74.73
NM	PW	21	3	a	2.13	40.49
M	WW	21	4	a	1.89	43.83
M	MW	21	5	a	2.13	43.95
M	PW	21	6	a	3.94	67.74
M	WW	21	7	a	1.73	40.22
nv	WW	21	8	a	5.55	100
nv	PW	21	9	a	5.78	96.86
NM	WW	21	10	b	1.82	41.38
NM	MW	21	11	b	4.46	79.49
NM	PW	21	12	b	3.64	62.21
M	WW	21	13	b	0.49	20.66
M	MW	21	14	b	1.75	36.14
M	PW	21	15	b	2.78	48.43
NM	MW	21	16	b	4.41	77.61
nv	WW	21	17	b	5.63	99.41
nv	MW	21	18	b	5.46	92.83
NM	WW	21	19	c	1.97	43.25
NM	MW	21	20	c	4.55	78.45
NM	PW	21	21	c	4.07	73.14
M	WW	21	22	c	1.31	34.87
M	MW	21	23	c	3.74	70.7
M	PW	21	24	c	3.33	60.07
M	PW	21	25	c	3.68	66.08
nv	MW	21	26	c	5.88	100
nv	PW	21	27	c	5.95	100
NM	WW	21	28	d	1.18	32.57
NM	MW	21	29	d	4.09	75.09
NM	PW	21	30	d	4.68	80.02
M	MW	21	32	d	3.09	57.94
M	PW	21	33	d	3.51	61.56
NM	PW	21	34	d	4.31	74.52
nv	WW	21	35	d	5.48	99.82
nv	MW	21	36	d	5.78	100

Myco	Water regime	Time	Plant number	Table	P (mg L^{-1})	%Pi
NM	WW	21	37	e	2.21	51.18
NM	MW	21	38	e	4.01	72.98
NM	PW	21	39	e	4.28	73.32
M	WW	21	40	e	1.13	31.32
M	MW	21	41	e	4.25	76.97
M	PW	21	42	e	2.9	51.68
NM	WW	21	43	e	1.94	43.89
M	MW	21	44	e	3.06	57.13
nv	PW	21	45	e	6.05	100
NM	WW	42	1	a	0.06	21.71
NM	MW	42	2	a	0.9	35.49
NM	PW	42	3	a	1.12	36.35
M	WW	42	4	a	0.14	26.94
M	MW	42	5	a	0.09	22.6
M	PW	42	6	a	1.25	40.47
M	WW	42	7	a	0.06	25.92
nv	WW	42	8	a	4.86	100
nv	PW	42	9	a	5.14	100
NM	WW	42	10	b	0.1	26.02
NM	MW	42	11	b	0.48	28.06
NM	PW	42	12	b	0.85	33.66
M	WW	42	13	b	0.02	24.46
M	MW	42	14	b	0.04	20.75
M	PW	42	15	b	0.25	24.12
NM	MW	42	16	b	2.56	59.88
nv	WW	42	17	b	4.91	100
nv	MW	42	18	b	4.88	94.93
NM	WW	42	19	c	0.16	26.3
NM	MW	42	20	c	1.23	38.68
NM	PW	42	21	c	1.05	38.97
M	WW	42	22	c	0.02	24.27
M	MW	42	23	c	1	37.65
M	PW	42	25	c	0.69	32.81
nv	MW	42	26	c	5	100
nv	PW	42	27	c	5.19	100
NM	WW	42	28	d	0.1	26.92
NM	MW	42	29	d	0.65	31.36
NM	PW	42	30	d	1.75	48.68
M	MW	42	31	d	0.75	32.44
M	PW	42	32	d	0.49	28.64
NM	PW	42	33	d	1.29	41.66

Myco	Water regime	Time	Plant number	Table	P (mg L^{-1})	%Pi
nv	WW	42	34	d	4.75	100
nv	MW	42	35	d	5.1	100
NM	WW	42	36	d	0.12	26.37
NM	MW	42	37	e	0.98	36.31
NM	PW	42	38	e	1.38	42.62
M	WW	42	39	e	0.03	26.64
M	MW	42	40	e	1.29	41.26
M	PW	42	41	e	0.27	25.14
NM	WW	42	42	e	0.08	25.95
M	MW	42	43	e	0.44	27.27
nv	PW	42	44	e	5.21	100

