# Arbuscular mycorrhizal (AM) fungal diversity of arid lands:

## From AM fungal species to AM fungal communities

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## Summary

One of the widespread constituents of soil communities are the symbiotic arbuscular mycorrhizal (AM) fungi which associate in a mutualistic symbiosis with 80% of all land plants (Parniske, 2008).They were shown to positively influence plant nutrition (Smith & Read, 2008), plant productivity (Klironomos *et al.*, 2000; Wilsey & Potvin, 2000) and improve their host plants' tolerance to biotic and abiotic stresses (Augé, 2001; Hildebrandt *et al.*, 2007; Porcel *et al.*, 2011). It was shown that AM fungal communities vary among broadly defined habitat types and further, that differences in AM fungal communities occur between different continents and climatic zones (Öpik 2006, 2013). This thesis highlights, for the first time, the particularity of the AM fungal communities in one location including four different habitat types of Southern Arabia. Using morphological and molecular methods, three AM fungal species new-to-science were described. These are *Diversispora omaniana*, *Septoglomus nakheelum* and *Rhizophagus arabicus*. In addition, four previously described AM fungal species were detected and isolated from the Arabian Peninsula for the first time. These are *Claroideoglomus drummondii*, *Diversispora aurantia*, *Diversispora spurca* and *Funneliformis africanum.*

The isolated and characterized AM fungal species were then used in two different research approaches. The first approach was to gain basic knowledge on physiological aspects of the isolated species through two experiments.

The first experiment was conducted in experimental microcosms with sorghum as host plant to evaluate the interacting effects of water regime and the presence of a potentially invasive AM fungal species, *Rhizophagus irrigularis*, in an assemblage of our AM fungal species (called native AM fungal assemblage). The community structure of the AM fungi colonizing the roots was assessed by determining the transcript abundance of the large ribosomal subunit (rLSU) of each individual AM fungus, using real-time PCR and species-specific primers. Our results showed that both water regime and the introduction of an invasive AM fungal species strongly altered the structure of the native AM fungal assemblage accompanied by a reduction of the plants' drought tolerance as reflected by a lower accumulation of plant biomass and reduced amount of extraradical mycelium. Especially in arid environments, where the occurring AM fungal species are adapted to the environmental conditions, such changes can have a great impact on ecosystem functioning.

In a second experiment, we wanted to investigate functional characteristics of *R. arabicus*, one of our newly discovered AM fungal species endemic to the desert ecosystem, in comparison to *R. irregularis*, a well-studied and established lab AM fungus isolated from an agicultural field in Switzerland. As the plants' drought tolerance is highly dependent on the associated AM fungal species we tested the two closely related AM fungi of contrasting climates for their ability to promote drought tolerance of sorghum

by comparing transpiration rates, growth and nutrition of sorghum plants. Additionally, we estimated their ability to produce extraradical hyphae and their efficiency to extract nutrients from moist and dry soil to reveal their performance under contrasting conditions. Functional traits as extraradical hyphal formation and nutrient uptake efficiency, which were affected by the applied stress conditions, we observed huge differences between the two AM fungi where *Rhizophagus arabicus* seems to improve the plants' drought tolerance more than *R. irregularis*. These differences might have resulted from specific adaptations to environmental conditions prevalent at the place where the AM fungal species originated from. *R. arabicus* was isolated from a hyper-arid environment and therefore might be better adapted to dry conditions.In a second part of this experiment, the aquaporin (AQPs) genes of *Sorghum bicolor*, our model plant were investigated. AQPs are pore-forming integral membrane proteins, located in various membranes of plant cells, and were recognized to play an important role in root water transport. First, we identified and characterized all AQP encoding genes in the fully-sequenced genome of sorghum. Additionally, the regulation of AQP transcript expression of mycorrhizal and non-mycorrhizal sorghum under well-watered and drought condition was assessed. Bioinformatic analyses of the *S. bicolor* genome revealed 35 genes coding for AQPs. Gene expression studies showed that the selected AQP genes were differentially regulated, either by mycorrhization, by water regime or by their interaction.

The last part of this thesis describes experiments that have been conducted to evaluate the abilities of some of the propagated AM fungal strains in promoting the seedling growth of two important plant species: *Phoenix dactylifera* (Nakhal) and *Prosopis cineraria* (Ghaf). The application of AM fungal inocula in horticulture, agriculture and revegetation programs became more prominent within the last decades as the number of studies demonstrating improved plant growth after inoculation has steadily increased. Especially in arid and semiarid ecosystems, the use of AM fungal inocula is of particular interest due to additional challenges the plants have to face in these climates such as drought, soil salinity and low nutrient availability. Furthermore, it was shown that the mycorrhizal potential in those soils is comparatively low and the additional application of AM fungi may lead to an improved establishment and functionality of the symbiosis. In our experiments some of the isolated AM fungal species or combinations of species were able to enhance the plants' growth under nursery conditions. The plants have been transplanted to a desert field site in Oman for continuous long term observations.

This PhD thesis displays a full circle of isolation of AM fungi from nature, over their identification and propagation, with their subsequent application in basic and applied experiments. Further experiments assessing functional traits and characteristics of those particular AM fungi would be of high interest. Furthermore, the isolated AM fungi can open new doors in the field of conservation and revegetation in that unique and fascinating part of the world.

# 1 General Introduction

## 1.1 Mycorrhizal Symbiosis

Symbiosis derived from the Greek meaning “living together” describes a close relationship between two or more different species and may be found between organisms of all five kingdoms. The term symbiosis can be used to describe interactions between symbiotic partners ranging from mutualistic to parasitic.
Symbiosis played a crucial role in the evolution of eukaryotic cells. As mitochondria and chloroplasts of eukaryotic cells were found to derive from free-living bacteria, it is believed that the early eukaryotic cell incorporated prokaryotic cells which led to the formation of mitochondria and chloroplast as we find them today. This finding illustrates the importance of symbiosis in the development of eukaryotic cells.
Among all existing types of symbiosis, the mycorrhiza is the most commonly found and wide-spread association formed between fungi and the roots of vascular plants. The term mycorrhiza literally means “fungus-root”. In the mycorrhizal symbiosis the plant delivers photosynthetically fixed carbohydrates to its fungal partner, whereas in return the plant is provided with soil nutrients as well as water collected by the fungus. Due to the extensive mycelium with which the fungus is colonizing the soil, the plants get access to a larger proportion of soil volume, resulting in an increased availability of soil nutrients. Thus, it is believed that a huge proportion of the plant’s mineral nutrients derive from their fungal partners (Selosse *et al.*, 2006). This may explain why about 90% of all plant species are living in association with mycorrhizal fungal partners. Mycorrhizas are formed between fungi from different taxa (Glomeromycota, Ascomycota and Basidiomycota) and an even wider range of plants including mosses (Anthocerophyta, Marchantiophyta and Bryophyta), ferns (Pteridophyta) and higher plants (Spermatophyta) (Smith & Read, 2008). Over the time, different types of mycorrhizal associations have evolved and are commonly classified into seven groups based on the fungal taxa involved and specific structural characteristics observed during the formation of the symbiosis. An overview about the different types of mycorrhizas is given in table 1.1. Several species of plants and fungi are able to form different types of mycorrhizas dependent on their associated partner (Smith & Read, 2008). Despite their division into different groups, mycorrhizas share some common characteristics although the nomenclature of the symbiotic structures differs dependent on the mycorrhizal symbiosis. First, the fungi penetrate and further colonize the roots of their host plants either extracellular or intracellular. Secondly, they build up specific organs for the exchange of goods between the symbiotic partners. Thirdly, they form an extensive external hyphal network to explore the soil for nutrients and new hosts.

Table 1.1 Characteristics of the important mycorrhizal types

| Types of mycorrhiza | Arbuscular | Ecto | Ectendo | Arbutoid | Monotropoid | Ericoid | Orchid |
|---|---|---|---|---|---|---|---|
| Fungal taxa | Glomero | Basidio/Asco (Glomero) | Basidio/Asco | Basidio | Basidio | Asco | Basidio |
| Plant taxa | Bryo, Pterido, Gymno, Angio | Gymno, Angio | Gymno, Angio | Ericales | Monotropoidales | Ericales, Bryo | Orchidales |
| Fungi +/- septae | – | + | + | + | + | + | + |
| Intracellular structures | + | – | + | + | + | + | + |
| Hartig net | – | + | + | + | + | – | – |

The fungal taxa are abbreviated from Glomeromycota, Ascomycota and Basidiomycota; the plant taxa from Bryophyta, Pteridophyta, Gymnospermae and Angiospermae. Modified from Smith and Read (2008).

A commonly shared characteristic of all mycorrhizas is the intracellular colonization of host roots, the only exception here are ectomycorrhizas (EM). With a structure called the mantle (or sheath) the fungi densely enclose the roots of their hosts. With their hyphae they also grow inwards and form a dense intraradical hyphal network called the Hartig net. Thereby, the fungus extracellularly surrounds the epidermal and cortical root cells where the nutrient exchange between the symbiotic partners takes place. The other types of mycorrhizas all form intracellular structures for the exchange of nutrients. Thy hyphae directly penetrate the root cells and form coils (found in arbuscular -, arbutoid – and ericoid mycorrhizas), coil-like structures called peletons (found in orchid mycorrhiza), highly specialized haustorium-like structures called “fungal pegs” (found in monotropoid mycorrhizas) or tree-like structures called arbuscules (typical for arbuscular mycorrhizas). The most important structures of the different mycorrhizal types are illustrated in figure 1.1.