AM fungal colonization at harvest

Water regime /condition	Plant number	table	T%	A%	V%	H%	arcsinT	arcsinA	arcsinV
MW	5	a	64	44	30	17	0.69	0.46	0.3
MW	23	c	56	44	40	5	0.59	0.46	0.41
MW	41	e	82	58	25	20	0.96	0.62	0.25
MW	44	e	70	58	47	3	0.78	0.62	0.49
MW	32	d	47	40	21	5	0.49	0.41	0.21
MW	14	b	97	59	60	5	1.33	0.63	0.64
PW	6	a	31	30	24	2	0.32	0.3	0.24
PW	25	c	80	57	46	16	0.93	0.61	0.48
PW	24	c	51	49	16	12	0.54	0.51	0.16
PW	33	d	66	63	16	2	0.72	0.68	0.16
PW	42	e	79	65	22	12	0.91	0.71	0.22
PW	15	b	73	61	25	8	0.82	0.66	0.25
WW	40	e	49	48	31	5	0.51	0.5	0.32
WW	4	a	77	62	56	9	0.88	0.67	0.59
WW	13	b	93	89	64	1	1.19	1.1	0.69
WW	7	a	76	72	44	5	0.86	0.8	0.46
WW	31	d	85	69	41	7	1.02	0.76	0.42
WW	22	c	82	72	45	5	0.96	0.8	0.47
PM	1	f	97	69	35.5	23.5	1.33	0.76	0.36
PM	2	f	94.5	60.5	48	18.5	1.24	0.65	0.5
PM	3	f	98	73	42	16.5	1.37	0.82	0.43

PM: premycorrhized plants at the time of transfer to the perlite

T, A,V, H%: total, arbuscular, vesicular, hyphal colonization percentage

Plant size and number of leaves at harvest

Myco	Water regime	Table	Plant number	Plant size (cm)	Turgid leave number	Dry leaves number	Flower number	Ear number
NM	WW	a	1	105.8	8	5	1	0
NM	MW	a	2	89.9	6	5	0	0
NM	PW	a	3	74.4	6	2	0	0
M	WW	a	4	86.9	8	4	1	1
M	MW	a	5	65.7	8	4	1	0
M	PW	a	6	79.9	6	2	0	0
M	WW	a	7	81.4	7	3	1	0
NM	WW	b	10	106.1	5	4	1	0
NM	MW	b	11	80.9	6	3	0	0
NM	PW	b	12	93.9	6	3	1	0
M	WW	b	13	122.1	7	4	1	1
M	MW	b	14	87.9	6	2	0	0
M	PW	b	15	94.9	7	3	0	0
NM	MW	b	16	89.2	6	3	1	0
NM	WW	c	19	96	5	3	1	1
NM	MW	c	20	73.4	6	3	1	0
NM	PW	c	21	78.9	4	4	1	0
M	WW	c	22	97	6	2	1	0
M	MW	c	23	70.9	6	4	0	0
M	PW	c	24	64.1	6	4	0	0
M	PW	c	25	74.4	7	4	0	0
NM	WW	d	28	116	6	4	1	1
NM	MW	d	29	100	6	4	0	0
NM	PW	d	30	87	6	3	0	0
M	WW	d	31	105	6	3	1	0
M	MW	d	32	82	6	3	0	0
M	PW	d	33	87	6	2	0	0
NM	PW	d	34	88	5	3	1	0
NM	WW	e	37	116	6	3	1	0
NM	MW	e	38	96	7	3	1	0
NM	PW	e	39	89	4	5	1	0
M	WW	e	40	91	8	3	1	0
M	MW	e	41	89	6	3	0	0
M	PW	e	42	94	6	3	0	0
NM	WW	e	43	118	7	3	1	0
M	MW	e	44	98	7	3	1	0