Ecto-, ectendo- and ericoid mycorrhizas are usually found in forest ecosystems in association with autotrophic trees, shrubs and rarely, herbs (Smith & Read, 2008). Further, the arbutoid mycorrhizas are also formed by trees and shrubs, but additionally they can be found in association with herbs which are partially achlorophyllous. All plants species forming arbutoid mycorrhizas belong to the order of Ericaceae. The monotropoid mycorrhizas are restricted to the Monotropoidaea, a subfamily of the Ericaceae, which are all achlorophyllous and therefore dependent on the symbiosis for their growth. Also members of the Orchidaceae depend on the association with fungal partners, especially at the seedling stage, when the plants are still achlorophyllous and need to form orchid mycorrhizas (Smith & Read, 2008).

Arbuscular mycorrhizas are the most widespread symbiosis. In temperate regions they occur mainly in association with herbs, whereas in tropical, semiarid and arid regions they are also predominant in the roots of trees and shrubs. The occurrence of the different types of mycorrhizas is given in figure 1.2.

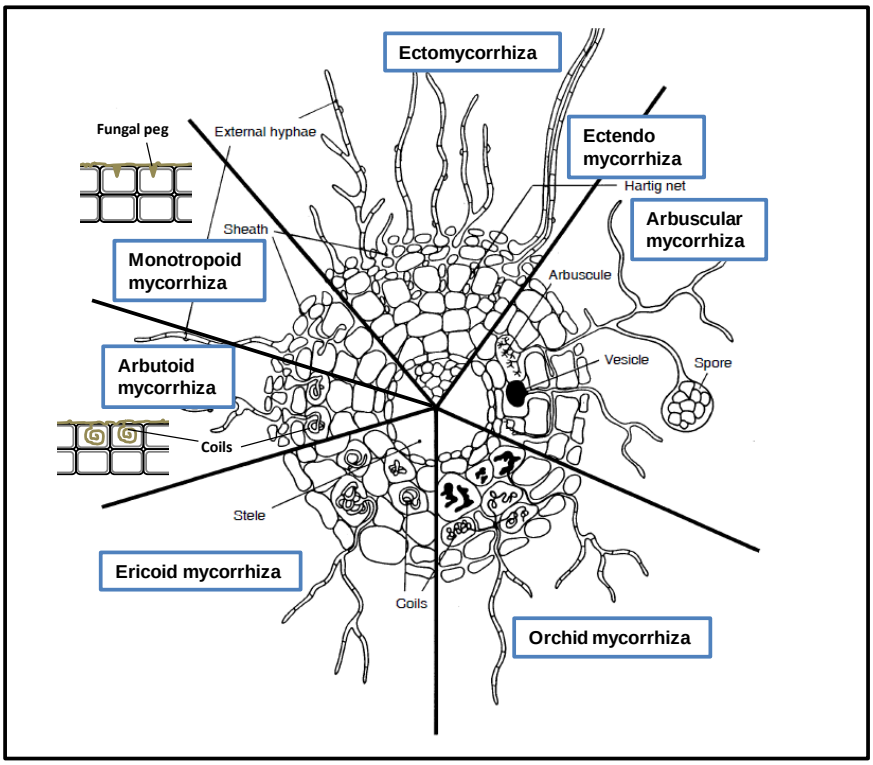


Fig. 1.1 Growth patterns and characteristic structures of important mycorrhiza types. Modified from Selosse and Le Tacon (1998).

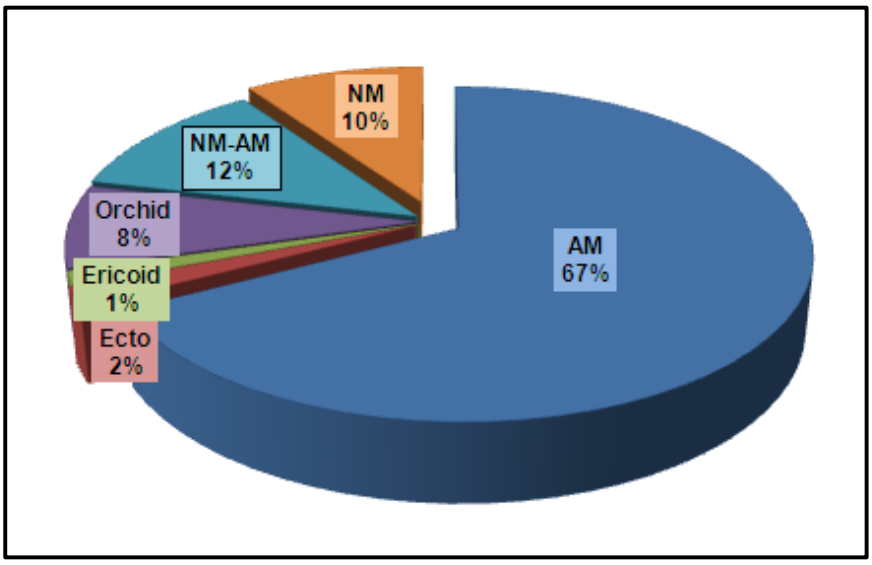


Fig. 1.2 The relative diversity of mycorrhizal and non-mycorrhizal (NM) plants for all vascular plants. AM (arbuscular mycorrhiza), Ecto (ectomycorrhiza), Orchid (orchid mycorrhiza), Ericoid (ericoid mycorrhiza), NM-AM (plants which occationally form AM). Arbutoid- and Monotropoid mycorrhiza integrated into Ecto; Ectendo mycorrhiza into Ecto or AM. Modified from Brundrett (2009).

## 1.2 Arbuscular Mycorrhiza (AM)

### 1.2.1 Introduction

The arbuscular mycorrhiza (AM) is the most commonly found mycorrhizal symbiosis, formed between a wide range of plant taxa including a large number of species, and the obligate symbiotic soil fungi belonging to the monophyletic Phylum of Glomeromycota. The Phylum comprises only about 250 described species (Blaszkowski, 2012). The existence of the AM symbiosis is dated back to the time when the first plants, mostly Bryophyta, started to colonize the land 460 million years ago. Findings of fossilized hyphae and spores together with molecular analyses support their ancient existence and lead to the assumption that the early AM symbiosis significantly contributed to the colonization of land by plants (Simon *et al.*, 1993; Redecker *et al.*, 2000). The name arbuscular mycorrhiza derives from the distinct tree-like structure, the arbuscule, which the AM fungi form inside the roots of their host plant for the nutrient exchange between the symbiontic partners. The central role of the AM symbiosis is the exchange of photosynthetically fixed carbon derived from the plant and soil nutrients delivered by the AM fungi. As the AM fungi are obligate symbionts, they are completely dependent on their plant partner for their growth and reproduction.

### 1.2.2 Arbuscular mycorrhizal fungi

Recently, the taxonomy of the AM fungi was newly structured, resulting in a new monophyletic clade, the Glomeromycota (Schüßler *et al.*, 2001; Redecker *et al.*, 2013). Before this new restructuring, the AM fungi were placed in the clade of Zygomycetes until molecular analyzes revealed their distinct positions within their own monophyletic clade. All members of the Glomeromycota are believed to have an asexual and clonal lifecycle, as so far no evidence for sexual reproduction was found. The AM fungi form large spores (up to 500µm in diameter) containing a huge number of nuclei with an estimated range from 800 up to 35000 depending on the AM fungal species (Smith & Read, 2008). The nuclei within one spore show a high genetic variability revealed by the occurrence of different ITS sequences in the nuclear rDNA obtained from DNA extracts of single spores (Pringle *et al.*, 2000; Rodriguez *et al.*, 2001; Jansa *et al.*, 2002). There is an ongoing discussion about the arrangement of the variant sequences among nuclei within the spore being either heterokaryotic with genetically different nuclei or homokaryotic with identical nuclei each containing all variant sequences (Pawlowska & Taylor, 2004). Genetic exchange despite the lack of sexual reproduction can occur by hyphal anastomosis (the fusion of two independent

hyphae of the same or genetically close AM fungal isolates) resulting in cytoplasmic continuity and nuclear migration (Giovannetti *et al.*, 2003; Croll *et al.*, 2009; Sbrana *et al.*, 2011; Tisserant *et al.*, 2014).

### 1.2.3 Plants forming arbuscular mycorrhiza

Plant species forming the AM symbiosis can be found within almost all plant phyla, ranging from the ancient phylum of bryophyta over almost all groups of Pteridophyta and Gymnosperms and the majority of families in the Angiospermes. Within the Angiosperms, the AM symbiosis can be found in around 217 plant families including more than 200'000 species (Brundrett, 2009). AM plants can be "facultatively mycorrhizal" including those species which are only sometimes colonized and "obligatorily mycorrhizal" including species which are consistently colonized. However, the extent to which a plant is responsive to colonization by AM fungi and the degree of benefit resulting from the symbiosis is highly dependent on AM fungus involved in the symbiosis and the environmental conditions (Smith & Read, 2008).