Leaf gas exchange parameters

Myco	Table	Plant number	tm	eref	^e	cref	^c	Qleaf	Tch	Tl	u	p	ci	E	gs	A
NM	a	1	09:27:14	11.7	1.2	441	4	174	30.9	31.2	202.3	1018	203	0.37	0.01	1.58
NM	a	1	09:30:00	11.9	0.3	444	4	178	31	31.3	202.5	1018	-35	0.16	0	1.4
NM	a	1	09:30:10	11.9	0.3	444	3	180	31	31.3	201.8	1018	-206	0.1	0	1.24
NM	a	2	09:24:05	11.7	0.6	446	1	167	30.9	31.1	202.1	1018	242	0.18	0	0.65
NM	a	2	09:24:15	11.7	0.5	446	1	168	30.9	31.1	201.6	1018	296	0.2	0	0.51
NM	a	2	09:24:24	11.8	0.5	447	2	170	30.9	31.2	202.6	1018	155	0.15	0	0.8
NM	a	3	09:04:30	12	0.7	445	2	135	30.5	30.7	202.5	1017	286	0.27	0	0.76
NM	a	3	09:04:37	12.1	0.5	445	2	133	30.5	30.7	201.6	1018	256	0.25	0	0.85
NM	a	3	09:04:48	12	0.7	446	2	133	30.5	30.7	202.5	1018	217	0.21	0	0.91
M	a	4	09:19:52	11.9	0.7	447	4	159	30.8	31.1	202.2	1017	112	0.22	0	1.4
M	a	4	09:20:12	11.8	0.6	448	4	159	30.8	31.1	202.5	1018	62	0.22	0	1.58
M	a	4	09:21:07	11.9	1	446	4	159	30.8	31.1	202	1018	225	0.43	0.01	1.7
M	a	5	09:12:20	11.9	0.6	445	0	151	30.6	30.9	201.7	1018	407	0.16	0	0.05
M	a	5	09:12:31	11.9	0.5	445	0	150	30.6	30.9	202.6	1018	421	0.14	0	0
M	a	5	09:12:41	12	0.4	445	0	151	30.7	30.9	201.7	1018	401	0.15	0	0.06
M	a	6	08:48:57	12.8	0.6	448	1	140	30.4	30.6	202.2	1017	357	0.28	0	0.41
M	a	6	08:49:02	12.8	0.6	449	1	141	30.4	30.6	202.6	1017	303	0.24	0	0.63
M	a	6	08:49:15	12.8	0.6	449	2	143	30.4	30.6	201.7	1018	216	0.2	0	0.91
M	a	7	08:53:19	12.6	0.6	446	2	135	30.5	30.7	202	1018	198	0.17	0	0.82
M	a	7	08:53:26	12.7	0.4	446	2	135	30.5	30.7	201.5	1017	189	0.15	0	0.76
M	a	7	08:53:39	12.7	0.4	447	2	134	30.5	30.7	202.1	1018	116	0.14	0	0.92
NM	b	10	08:41:28	12.6	0.3	448	2	109	30.2	30.3	202.1	1018	-286	0.06	0	0.95
NM	b	10	08:41:34	12.8	0.1	448	2	108	30.2	30.3	202	1017	-26	0.1	0	1
NM	b	10	08:41:41	12.7	0.1	448	3	109	30.2	30.4	202.8	1017	-937	0.03	0	1.12
NM	b	11	08:31:23	12.3	0.6	446	0	92	29.9	30	202	1017	400	0.24	0	0.13
NM	b	11	08:31:32	12.3	0.6	447	0	93	29.9	30.1	202.1	1017	370	0.21	0	0.26
NM	b	11	08:31:43	12.3	0.5	447	0	92	29.9	30.1	202.1	1017	359	0.16	0	0.23
NM	b	12	08:25:33	13.6	0.5	449	3	118	30.1	30.3	201.9	1017	104	0.19	0	1.36

Myco	Table	Plant number	tm	eref	^e	cref	^c	Qleaf	Tch	Tl	u	p	ci	E	gs	A
NM	b	12	08:25:39	13.4	0.6	449	3	119	30.1	30.3	202.1	1018	104	0.19	0	1.36
NM	b	12	08:25:47	13.4	0.5	449	3	118	30.1	30.3	202	1017	149	0.21	0	1.31
M	b	13	08:35:24	12.4	0.2	447	2	118	29.9	30.1	202.3	1017	-15	0.07	0	0.74
M	b	13	08:35:35	12.5	0.1	447	2	117	29.9	30.1	201.5	1017	66	0.09	0	0.7
M	b	13	08:36:50	12.4	0.2	447	2	116	30	30.2	202.3	1018	-208	0.07	0	0.99
M	b	14	08:28:05	13	0.5	447	1	116	30	30.2	202.5	1017	299	0.23	0	0.65
M	b	14	08:28:15	12.8	0.7	447	1	117	30	30.2	201.9	1017	327	0.26	0	0.57
M	b	14	08:28:25	12.9	0.5	447	1	119	30	30.2	202.5	1017	330	0.19	0	0.41
M	b	15	08:19:29	14.1	0.4	452	1	102	30.2	30.4	201.5	1017	234	0.14	0	0.63
M	b	15	08:19:41	14.3	0.2	452	1	102	30.2	30.4	202.3	1017	165	0.09	0	0.56
M	b	15	08:19:51	14.3	0.2	452	1	101	30.2	30.4	202	1017	178	0.11	0	0.63
NM	b	16	08:16:53	14.2	0.4	453	2	106	30.3	30.5	202.5	1017	58	0.11	0	0.95
NM	b	16	08:17:03	14.3	0.2	453	2	105	30.3	30.4	201.9	1017	170	0.13	0	0.8
NM	b	16	08:17:13	14.2	0.3	453	2	104	30.3	30.4	202.2	1017	-99	0.07	0	0.91
NM	c	19	07:56:56	13.9	0.6	456	3	86	29.7	29.8	202.1	1017	175	0.21	0	1.28
NM	c	19	07:57:15	13.9	0.5	456	3	84	29.7	29.8	202.5	1017	163	0.19	0	1.21
NM	c	19	07:57:26	14	0.3	456	3	83	29.7	29.9	201.9	1017	111	0.15	0	1.14
NM	c	20	08:02:03	14.1	0.2	458	1	93	29.9	30	201.9	1017	201	0.12	0	0.67
NM	c	20	08:02:10	14.1	0.2	457	1	94	29.9	30	202.4	1017	223	0.08	0	0.42
NM	c	20	08:02:21	14	0.2	457	1	94	29.9	30	201.6	1017	158	0.06	0	0.44
NM	c	21	08:09:42	14.2	1	454	3	105	30.2	30.4	201.8	1017	278	0.36	0.01	1.26
NM	c	21	08:09:52	14.2	0.8	454	3	107	30.3	30.4	202.4	1017	262	0.31	0.01	1.21
NM	c	21	08:10:14	14.4	0.5	454	3	108	30.3	30.5	202.1	1017	206	0.23	0	1.2
M	c	22	07:59:22	13.9	1	455	3	89	29.8	29.9	202.6	1017	240	0.3	0.01	1.38
M	c	22	07:59:33	13.9	0.9	455	3	92	29.8	29.9	201.8	1017	259	0.31	0.01	1.28
M	c	22	07:59:55	13.9	0.7	457	2	92	29.8	30	201.9	1017	264	0.21	0	0.85
M	c	23	08:03:56	14	1.2	455	2	95	29.9	30.1	202.1	1017	342	0.43	0.01	0.93
M	c	23	08:04:05	14	0.9	455	2	94	29.9	30.1	201.7	1017	308	0.34	0.01	0.99
M	c	23	08:04:15	14.1	0.6	455	2	93	29.9	30.1	202.6	1017	284	0.25	0	0.88
M	c	24	08:06:53	14.2	1.2	454	2	91	30	30.2	202	1017	341	0.43	0.01	0.92

Myco	Table	Plant number	tm	eref	^e	cref	^c	Qleaf	Tch	Tl	u	p	ci	E	gs	A
M	c	24	08:07:03	14.2	1.1	454	2	91	30	30.2	202.3	1017	344	0.43	0.01	0.89
M	c	24	08:07:14	14.2	1.1	454	2	91	30.1	30.2	201.9	1017	354	0.38	0.01	0.7
M	c	25	08:14:30	14.3	0.5	453	2	111	30.3	30.5	202	1017	201	0.19	0	1
M	c	25	08:14:43	14.4	0.4	453	3	111	30.4	30.6	201.9	1017	106	0.15	0	1.14
M	c	25	08:14:48	14.4	0.4	453	3	110	30.3	30.6	202.4	1017	106	0.15	0	1.14
NM	d	28	07:38:11	13.6	1.2	453	3	69	28.3	28.4	201.9	1017	310	0.39	0.01	1.3
NM	d	28	07:38:21	13.7	1	453	3	69	28.3	28.4	202.2	1017	311	0.39	0.01	1.27
NM	d	28	07:38:35	13.7	1	453	4	71	28.3	28.4	201.7	1017	288	0.39	0.01	1.52
NM	d	29	07:46:45	13.7	0.5	455	0	78	29	29.1	202.3	1017	384	0.18	0	0.23
NM	d	29	07:46:56	13.8	0.4	455	0	78	29	29.1	201.8	1017	396	0.18	0	0.18
NM	d	29	07:47:07	13.8	0.4	455	0	77	29	29.1	202.2	1017	392	0.18	0	0.2
NM	d	30	07:54:09	13.9	0.6	455	1	85	29.6	29.7	202.2	1017	258	0.15	0	0.62
NM	d	30	07:54:18	13.9	0.5	455	2	84	29.6	29.7	201.9	1017	173	0.16	0	1.03
NM	d	30	07:54:28	13.9	0.5	455	2	83	29.6	29.8	202.3	1017	210	0.16	0	0.89
M	d	31	07:35:29	13.6	0.8	451	5	70	28	28.1	201.9	1017	199	0.29	0.01	1.87
M	d	31	07:35:58	13.7	0.6	452	6	68	28.1	28.2	202.3	1017	89	0.24	0.01	2.23
M	d	31	07:36:08	13.6	0.6	452	5	68	28.1	28.2	201.5	1016	170	0.25	0.01	1.78
M	d	32	07:44:11	13.7	0.2	455	0	73	28.8	28.9	201.6	1017	350	0.12	0	0.27
M	d	32	07:44:28	13.7	0.2	455	0	73	28.9	29	201.8	1017	364	0.09	0	0.17
M	d	32	07:44:40	13.8	0.1	455	0	73	28.9	29	202.3	1017	286	0.07	0	0.26
M	d	33	07:41:11	13.6	0.6	453	2	72	28.6	28.7	202.5	1017	273	0.2	0	0.82
M	d	33	07:41:30	13.6	0.6	453	2	72	28.6	28.7	202.3	1017	263	0.2	0	0.89
M	d	33	07:41:42	13.6	0.6	453	3	72	28.6	28.7	202	1017	206	0.21	0	1.24
NM	d	34	07:49:26	13.9	0.9	454	1	81	29.2	29.3	201.6	1017	375	0.31	0.01	0.46
NM	d	34	07:50:33	13.8	0.6	455	2	81	29.3	29.4	201.9	1017	259	0.21	0	0.93
NM	d	34	07:50:48	13.9	0.5	455	2	81	29.3	29.5	201.6	1017	213	0.18	0	0.98
NM	e	37	07:20:20	13.2	0.8	454	2	54	25.3	25.3	201.6	1016	353	0.28	0.01	0.86
NM	e	37	07:20:30	13.3	0.7	454	2	55	25.3	25.4	202.1	1016	349	0.27	0.01	0.85
NM	e	37	07:20:41	13.3	0.7	454	2	56	25.3	25.4	201.7	1016	325	0.26	0.01	1.02
NM	e	38	07:25:26	13.4	0.6	452	4	66	26.4	26.5	202.2	1016	243	0.23	0.01	1.41