Arbuscular mycorrhizas are found in almost all biomes around the world, although with different frequencies. In the tropics, they represent the major type of mycorrhizal symbiosis. In temperate regions they are predominantly found in herbaceous plants, but also show a widespread distribution in woody plants, except the family of Pinaceae. Some tree species as e.g. *Quercus, Populus* and *Salix* were found to form tripartite symbiotic associations with AM fungi and EM fungi. Remarkably, oak trees were shown to be able to shift between AM – and EM symbiosis as a response to interannual rainfall variability (Querejeta *et al.*, 2009).

Only very few plant families including Brassicaceae, Caryophyllaceae, Chenopodiaceae, Cyperaceae, Juncaceae, Polygonaceae and Proteaceae do not form mycorrhizal associations of any type, although even in the roots of these families mycorrhizal structures are occasionally observed. Generally, it is easier to list the plant families observed to be non-mycorrhizal instead of those forming the AM symbiosis.

### 1.2.4 Characteristics and establishment of the AM symbiosis

Arbuscular mycorrhizas are composed of three major components: the host root, the intraradical fungal structures and the extraradical mycelium. There are three main sources of inocula to initiate the colonization of a new host root: spores, infected root fragments, or hyphae of an already established mycelium. The infection process and the establishment of the AM symbiosis are illustrated in figure 1.3. AM spores are thick-walled and resistant propagules which can rest for a long period in the soil until signals from a potential host root are perceived. Strigolactones are exuded by the host root and after their perception, the spore starts to germinated and hence the presymbiotic phase is initiated. This phase further

includes hyphal growth and branching together with an increased physiological activity including the release of fungal signaling molecules (generally called Myc-factors) which trigger symbiosis-specific responses in the host root. After the hyphopodium (the AM-specific appressoria) attaches to the root epidermis, the plant cell forms a prepenetration apparatus (PPA) guiding the hypha which extends from the hyphopodium through root cells towards the cortical cells. Further growth of the fungus takes place in the apoplast, laterally along the root axis. The development of PPA-like structures in cells of the inner cortex serves as preparation for invasion by the fungus. The hypha enters the cell and starts to branch to form the short-living arbuscule, a tree-shaped structure where the nutrient exchange occurs (Dickson *et al.*, 2007). The strictly obligate biotrophic AM fungus receives between 4 and 20% of the plant's total photosynthetic products (Douds *et al.*, 2000). By entering the cell, the hypha always stays surrounded by the periarbuscular membrane, a plant-derived membrane that separates the fungus from the symplasm of the plant cell, harboring specific transporters mediating the exchange of metabolites between the symbiotic partners (Balestrini & Bonfante, 2005). Besides the short-living arbuscules, some AM fungi also form thick-walled, inter- or intracellular vesicles containing lipids and nuclei and serving as storage organs as well as infection units within root fragments (Smith & Read, 2008). Once the symbiosis has been established, hyphal growth proceeds both within roots and in the soil leading to the formation of new spores and terminating the lifecycle of the AM fungus. Characteristic structures found in the AM symbiosis are illustrated in figure 1.4.

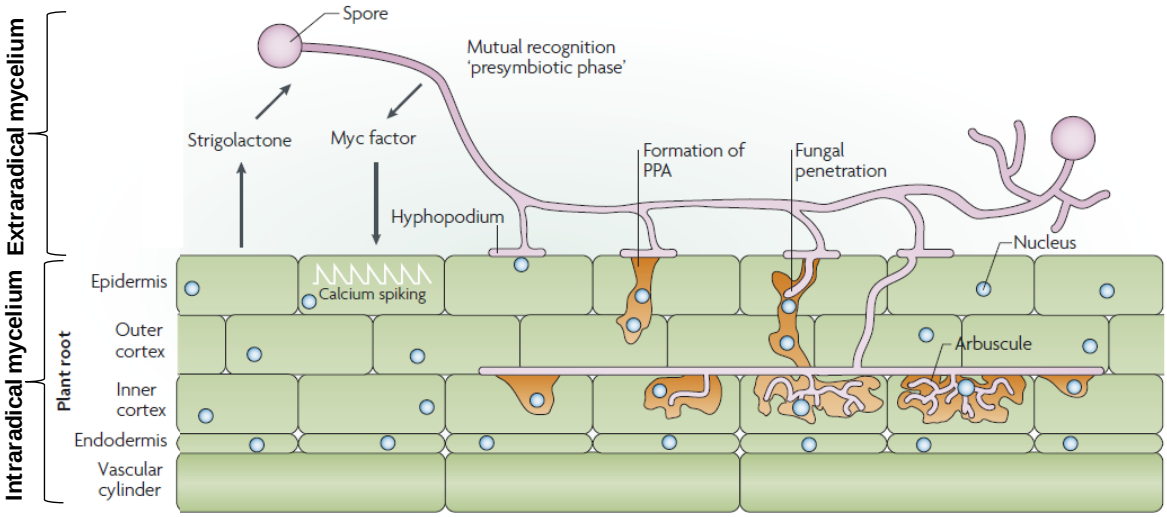


Fig. 1.3 Steps in arbuscular mycorrhiza development. Colonization process is displayed from left to right. The plant exudes strigolactones which are perceived by the AM fungus. The AM fungus increases its physiological activity and responds by producing "Myc-factors". Consequently, the root prepares for establishment of the symbiosis. After attachment and hyphopodium formation, the AM fungus enters the root to establish the intraradical mycelium and the formation of arbuscules. After establishing the intraradical mycelium, the AM fungus starts to build up the extraradical mycelium to forage for nutrients and new hosts. Modified from Parniske (2008).

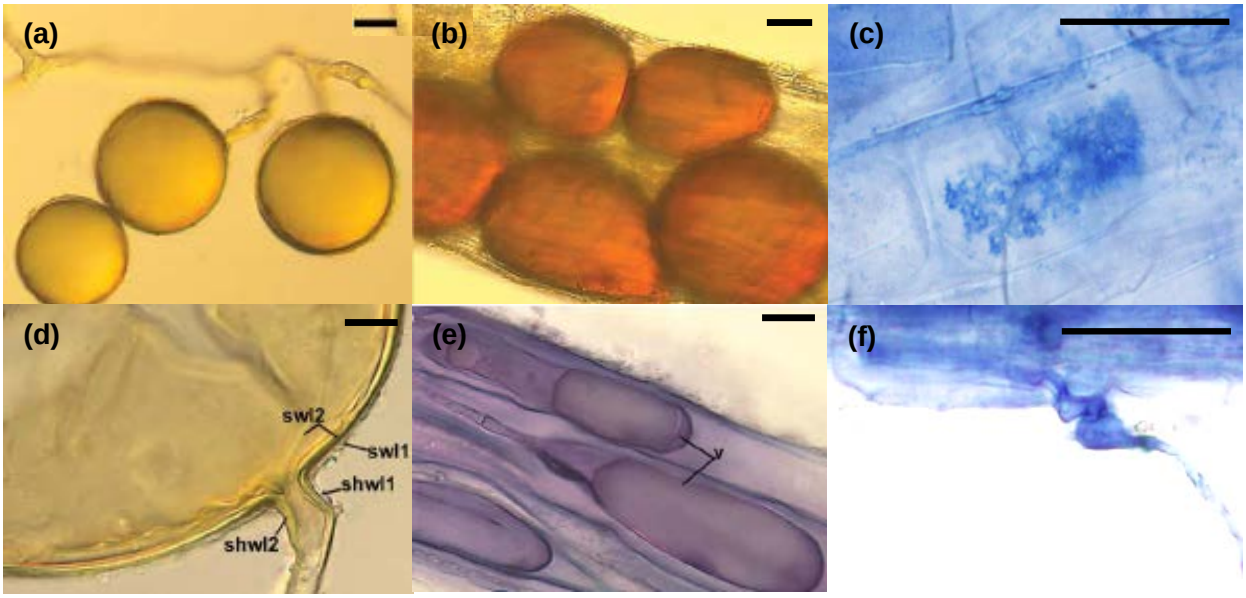


Fig. 1.4 Characteristic structures found in the AM symbiosis. (a) Spores of *Rhizophagus arabicus*. (b) Spores of *R. arabicus* in roots of *Allium porrum*. (c) Arbuscule of *Rhizophagus irregularis* in roots of *A. porrum*. (d) Spore of *R. arabicus* with spore wall layers (swl) and subtending hyphal wall layers (shwl). (e) Vesicles (v) of *R. arabicus* in roots of *Allium porrum*. (f) Appressoria of *R. irregularis* in roots of *A. porrum*. Scale bars: a,b = 20µm; c = 50µm; d,e = 10µm; f = 100µm.