Myco	Table	Plant number	tm	eref	^e	cref	^c	Qleaf	Tch	Tl	u	p	ci	E	gs	A
NM	e	38	07:25:36	13.5	0.5	452	4	67	26.4	26.5	201.8	1016	135	0.18	0	1.69
NM	e	38	07:25:45	13.4	0.5	451	3	68	26.4	26.6	202.1	1016	194	0.17	0	1.31
NM	e	39	07:22:36	13.3	0.5	453	1	65	25.8	25.9	202.6	1016	322	0.17	0	0.66
NM	e	39	07:22:49	13.3	0.5	453	1	64	25.8	25.9	202	1016	326	0.16	0	0.61
NM	e	39	07:22:57	13.4	0.4	453	1	65	25.8	25.9	202.1	1016	305	0.12	0	0.53
M	e	40	07:18:14	13.1	0.8	456	2	54	24.8	24.9	201.6	1016	367	0.31	0.01	0.85
M	e	40	07:18:24	13.2	0.6	456	2	53	24.8	24.9	202.6	1016	350	0.23	0.01	0.78
M	e	40	07:18:35	13.1	0.6	456	2	54	24.9	25	201.6	1016	341	0.23	0.01	0.84
M	e	41	07:15:48	13	0.2	460	0	58	24.2	24.2	202	1016	416	0.05	0	0.06
M	e	41	07:16:29	13.1	0.1	460	0	56	24.4	24.4	201.7	1016	377	0.05	0	0.14
M	e	41	07:16:50	13.1	0.1	459	0	55	24.4	24.5	201.5	1016	357	0.07	0	0.23
M	e	42	07:32:49	13.7	0.7	450	1	70	27.7	27.8	202.1	1017	357	0.31	0.01	0.66
M	e	42	07:33:09	13.7	0.5	451	1	72	27.8	27.9	202.4	1017	318	0.21	0	0.69
M	e	42	07:33:19	13.7	0.5	451	2	72	27.8	27.9	201.8	1017	299	0.2	0	0.74
NM	e	43	07:30:15	13.6	0.6	451	3	73	27.4	27.5	201.9	1016	268	0.25	0.01	1.2
NM	e	43	07:30:33	13.6	0.5	451	2	73	27.4	27.5	202	1016	240	0.18	0	1.02
NM	e	43	07:30:43	13.7	0.4	452	3	73	27.4	27.6	202.5	1016	233	0.19	0	1.12
M	e	44	07:27:46	13.5	0.4	451	1	68	26.9	27	202	1016	359	0.2	0	0.46
M	e	44	07:28:00	13.5	0.5	451	1	68	26.9	27.1	202.1	1016	353	0.17	0	0.41
M	e	44	07:28:12	13.5	0.4	451	0	68	27	27.1	202.2	1016	346	0.12	0	0.31
NM	a	1	09:19:03	12.7	2.2	422	6	95	29.8	30	201.9	1018	267	0.78	0.02	2.4
NM	a	1	09:18:59	12.7	2.3	422	6	98	29.8	30	201.6	1018	267	0.78	0.02	2.4
NM	a	1	09:18:44	12.8	2.3	421	3	102	29.8	30	201.9	1018	337	0.89	0.03	1.34
NM	a	2	09:21:46	12.7	3.4	420	7	102	29.8	30	201.7	1018	310	1.17	0.04	2.49
NM	a	2	09:22:09	12.7	3.2	421	6	108	29.8	30	201.5	1018	312	1.12	0.04	2.33
NM	a	2	09:22:19	12.8	3	418	3	108	29.8	30	202.5	1018	350	1.09	0.04	1.26
NM	a	3	09:10:14	12.7	3.6	424	9	131	29.5	29.7	201.8	1017	292	1.28	0.05	3.43
NM	a	3	09:10:00	12.6	3.8	426	13	133	29.5	29.7	202.1	1017	260	1.35	0.05	4.71
NM	a	3	09:10:04	12.7	3.7	424	11	133	29.5	29.7	202.1	1017	276	1.32	0.05	4.04
M	a	4	09:26:00	12.7	0.8	420	4	94	29.9	30	201.8	1018	103	0.24	0	1.62