## 1.3 Classification of AM fungi

### 1.3.1 Classification and Phylogeny

Before the Glomeromycota were placed into their own monophyletic phylum, they had been classified within the Endogonaceae of the Zygomycota as they share some common characteristics regarding their spores and hyphae. The observation of differences in spore characteristics between AM fungal spores and zygospores together with the recognition of the obligate symbiotic lifecycle of AM fungi, a new order, the Glomales, were separated from the Endogonales (Smith & Read, 2008). Finally Schüssler et al. (2001) reclassified the AM fungi on the basis of phylogenetic analyses, using the small ribosomal subunit (SSU) of ribosomal RNA genes including also higher fungal taxa, into a separate fungal phylum, the Glomeromycota. In the resulting phylogenetic tree the Glomeromycota are a sister clade to the Basidio- and Ascomycota (Fig. 1.5). In contrast, molecular analyzes of protein-encoding genes (alpha- and beta-tubulin, RNA polymerase II subunits rpb1 and rpb2) place the Glomeromycota next to Mortierella as closest neighbour (Redecker & Raab, 2006).

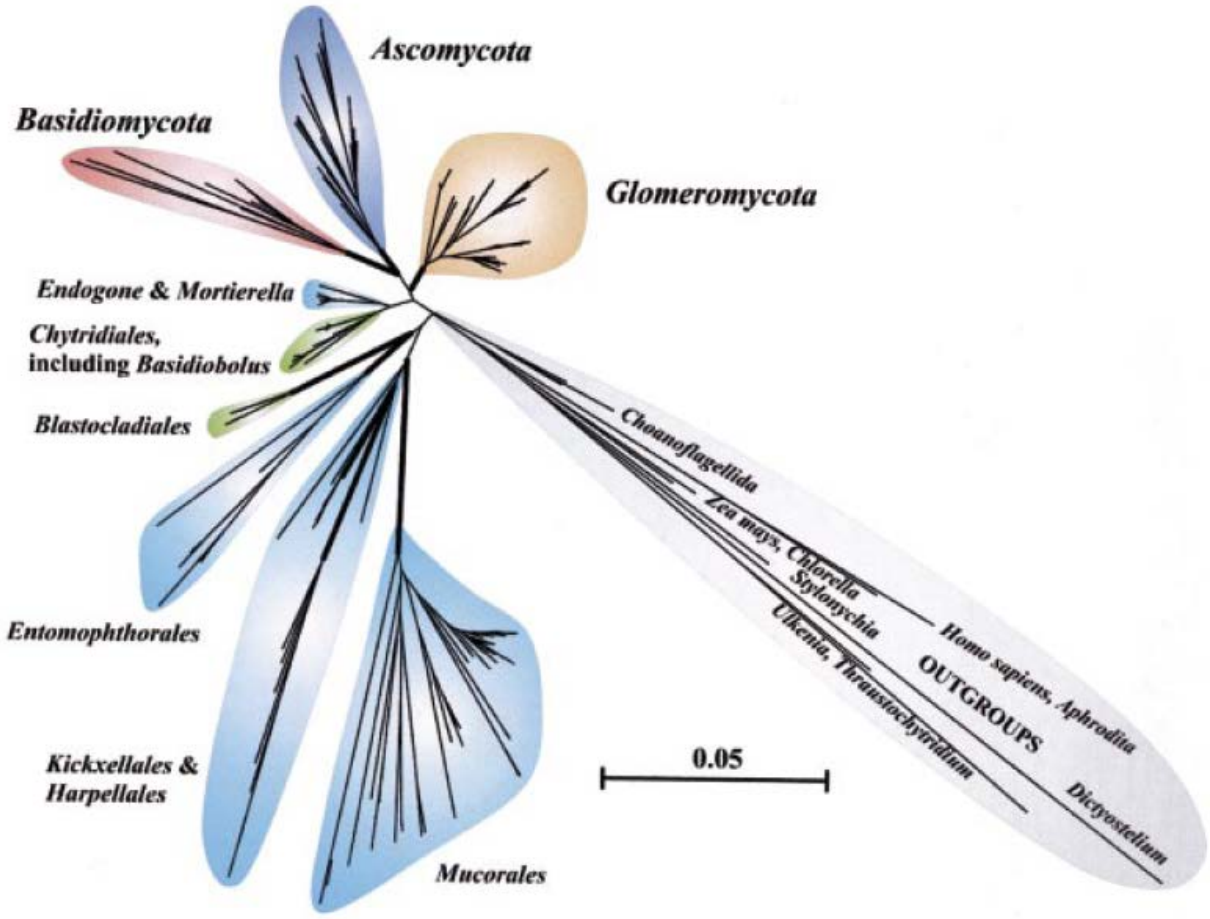


Fig 1.5 Phylogeny of fungi based on SSU rRNA sequences. Thick lines delineate clades supported by bootstrap values above 90%. The *Zygomycota* and *Chytridiomycota* do not form monophyletic clades and therefore are shown as the respective taxa representing the clade. From Schüssler *et al*. (2001).

Recently, Redecker et al. (2013) published an evidence-based consensus for the classification of Glomeromycota (Fig. 1.6) to provide a stable and robust systematics of the Glomeromycota which was often under discussion during the last decade. The phylum Glomeromycota (class: Glomeromycetes) includes the four orders Glomerales, Diversisporales, Paraglomerales, and Archaeosporales. Further, the four orders are divided into 11 families, 25 genera and so far 252 species. A complete species list can be found on the web page of the Schüssler group (http://schuessler.userweb.mwn.de/amphylo/).

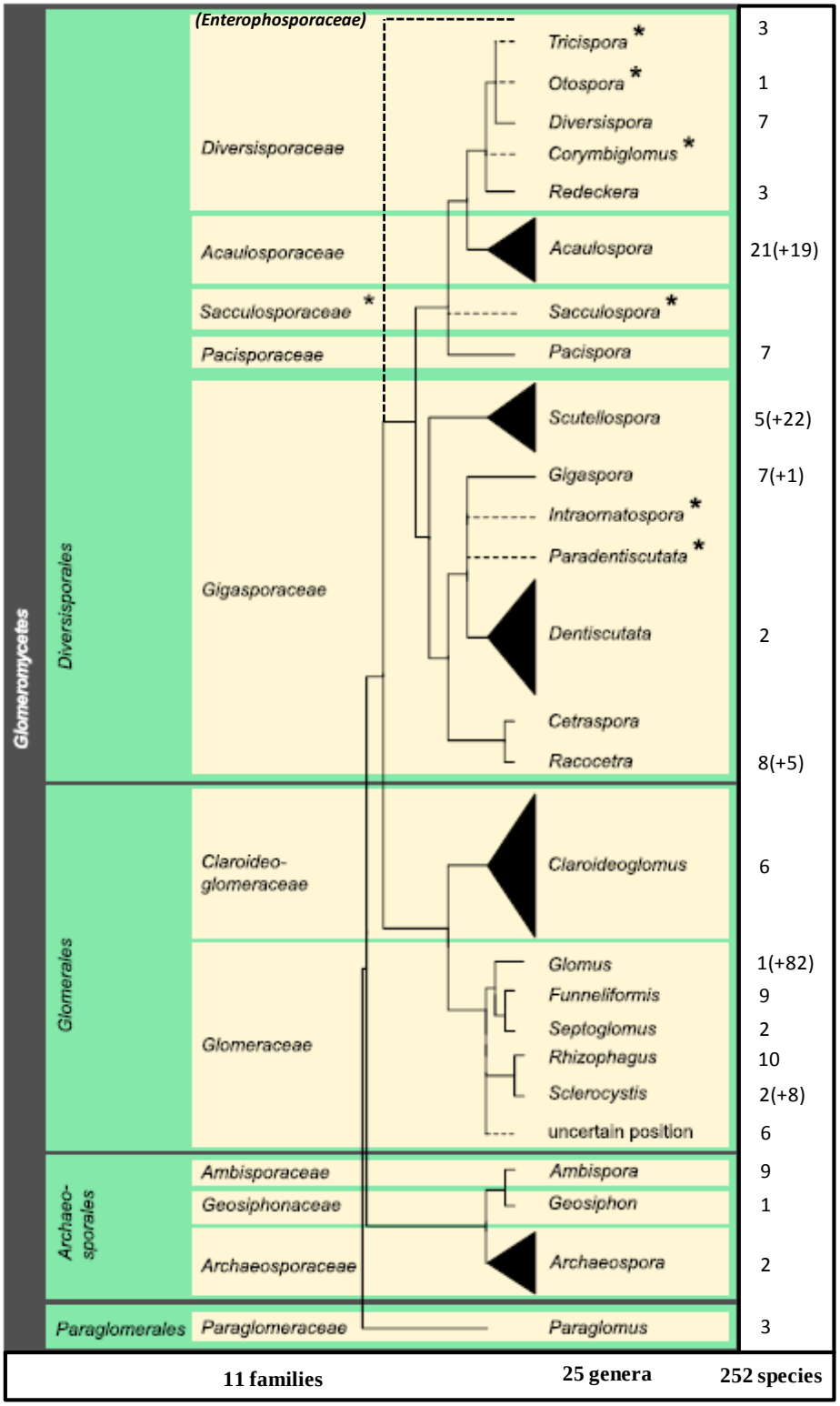


Fig. 1.6 Consensus classification of the Glomeromycota. *Dashed lines* indicate genera of uncertain position, *asterisks* indicate insufficient evidence, numbers in the last panel indicate the quantity of AM fungal species within each genera. *Enterophosporaceae* currently unclassified. Modified from Redecker *et al.* (2013).

### 1.3.2 Identification of AM fungi

Traditionally, the taxonomy of AM fungi has been based on spore morphology whereby families and genera were distinguished mainly by considering modes of spore formation, and species on the basis of spore color, size and subcellular structures, in particular phenotypic and histochemical characteristics of spore wall components (Blaszkowski, 2012). The international Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) provides comprehensive information on classification, nomenclature, species description and diagnosis (http://invam.wvu.edu/the-fungi). However, characterization of AM fungal spores might be challenging when spores are degraded, showing a dimorphic character or if they are in different developmental stages. To overcome the limitations of morphological identification, molecular methods were developed for species characterization.

The application of DNA-based molecular methods turned out to be best suitable to study genetic diversity among and within AM fungal species. Consequently, these techniques were continuously improved. Sequences of the nuclear-encoded ribosomal DNA (rDNA) are widely used in taxonomy and molecular phylogeny. This multicopy rDNA region includes three genes coding for structural RNAs (small subunit (SSU), 5.8S and large subunit (LSU)) which are separated by internal transcribed spacers (ITS) (Fig. 1.7). The SSU and LSU genes are conserved regions and show only little variation among closely related species. Therefore, they can only be used to reveal distant relationships on the species and genus level (Helgason *et al.*, 1999; Wu *et al.*, 2007). Incorporation of the ITS region allows robust phylogenetic analyzes and species level resolution due to its high degree of polymorphism (Gamper *et al.*, 2009). Variation of the ITS region is even present within single spores (Sanders *et al.*, 1995) (section 1.2.2). It was suggested that the high intraspecific variability derived from the long asexual evolution history of the Glomeromycota which led to significant genetic diversity within single species (Stockinger *et al.*, 2009). Another reason for the high degree of polymorphism is the low evolutionary pressure on the ITS region as it is spliced during rRNA maturation. However, the secondary structure of the ITS region and signals within its sequence are highly important for processing the rRNA transcripts, implying some degree of conservation, making it a good candidate as DNA marker (Hillis & Dixon, 1991).

Recently, Krüger et al. (2009) designed AM fungal-specific PCR primers amplifying a SSU-ITS-LSU fragment that allows phylogenetic analyses with species level resolution (Fig. 1.7). Thus, these primers are suitable to monitor entire AM fungal field communities, based on a single rDNA marker region.

Currently, the most applied and trustful method for AM fungal species description is the combination of DNA-based phylogenetic methods together with morphological observations (Redecker *et al.*, 2013).


Fig 1.7 Small subunit (SSU) rDNA, internal transcribed spacer (ITS) region and large subunit (LSU) rDNA with the binding sites of the newly designed forward (SSUmAf and SSUmCf) and reverse (LSUmAr and LSUmBr) primer mixtures are illustrated. From Krüger *et al*. (2009).

### 1.3.3 "Species concept" in the Glomeromycota

The "biological species concept" defines a biological species as a group of individuals which can breed together and produce fertile offspring. Regarding the Glomeromycota, this concept is not applicable because of their asexual lifecycle (despite their ability of anastomoses formation and the resulting exchange of nuclei). Therefore the species concept of AM fungi is based on morphological (morphospecies)and molecular investigation. The low number of AM fungal species (around 252) might imply a low diversity in the Glomeromycota, being a species-poor group. However, the presence of numerous sequences of AM fungi from environmental studies, which cannot be assigned to known species, suggests a so far underestimated AM fungal species richness (Opik *et al.*, 2006).

Within the last decade, the number of molecular investigations stuying the distribution and diversity of AM fungi in natural ecosystems steadily increased. Molecularly identified AM fungal species were referred to as phylotype, phylogroup or operational taxonomic unit (OTU), independently within each case study. The lack of a commen nomenclature for molecularly identified AM fungi complicated analyses on the molecular diversity of the Glomeromycota. Recently, Öpik *et al*. (2009) have established a new database, MaarjAM (http://maarjam.botany.ut.ee), in which all publicly available Glomeromycota DNA sequence data (SSU rRNA gene sequences) and associated metadata were summarized. In the MaarjAM database, molecularly different AM fungi are differentiated as virtual taxa (VT) on a common basis to facilitate the description of distribution and richness patterns withinthe Glomeromycota.

## 1.4 Functional aspects of the AM symbiosis

It is generally accepted that the AM symbiosis involves both benefits and costs to the plant. The plant delivers photosynthetic products to the fungal partner representing the cost of the symbiosis for the plant

(Douds *et al.*, 2000). In return the AM fungus provides several functions benefiting the plant partner. Under conditions where the carbon source is limited as under low light intensities, in which the costs for the plant exceed the benefits gained by the symbiosis, the maintenance of the AM fungus becomes a burden and the association tends towards parasitism. However, the plant is continuously exposed to different environmental stresses which are in many cases alleviated by the AM symbiosis and therefore result in an overall benefit for the plant.

Natural and agricultural soils are often affected by mineral deficiency. Particularly, this accounts for phosphorous (P), which is an essential element classified as a macronutrient because of the relatively large amounts required by plants. Hence, the plants are not able to cover their demand required for optimal growth and therefore, P deficiency is one of the most common abiotic stresses experienced by plants. Besides P, other mineral nutrients such as zinc [Zn] and iron [Fe] are often present in only low concentrations, are poorly mobile and mainly they are not directly available for the plant root due to several factors such as soil moisture, pH, fixation or microbial activity (Marschner, 1995). AM hyphae associated with the plant root expand the root absorption zone by several millimeters, to as much as 10 cm whereby a larger soil volume can be exploited for mineral nutrients (Smith & Read, 2008). This is achieved on the one hand by the smaller diameter of the AM hyphae (averaging 3-4 µm), which can penetrate soil pores and contact soil particles that are inaccessible to the host roots and on the other hand they extend into the soil further away from the root zone. Consequently, mycorrhized plants possess increased mineral uptake with enhanced concentrations in tissue nutrients such as P, Cu and Zn (Smith & Read, 2008). This mycorrhizal growth response, often referred as the 'big and little plant effect' has been shown for a wide range of plant species. Recently, the potential of the AM symbiosis in plant nitrogen (N) nutrition was recognized to play an important role (Hawkins *et al.*, 2000; Hodge *et al.*, 2001). Jin *et al*. (2005) provided a model where the uptake, metabolism, transport and transfer of labeled $^{15}$N-ammonium in the AM symbiosis were demonstrated. N is also a macronutrient as it is required in large amounts for plant growth. Besides the organic N pool in the soil, inorganic N occurs either as poorly available ammonium or nitrate, which becomes highly immobile when soil dries. To get access to this low available N source, hyphal N uptake similarly as for P uptake becomes more important and illustrates the potential of the AM symbiosis in N nutrition of the host plant.

Besides the stress of mineral deficiency, the AM symbiosis was shown to alleviate a broad range of environmental stresses such as biotic stresses (Pozo *et al.*, 2010), heavy metal stress (Hildebrandt *et al.*, 2007), salt stress (Evelin *et al.*, 2009), soil compaction stress (Yano *et al.*, 1998) and temperature stress (Bunn *et al.*, 2009). Furthermore, several studies have demonstrated an increased drought tolerance of mycorrhizal plants (for more details see chapter 1.6.2).

Generally it is assumed that the AM symbiosis leads to an increased growth and improved nutrition of their host plant, explaining the success of this symbiosis and their wide distribution, making it the most abundant symbiosis in terrestrial ecosystems.

However, such well-established and successful systems can be exploited by "cheaters". The most obvious case for this are the mycoheterotrophic plants. Achlorophyllous plants establish a mycorrhizal association and receive the required carbon from their fungal partners, which are further associated with neighboring autotrophic plants. The mycoheterotrophic mycorrhiza represents the most extreme example of parasitism among all mycorrhizal associations. A continuum from parasitism to mutualism exists in AM associations and the outcome of a certain association is strongly influenced by the identity of the symbiotic partners and the environmental conditions (Jones & Smith, 2004). Klironomos (2003) illustrated the importance of fungal origin on plant growth performance for a wide range of AM fungal species and plant species ranging from highly mutualistic to parasitic.

## 1.5 AM fungi and ecosystem functioning

### 1.5.1 Importance of AM fungal diversity and co-occurrence of AM fungi

The root system of a single host plant can be colonized simultaneously with several different AM fungal species (Vandenkoornhuyse *et al.*, 2002; Rosendahl & Stukenbrock, 2004). The reason why these species can coexist in the roots of their host plant was described as functional diversity or functional complementarity of AM fungi (Koide, 2000; Fitter, 2005). The term functional complementarity comes from the field of community ecology and describes the phenomenon that co-occurring organisms have to use different resources or perform different ecological functions to be able to coexist. As already mentioned in the previous sections, the AM symbiosis is multifunctional in terms of improved nutrient uptake and enhanced tolerance to different biotic and abiotic stresses. However, these functions can be incompatible e.g. an AM fungus good in acquiring P by an extended extra-radical mycelium growing remote from the root cannot be good in attaching the roots to the soil where an extensive extra-radical mycelium in the rhizosphere is needed (Fig. 1.8) (Fitter, 2005; Finlay, 2008). Hart *et al*. (2002) have suggested that functional traits as the production of intraradical versus extraradical mycelium may be phylogenetically conserved. The authors have shown that AM fungal species from the Gigasporaceae invest more into the extraradical mycelium whereas species from the Glomeraceae invest more in the production of hyphae inside the root. The study of Maherali *et al*. (2007) further outlined that these conserved functional traits correlate with different functional roles of the different AM fungal taxa: the

pronounced intraradical mycelium of the Glomeraceae correlated with enhanced pathogen protection whereby the extensive extraradical mycelium of the Gigasporaceae correlated with increased shoot P concentrations of the host plant. Furthermore it was shown that dual inoculation with AM fungal species from both families best promoted plant growth. In contrast to the previously mentioned studies in which interspecific differences in functional traits were demonstrated, Munkvold *et al*. (2004) observed also high intraspecific variations in mycelium growth and improvement of P uptake when cucumber was inoculated with 24 different isolated of AM fungi. The authors stated that even AM fungal communities of low species diversity may still contain intraspecific AM fungal variants with considerable functional diversity.