Myco	Table	Plant number	tm	eref	^e	cref	^c	Qleaf	Tch	Tl	u	p	ci	E	gs	A
M	a	4	09:27:25	12.8	0.6	419	1	101	29.9	30.1	201.5	1018	322	0.24	0	0.41
M	a	4	09:27:37	12.7	0.8	418	0	104	29.9	30.1	202	1018	365	0.24	0	0.18
M	a	5	09:16:34	12.7	1.8	424	9	127	29.7	29.9	201.6	1018	171	0.6	0.02	3.19
M	a	5	09:16:42	12.7	1.7	422	6	128	29.7	29.9	201.8	1018	222	0.56	0.02	2.29
M	a	5	09:16:54	12.8	1.5	422	7	133	29.7	30	201.8	1018	201	0.54	0.01	2.47
M	a	6	09:13:02	12.6	6.2	424	14	123	29.6	29.8	201.6	1018	310	2.1	0.09	5.01
M	a	6	09:12:51	12.7	6.2	424	14	128	29.6	29.8	202.4	1018	309	2.13	0.09	5.1
M	a	6	09:12:43	12.6	6.4	424	14	131	29.6	29.8	201.7	1018	316	2.22	0.1	4.95
M	a	7	08:59:45	12.3	0.7	427	6	75	29.1	29.2	201.8	1018	30	0.25	0	2.22
M	a	7	09:00:58	12.3	0.6	427	4	75	29.1	29.3	202.1	1017	161	0.27	0.01	1.55
M	a	7	08:58:05	12.3	0.9	426	3	89	29.1	29.2	201.8	1017	245	0.32	0.01	1.21
NM	b	10	08:55:34	12.3	1.9	426	2	56	29.1	29.2	201.6	1018	355	0.69	0.02	0.87
NM	b	10	08:55:43	12.1	1.9	426	2	57	29.1	29.2	202.3	1017	351	0.63	0.02	0.85
NM	b	10	08:55:54	12.2	1.8	426	2	58	29.1	29.2	201.7	1018	355	0.6	0.02	0.75
NM	b	11	08:50:04	12.2	1.5	427	2	44	29.1	29.2	201.8	1018	340	0.51	0.01	0.83
NM	b	11	08:50:11	12.2	1.4	427	2	44	29.1	29.2	202.1	1017	340	0.51	0.01	0.83
NM	b	11	08:49:38	12.2	1.8	427	1	46	29.1	29.2	201.8	1017	385	0.61	0.02	0.34
NM	b	12	08:46:00	12.4	2.7	426	0	57	29.1	29.2	202	1017	401	0.96	0.03	0.18
NM	b	12	08:46:17	12.3	2.8	428	4	59	29.1	29.2	202	1017	346	0.96	0.03	1.47
NM	b	12	08:46:59	12.3	2.6	429	4	59	29.1	29.2	202.5	1017	331	0.85	0.03	1.62
M	b	13	08:52:32	12.2	0.9	427	2	49	29.1	29.2	201.9	1018	278	0.33	0.01	1.01
M	b	13	08:52:16	12.2	1	426	3	50	29.1	29.2	202.6	1018	275	0.38	0.01	1.17
M	b	13	08:51:57	12.3	1	426	2	52	29.1	29.2	202.5	1018	323	0.4	0.01	0.79
M	b	14	08:43:24	12.5	0.9	429	5	67	29.1	29.2	201.9	1017	196	0.36	0.01	1.79
M	b	14	08:43:47	12.3	0.9	428	3	68	29.1	29.2	201.8	1018	260	0.31	0.01	1.1
M	b	14	08:43:52	12.3	1	428	3	69	29.1	29.2	202.2	1017	260	0.31	0.01	1.1
M	b	15	08:40:30	12.5	1.1	427	5	74	29.1	29.2	201.6	1017	194	0.37	0.01	1.86
M	b	15	08:40:19	12.4	1.1	427	6	75	29.1	29.2	202.1	1018	195	0.43	0.01	2.14
M	b	15	08:40:04	12.4	1.2	428	6	76	29.1	29.2	202.1	1017	199	0.46	0.01	2.27
NM	b	16	08:34:49	12.4	1.3	429	4	83	29.1	29.2	201.6	1017	274	0.48	0.01	1.55