Functional complementarity in the AM symbiosis by exhibiting different functions for the host plant was demonstrated for a wide range of AM fungal species (Smith *et al.*, 2004; Facelli *et al.*, 2010). Hence, this implicitly explains the importance of AM fungal diversity for host plant performance, especially by buffering the system against diverse stresses.

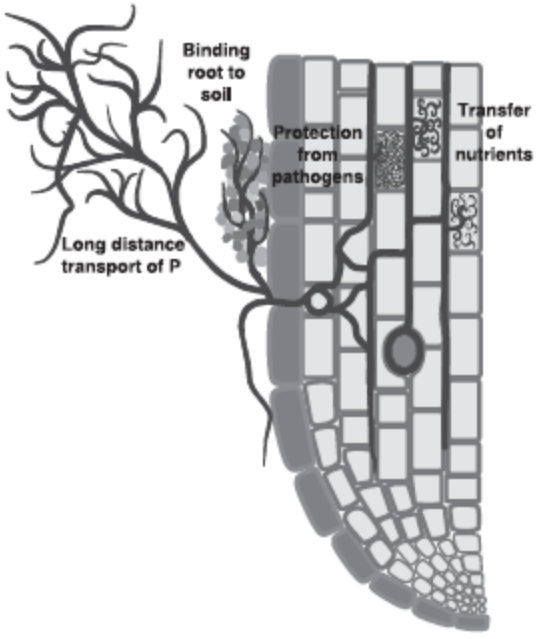


Fig. 1.8 Diagram to illustrate the incompatible morphological requirements of different mycorrhizal functions: P transport necessitates extensive development of the extra-radical mycelium remote from the root (beyond the phosphate depletion zone); improved water relations is based on maintaining the root– soil bond and hence water pathway as soil dries, and involves extra-radical mycelial development in the rhizosphere; protection from pathogens and P transfer both depend on the intra-radical mycelium, but the former involves the hyphae, the latter the arbuscules. Figure from Fitter (2005).

### 1.5.2 AM fungal community composition

As previously mentioned, the arbuscular mycorrhiza is the most widespread symbiosis in terrestrial ecosystems. However, it was shown that AM fungal communities vary among broadly defined habitat types and further, that differences in AM fungal communities occur between different continents and climatic zones (Opik *et al.*, 2006, 2013). Several studies have revealed the importance of environmental factors in shaping AM fungal communities. Klironomos *et al*. (2001) observed seasonal differences in the occurrence ofspores of different AM fungal taxa in a Canadian old-field meadow where soil freezes during winter and droughts prevail during summer. *Scutellospora* spp. were mainly present in summer and rarely found in winter, whereas the opposite was observed for *Glomus* spp.. In contrast, Egerton-Warburton *et al.* (2007) and Querejeta *et al.* (2009) reported about *Glomus* dominance within AM fungal communities in soils of xeric sites, whereas *Scutellospora* spp., *Gigaspora* spp. and *Acaulospora* spp. predominated mesic sites. The mechanisms behind such observed differences are difficult to interpret and are most likely the result of a combination of several factors. Other factors including host plant identity and plant community composition (Bever *et al.*, 1996; Helgason *et al.*, 1998; Öpik *et al.*, 2003), soil type (Landis *et al.*, 2004; Lekberg *et al.*, 2007) and soil management practices (Jansa *et al.*, 2003; Oehl *et al.*, 2004; Al-Yahya'ei *et al.*, 2011) were also shown to influence AM fungal community composition.

Furthermore, Klironomos *et al*. (2001) suggested that seasonal variations as observed in the old-field meadow can be explained by differing tolerances to freezing and drought among AM fungal species. Such tolerances might have resulted from adaptation of AM fungal species to certain environmental conditions. Adaptations of AM fungi to temperature or water availability were demonstrated by assessing colonization abilities, mycelia production or plant growth performance after inoculation with two AM fungal strains from contrasting climates. Antunes et al. (2011) observed that growth promotion was best when the experimental conditions (in this case temperature) were closest to the environmental conditions, from where the AM fungus was isolated from. Similarly, Marulanda *et al*. (2007) and Lekberg&Koide (2008) demonstrated adaptations to water availability of contrasting AM fungal strains.

Despite the fact of adaptation, some AM fungal species appear to have worldwide distributions, while others have been recorded so far from only a single site (Opik *et al.*, 2006). Thus, it was predicted that within the Glomeromycota, AM fungal species might be either generalists or specialist (Oehl *et al.*, 2003). Also Lekberg *et al*. (2007) suggested that AM fungal species differ in niche breadth as *G. intraradices* occurred in almost all sampling sites independent of soil type whereas *G. mosseae* was restricted to clay soils. Similarly, the predominant occurrence of *G. mosseae* in finer textured soils was observed in other studies (Johnson *et al.*, 1992; Landis *et al.*, 2004) underlining the conclusion of specialization respectively restriction within the Glomeromycota.

Another aspect in terms of community composition is competition. As mentioned previously, co-occurrence inside a single root system and further inside the soil is presumably based on functional complementarity among co-existing AM fungal species. Hence, competition may predominantly occur between AM fungi with similar functional characteristics. Engelmoer *et al*. (2013) observed that the abundance of *Glomus aggregatum* was reduced for approximately 35% when grown in competition with *R. irregularis* under different phosphorus availabilities, as revealed by molecular techniques analyzing the abundance of gene copy numbers of *G. aggregatum* inside the roots. This observation is in accordance with previous competition studies (Jansa *et al.*, 2008; Kiers *et al.*, 2011) and might be the result of similar colonization strategies of *Glomeracean* fungi which were shown to invest more into internal hyphae as mentioned previously. Maherali *et al*. (2007) gave evidence that phylogenetic relatedness which comprises functional relatedness influences community composition. By constructing experimental communities consisting of eight either closely related (within one family) or distinct (from three different families) AM fungal species, they found that species richness was highest in communities including AM fungal species from all three families. Therefore, phylogenetic relatedness displays another driver for AM fungal community composition.

In summary, AM fungal distribution and thus AM fungal community composition is the product of environmental conditions and interspecific competition among AM fungal species creating distinct AM fungal communities adapted to particular ecosystems.

Generally, it is important to distinguish between the “active” community and “passive” community of AM fungi. The “passive” community can be analyzed based on AM fungal spore abundance in the soil while the “active” community can be detected based on RNA extracts from plant roots. An AM fungus present in the soil as resting spore does not necessarily colonize a host root at a given time. When conducting AM fungal community surveys, these factors need to be taken into account.

### 1.5.3 Invasion in AM fungal communities

Nowadays, the use of commercial AM inoculants is growing and certain AM fungal species (mainly *R. irregularis*) are traded globally and used in agriculture and revegetation programs (Gianinazzi-Pearson, 2002) even if they are not native in the applied soil. How the introduced AM fungal species would alter the existing native AM fungal community is still poorly understood as only few greenhouse studies have addressed this question. Koch *et al*. (2010) studied the impact of inoculation with two stains of *R. irregularis* on the structure of a native Canadian AM fungal community inhabiting *Sorghum bicolor* roots. They detected a dramatic decrease in the diversity of the native AM fungal community after

inoculation. Similarly, Mummey *et al*. (2009) found that pre-inoculation with two *Glomus* species reduced the AM fungal richness of another native AM fungal community in roots of *Leucanthemum vulgare* compared to uninoculated controls after exposure to the field soil. However, pre-inoculation with *Gigasporaceae* species had no impact on the AM fungal community richness. The authors explained their observations by differences in colonization strategies typically observed between the *Gigasporaceae* and the *Glomeraceae* (as mentioned in the previous section), as the field soil used in their study was dominated by *Glomeraceae* species. Therefore, competition between the native *Glomeraceae* species which were shown to invest more into the intraradical mycelium and the inoculated *Gigasporaceae* which invest more into the external mycelia, is less pronounced as for AM fungal -inoculants of the same taxon. Also, Antunes *et al*. (2008) observed no effect on the native AM fungal community of an agricultural soil colonizing maize roots after inoculation with *R. irregularis*. Long-term studies and AM fungal community surveys of field sites, where AM fungal inoculants have been applied in the past, might resolve the question about the impacts of commercial inocula application on native AM fungal communities.

## 1.6 AM symbiosis in arid environments such as desert ecosystems

### 1.6.1 Characteristics of desert ecosystems

Desert ecosystems are affected by several environmental extremes. Obviously, the most prominent features in desert ecosystems is drought, with low annual rainfall where precipitation is often less that 250mm/year. In addition, there are large seasonal and diurnal temperature contrasts. Consequently, the soil in the deserts possesses some particular characteristics challenging agriculture. The light-structured, sandy soils are mostly of infertile nature and prone to erosion. Due to the impact of evapotranspiration under drought, the soil accumulates salts in the upper layers and soil salinity is the result. A further consequence of drought is the high P fixation and the general inaccessibility of nutrients which normally reach the root by mass flow (Shen *et al.*, 2011).

### 1.6.2 Multifunctional role of AM fungi on plant drought tolerance

Knowledge on the multifunctional role of AM fungi on drought stress tolerance of plants steadily increased in the last decades. Several studies found altered rates of water movement into, through and out of host plants compared to uninoculated controls with consequent effects on tissue hydration and

physiology (as reviewed by Augé, 2001). It is generally assumed that the alleviation of drought stress by the AM symbiosis is the result of a combination of physical, nutritional, physiological and cellular effects.

Mycorrhizal plants were shown to maintain higher rates of gas exchange during soil drying compared to non-mycorrhizal plants of similar size and nutrient status (Ruiz-Lozano *et al.*, 1995; Duan *et al.*, 1996) and to perpetuate transpiration at lower leaf water potentials (Ruiz-Lozano *et al.*, 2006). Mycorrhizal *Rosmarinus officinalis* in association with *Glomus deserticola* showed a lower decrease in leaf and stem water potential as well as improved photosynthetic activity and stomatal conductance under drought stress condition compared to non-mycorrhizal controls (Sánchez-Blanco *et al.*, 2004). Additionally, mycorrhizal *R. officinalis* showed a more pronounced decrease in osmotic potential at full turgor, indicating the capacity of osmotic adjustment. Several studies have attributed the enhanced capacity of osmotic adjustment in AM symbiosis by detecting decreases in osmotic potentials or increases in proline accumulation in mycorrhizal plants (Kubikova *et al.*, 2001; Khalvati *et al.*, 2005; Ruiz-Lozano *et al.*, 2006). Further, the AM symbiosis was shown to protect plants against oxidative damage generated by drought (Fusconi & Berta, 2012).

The improved drought tolerance of mycorrhizal plants is further explained by enhanced water uptake through fungal hyphae. As mentioned ealier, the diameter of AM hyphae is a magnitude smaller as the diameter of root hairs and therefore, hyphae can penetrate soil pores which are not accessible to the roots and hence, absorb water which is not available for the plant. However, whether the absorbed water directly contributes to the improved drought tolerance of the plant is conversely discussed, as estimations on the amount of water delivered to the plant by hyphae were ambiguous (Smith *et al.*, 2009). Nevertheless, several studies investigating the role of the external mycelium of AM fungi demonstrated a significant contribution of the mycelium to the improved drought tolerance of their host plant (Ruiz-Lozano, 2003; Augé *et al.*, 2007; Neumann *et al.*, 2009). It involves mainly the uptake of nutrients from dry soil, especially those which are diffusion-limited. This is achieved, on the one hand, since hyphae increase the nutrient absorbing surface area available for the roots allowing access to a bigger soil volume and, on the other hand, they can exploit solution-filled soil pores which are not accessible by plant roots. Thus, hyphae can reach secluded nutrients not available for plants.

Another important aspect of the AM symbiosis are changes in soil water retention properties. It has been suggested that AM hyphae improve soil structure by their physical, chemical and biological actions. The formation of stable soil-aggregates is increased and consequently, the water infiltration of the soil is improved. Hence, soil moisture characteristics of colonized soils by AM hyphae are ameliorated (as reviewed by Fusconi and Berta, 2012). Mycorrhization by *R.irregularis*, for example, altered the characteristic soil moisture curve relative to non-mycorrhizal soils whereas the mycorrhizal soil had to

dry out more to reach the same soil water potential as the non-mycorrhiazl soil (Augé, 2001). Additionally, erosion of soils is alleviated due to the formation of stable soil-aggregates.

### 1.6.3 Impact of drought on AM fungi

Only few studies have focused on the impact of drought on the fungal partner in terms of percentage root colonization, hyphal production or survival; they found a wide range of possible outcomes. While in some studies root colonization decreased upon drought treatment, other studies found even an increased abundance inside the host root, depending on the identity of the AM fungus (Augé 2001). Similar results were obtained by investigating the production of extraradical hyphae. Differences might result from adaptations of AM fungi towards environmental conditions prevailing at their place of origin (see chapter 1.5.2). Another reason might be that AM fungi display differences in structural traits. AM fungal species with an extensive external mycelium might be less susceptible to drought due to enhanced access to soil water.

### 1.6.4 Application of AM fungi as biotechnological tool

The application of AM fungal inocula in horticulture, agriculture and revegetation programs became more prominent within the last decades as the number of studies demonstrating improved plant growth after inoculation has steadily increased (Requena *et al.*, 2001). Inoculation with *Claroideoglomus claroideum* or a mixture of native AM fungi improved growth of four shrub species compared to uninoculated controls, whereas the latter improved growth to a greater extent (Caravaca *et al.*, 2003). Also Alguacil et *al*. (2011) observed improved growth response after inoculation with native *R. irregularis* and *Glomus* sp. or a mix of both. Especially in arid and semiarid ecosystems, the use of AM fungal inocula is of particular advantage due to additional challenges the plants have to face in these climates as summarized in chapter 1.6.1. Furthermore, it was shown that the mycorrhizal potential in those soils is comparatively low (Requena *et al.*, 1996) for which reason the additional application of AM fungi may guarantee an improved establishment and functionality of the symbiosis. Particularly, the application of AM fungal inocula in horticulture which involves growing plants in the presence of AM fungi prior to planting them in the field is especially beneficial since the plants possess an already established AM symbiosis and colonization of the soil by fungal hyphae can start immediately after transplantation into the field. In addition, AM hyphae were shown to be important infection structures in arid and semi-arid ecosystems

(Requena *et al.*, 1996; Azcón-Aguilar *et al.*, 2003). Therefore, the nursery-application of AM fungi can increase the infectivity of soils in such habitats (Requena *et al.*, 2001).
Recently, Ceballos *et al*. (2013) demonstrated that the use of P fertilizers for cassava cultivation can be reduced by 50% by the application of AM fungal inocula, and that yields were even higher compared to non-inoculated cassava which received 100% of P fertilizers. Another important factor in terms of agricultural application of AM fungal inocula, is the ability of AM fungi to promote plant resilience to stress conditions (as summarized in chapter 1.5.1). In the view of climate change with predicted environmental alterations, AM fungi may serve as an additive to enhance crops' adaptation to altered environmental conditions and as a buffer against stress conditions that might develop as a result of global change.

Concerns regarding the effect of the application of AM fungal inocula on native AM fungal communities have been discussed in chapter 1.5.2. Mummey *et al*. (2009) have shown that the application of adequate AM fungal inoculants did not disturb the native AM fungal community, which suggests that it may be possible to select specific AM fungal species which do not decrease the local AM fungal diversity.

## 1.7 Significance of plant aquaporins: Role and regulation

### 1.7.1 Description

Root water uptake from the soil and its distribution within the plant is important for all physiological processes. In roots, water transport occurs via three major pathways: the apoplastic path around the protoplasts which facilitates the transport of water across tissues or organs; the symplastic path through the plasmodesmata where the water flows directly from cytoplasm to cytoplasm following a concentration gradient; and the transcellular path across the cell membranes (Steudle, 2000). After water is taken up from the soil, it passes the root cortex mainly via the apoplastic pathway. However, in the endodermis, the water is forced to pass the endodermal cells by the transcellular pathway due to the Casparian strip. As "gate keeper" cell layer, the endodermis functions as important regulatory organ where the plant is able to control the movement of water. Here, water movement occurs by a gradient-driven flow through membranes, a process which is mediated and regulated by aquaporins (AQPs) (Luu & Maurel, 2005).
AQPs are a family of small pore-forming integral membrane proteins. They have a well-conserved structure with six membrane-spanning α-helices which are connected by five loops (A-E) and an N- and C-terminus facing the cytosol (Murata *et al.*, 2000). The pore is formed by the interaction of the transmembrane helices and the two loops B and E which both carry a conserved asparagine-proline-

alanine (NPA) motif and dip from either side of the membrane into the center of the pore. The high selectivity of AQPs mainly results from the NPA motifs located in the center of the pore and an aromatic/arginine (Ar/R) formed by four amino acids which together form two major filter regions. A first size exclusion zone is provided by the NPA motifs and a second exclusion barrier derives from the Ar/R. Additional specificity is achieved by specific interactions of the substrate within the pore mediated by spatially defined H-bonding and hydrophobic interactions (Fig. 1.9). In plants they form a large family with 35 members in *Arabidopsis thaliana* (Johanson *et al.*, 2001) 33 members in *Zea mays* (Chaumont *et al.*, 2001) and *Oryza sativa* (Sakurai *et al.*, 2005) and 55 in *Populus trichocarpa* (Cohen *et al.*, 2013). Based on amino-acid sequence comparison, AQPs of most plant species can be divided into five subfamilies which are associated to their specific membrane localization: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs) first identified in the symbiosomes of legumes, but also present in the plasma membrane and endoplasmic reticulum (ER), small basic intrinsic proteins (SIPs) found in the ER and only in dicots, uncharacterized intrinsic proteins (XIPs) localized in the plasma membrane (Fig. 1.10) (Maurel *et al.*, 2008; Bienert & Chaumont, 2011; Lopez *et al.*, 2012). Among those groups, PIPs and TIPs are thought to be involved in the regulation of root water uptake under drought conditions (Ruiz-Lozano *et al.*, 2012). Beside their importance in plant water relations, AQPs have also been shown to facilitate the transport of small uncharged solutes as glycerol, ammonia, urea, boric acid, silicic acid carbon dioxide (Maurel *et al*., 2008). Thus, AQPs represent a large group of multifunctional transporters involved in whole plant water relations as well as plant metabolism, nutrition and signaling.