Myco	Table	Plant number	tm	eref	^e	cref	^c	Qleaf	Tch	Tl	u	p	ci	E	gs	A
NM	b	16	08:36:40	12.4	0.6	428	4	93	29.1	29.2	201.9	1017	171	0.25	0	1.4
NM	b	16	08:36:27	12.4	0.7	428	4	96	29.1	29.2	202.5	1017	162	0.24	0	1.39
NM	c	19	07:47:09	13.8	3	444	0	37	29.5	29.6	202	1017	422	1.03	0.04	0.12
NM	c	19	07:47:29	13.8	3	445	0	38	29.5	29.6	202.5	1017	418	1.02	0.04	0.23
NM	c	19	07:47:40	13.8	3	445	0	38	29.5	29.6	201.5	1017	417	1.01	0.04	0.26
NM	c	20	07:58:22	13.6	2.8	445	2	40	29.3	29.3	202.3	1017	393	0.98	0.04	0.86
NM	c	20	07:58:33	13.6	2.7	445	1	40	29.3	29.3	201.9	1018	408	0.94	0.03	0.46
NM	c	20	07:58:59	13.7	2.6	446	5	40	29.2	29.3	202.1	1017	351	0.91	0.03	1.75
NM	c	21	08:04:38	13.5	2.1	440	0	40	29.1	29.2	202.3	1018	412	0.72	0.02	0.18
NM	c	21	08:05:31	13.5	2	436	0	40	29.1	29.1	201.9	1018	405	0.73	0.03	0.25
NM	c	21	08:06:07	13.3	2	438	0	40	29	29.1	202.2	1018	418	0.65	0.02	0.05
M	c	22	07:51:32	13.7	1.7	448	2	41	29.5	29.5	201.7	1017	365	0.58	0.02	0.9
M	c	22	07:51:42	13.9	1.5	448	2	41	29.5	29.5	202.4	1017	354	0.57	0.02	1.03
M	c	22	07:51:54	13.7	1.5	448	3	42	29.5	29.5	201.9	1017	343	0.51	0.02	1.07
M	c	23	07:55:10	13.6	1.5	451	3	40	29.4	29.4	202.1	1017	328	0.51	0.02	1.28
M	c	23	07:55:43	13.5	1.3	451	3	40	29.3	29.4	201.6	1017	331	0.43	0.01	1.05
M	c	23	07:55:53	13.6	1.2	449	1	40	29.3	29.4	202.2	1017	390	0.41	0.01	0.4
M	c	24	08:02:11	13.6	1.4	440	1	39	29.2	29.2	202.3	1018	383	0.53	0.02	0.51
M	c	24	08:00:59	13.5	1.8	440	0	40	29.2	29.2	201.5	1017	401	0.63	0.02	0.33
M	c	24	08:01:40	13.6	1.7	440	1	40	29.2	29.2	201.8	1017	390	0.57	0.02	0.44
M	c	25	08:31:16	12.6	1.1	429	3	64	29.1	29.2	202.3	1018	310	0.45	0.01	1.06
M	c	25	08:31:35	12.6	1.1	428	2	64	29.1	29.2	202	1017	311	0.42	0.01	0.99
M	c	25	08:31:50	12.6	1	428	2	64	29.1	29.2	201.9	1017	293	0.36	0.01	0.99
NM	d	28	07:39:30	13.6	3.3	445	3	56	29.1	29.2	202.1	1017	380	1.11	0.04	1.34
NM	d	28	07:39:47	13.7	3.1	444	3	56	29.1	29.2	202.2	1017	382	1.07	0.04	1.23
NM	d	28	07:39:05	13.7	3.3	445	3	58	29.1	29.2	202.4	1017	382	1.11	0.04	1.29
NM	d	29	07:36:35	13.6	2.4	446	4	65	28.9	29	201.8	1017	354	0.87	0.03	1.63
NM	d	29	07:35:52	13.6	2.5	446	5	67	28.9	29	202.1	1017	354	0.94	0.04	1.79
NM	d	29	07:36:07	13.6	2.5	446	5	67	28.9	29	201.5	1017	352	0.92	0.03	1.77
NM	d	30	07:26:57	13.3	1.8	448	5	74	28.3	28.4	201.6	1017	309	0.61	0.02	1.95

Myco	Table	Plant number	tm	eref	^e	cref	^c	Qleaf	Tch	Tl	u	p	ci	E	gs	A
NM	d	30	07:27:22	13.4	1.5	447	5	77	28.3	28.4	201.6	1016	307	0.56	0.02	1.8
NM	d	30	07:27:31	13.3	1.6	447	4	79	28.3	28.5	202.1	1016	310	0.55	0.02	1.73
M	d	31	07:44:31	13.8	0	444	0	44	29.4	29.5	201.7	1017	216	0.03	0	0.15
M	d	31	07:43:07	13.8	0.1	443	0	46	29.4	29.4	201.9	1017	353	0.06	0	0.11
M	d	31	07:43:17	13.9	0	444	0	46	29.4	29.5	202.1	1017	-839	0	######	0.28
M	d	32	07:33:34	13.5	1.5	445	5	75	28.8	29	202.3	1017	291	0.53	0.02	1.81
M	d	32	07:33:10	13.5	1.5	445	4	76	28.8	28.9	202	1017	310	0.55	0.02	1.62
M	d	32	07:33:21	13.4	1.6	445	5	76	28.8	28.9	202	1017	291	0.52	0.02	1.8
M	d	33	07:30:03	13.4	2.7	446	5	77	28.6	28.7	202.3	1017	354	0.94	0.04	1.86
M	d	33	07:30:20	13.5	2.7	447	6	77	28.6	28.7	202.4	1017	347	0.96	0.04	2.09
M	d	33	07:29:27	13.4	2.7	447	5	80	28.5	28.6	202.1	1017	355	0.97	0.04	1.92
NM	d	34	07:24:50	13.3	1.8	449	2	50	28.1	28.2	201.7	1016	391	0.63	0.02	0.7
NM	d	34	07:24:57	13.3	1.8	449	2	51	28.1	28.2	201.9	1016	384	0.64	0.02	0.83
NM	d	34	07:25:05	13.4	1.7	448	1	51	28.1	28.2	202.1	1017	402	0.62	0.02	0.5
NM	e	37	07:07:14	12.4	0.2	457	0	26	25	25.1	202.6	1016	419	0.07	0	0.06
NM	e	37	07:08:27	12.4	0.3	456	0	28	25.3	25.3	201.9	1016	400	0.09	0	0.13
NM	e	37	07:08:48	12.5	0.2	455	0	29	25.3	25.4	201.8	1016	359	0.07	0	0.19
NM	e	38	07:15:31	12.9	0.8	453	3	60	26.6	26.7	201.7	1016	315	0.34	0.01	1.23
NM	e	38	07:15:39	12.9	0.7	451	2	60	26.7	26.7	202.3	1016	344	0.27	0.01	0.74
NM	e	38	07:16:00	12.9	0.7	452	3	60	26.7	26.8	202.4	1016	261	0.25	0.01	1.3
NM	e	39	07:11:59	12.6	0.2	455	0	41	26	26	201.7	1016	414	0.08	0	0.06
NM	e	39	07:12:19	12.7	0.1	454	0	44	26.1	26.1	201.6	1016	437	0.04	0	0
NM	e	39	07:13:01	12.7	0.1	454	0	51	26.2	26.3	201.5	1016	279	0.05	0	0.27
M	e	40	07:03:23	12	0.4	463	1	26	24.2	24.2	202.1	1016	355	0.13	0	0.43
M	e	40	07:04:17	12.2	0.2	463	0	28	24.4	24.4	201.9	1016	405	0.09	0	0.15
M	e	40	07:04:30	12.1	0.3	462	0	28	24.4	24.4	202.1	1016	442	0.11	0	0.02
M	e	41	07:01:17	11.9	0.2	468	2	23	23.7	23.7	202.4	1016	236	0.1	0	0.86
M	e	41	07:01:38	12	0.1	465	3	23	23.8	23.8	202	1016	-6	0.06	0	1.06
M	e	41	07:00:35	11.8	0.1	464	3	25	23.5	23.5	202.4	1016	-322	0.04	0	1.33
M	e	42	07:18:05	13.1	1.3	451	3	49	27.1	27.2	202.1	1016	356	0.48	0.02	1.11

Myco	Table	Plant number	tm	eref	^e	cref	^c	Qleaf	Tch	Tl	u	p	ci	E	gs	A
M	e	42	07:18:15	13	1.4	451	2	49	27.1	27.2	201.7	1016	368	0.48	0.02	0.92
M	e	42	07:18:00	13	1.3	451	3	51	27.1	27.1	202.1	1016	359	0.51	0.02	1.14
NM	e	43	07:20:02	13.1	0.8	450	0	39	27.4	27.5	202	1016	412	0.32	0.01	0.19
NM	e	43	07:20:11	13.1	0.8	449	0	39	27.5	27.5	202.3	1016	414	0.25	0.01	0.13
NM	e	43	07:20:43	13.2	0.6	449	0	43	27.5	27.6	201.5	1016	402	0.24	0.01	0.19
M	e	44	07:22:38	13.2	0.8	449	0	41	27.8	27.9	202.1	1016	390	0.3	0.01	0.34
M	e	44	07:22:28	13.2	0.9	449	1	42	27.8	27.9	201.8	1016	389	0.3	0.01	0.35
M	e	44	07:22:17	13.2	1	449	1	43	27.8	27.9	202.6	1016	364	0.34	0.01	0.62

Symbol	Description	Unit
tm	time of day	
eref	H2O Reference, as partial pressure	mBar
^e	Delta H2O (w'an-Wref), partial p	mBar
cref	CO2 reference	vpm
^c	delta CO2 (Cref-C'an)	vpm
Qleaf	P.A.R. incident on leaf surface	µmol m^{-2} s^{-1}
Tch	leaf chamber temperature	°C
Tl	leaf surface temperature	°C
u	ASU mass flow (measured)	µmol s-1
p	atmospheric pressure	mBar
ci	sub-stomatal CO2	vpm
E	transpiration rate	mmol m-2 s-1
gs	stomatal conductance of CO2	mol m-2 s-1
A	photosynthetic rate	µmol m-2 s-1
C'an	CO2 analysis (corrected for dilution)	vpm
Wref	H_2O reference	%RH
P.A.R.	Photosynthetic Active Radiation	