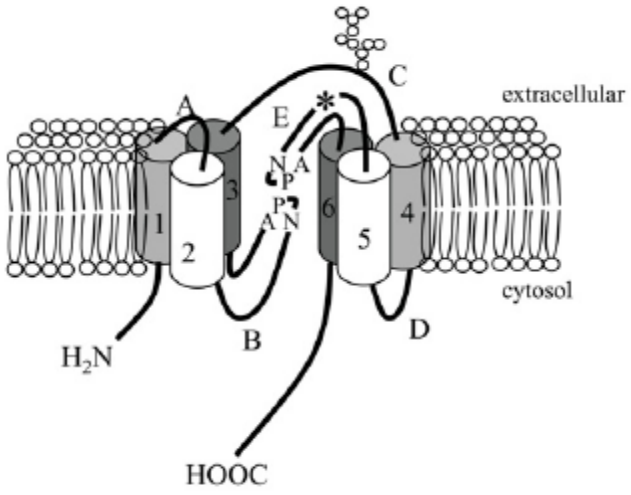


Fig. 1.9 The hourglass model. Six transmembrane domains (1-6) are connected by five loops (A-E) and forming the central pore. The N- and C-termini are located in the cytosol. Conserved NPA motifs interact with each other in the center of the pore. From Zardoya, 2005.

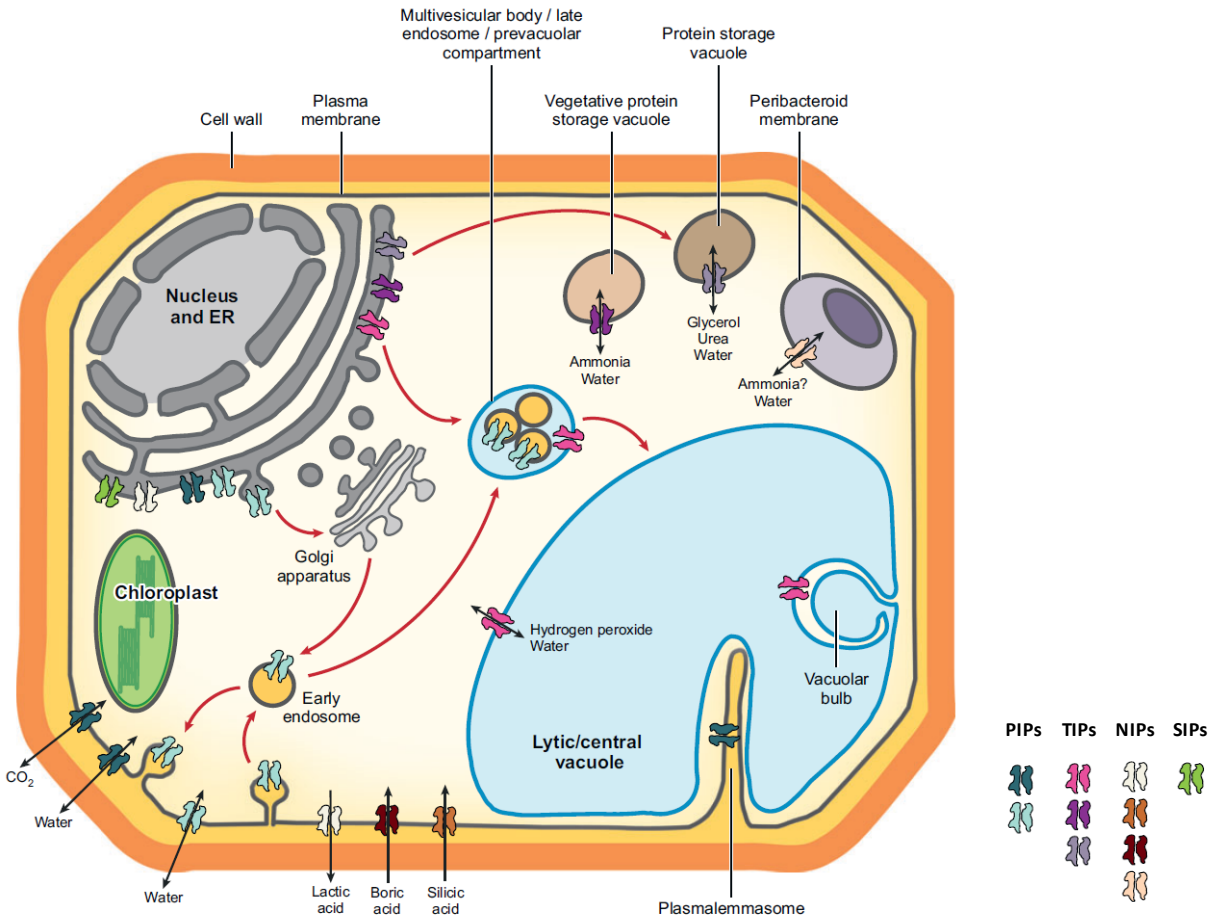


Fig. 1.10 Cellular localization of plant aquaporins (AQPs). The figure shows the subcellular localization of the different AQP subfamilies PIPs, TIPs, NIPs and SIPs and indicates the variety of their transport functions. Modified from Maurel *et al.*, 2008.

### 1.7.2 Aquaporin regulation upon mycorrhization

Since it has been demonstrated that the AM symbiosis regulates root hydraulic properties, including root hydraulic conductivity, it was hypothesized that the AM symbiosis might also be involved in the regulation of plant AQPs (Uehlein *et al.*, 2007; Ruiz-Lozano & Aroca, 2010). The first studies indicating the involvement of the AM symbiosis in the modulation of plant AQPs were performed by Roussel *et al.* (1997) and Krajinski *et al*. (2000), who found mycorrhiza-induced expression of TIPs in *Petroselinum crispum* (parsley) and *Medicado truncatula*, respectively. In ectomycorrhizal poplar plants, an increase in the water transport capacity of mycorrhizal roots was correlated with an upregulation of several PIP-encoding genes, giving further evidence for a mycorrhiza-based AQP regulation (Marjanović *et al.*,

2005). The importance of AQPs for the AM symbiosis was demonstrated in *NtAQP1* antisense tobacco mutants grown under drought conditions. While root colonization was unaffected, mycorrhizal plants had a reduced root and shoot fresh weight compared to wildtype plants, indicating the importance of AQPs for the efficiency of the AM symbiosis under drought conditions (Porcel *et al.*, 2005). However, the mechanisms behind those observations are still poorly understood.

Studies investigating the regulation of plant AQPs as affected by mycorrhization and drought stress gave controversial results. While in some cases, AM symbiosis led to an increased expression of AQP genes, other studies reported about downregulation. Opinions about the observed differences in AQP regulation patterns are still conflicting. One hypothesis supports the idea of facilitated water transport with increased membrane water permeability, which require up-regulation of AQPs under drought stress (Jang *et al.*, 2004; Yu *et al.*, 2005). Transgenic tobacco mutants, over-expressing *BnPIP1*, exhibited increased tolerance to water stress, while reduced water uptake and decreased tolerance to water stress was observed in *BnPIP1*-antisense mutants (Yu *et al.*, 2005). The second hypothesis is based on the idea of cellular water conservation which implies down-regulation of AQP genes to prevent water loss from the cell (Smart *et al.*, 2001; Aharon *et al.*, 2003). Transgenic tobacco plants over-expressing *AtPIP1b* wilted faster during water stress compared to wild-type tobacco plants (Aharon *et al.*, 2003).

In any case, particular AQP regulation patterns detected in colonized roots were related to an overall improvement of plants' drought tolerance as reflected by improved growth and water status of mycorrhizal plants (Ruiz-Lozano *et al.*, 2012). It was further suggested that the role of AQPs in the AM symbiosis might be more complex and not only involved in the regulation of plant water status. The AM-mediated induction of specific PIPs and NIPs, which were shown to transport water and ammonia, respectively, might be involved in symbiotic exchange processes between the fungus and the plant (Uehlein *et al.*, 2007). Recently, it was recognized that *R. irregularis*-inoculated tomato and maize plants are able to switch between apoplastic and cell-to-cell (mediated by AQPs) water transport pathways, which implies a higher flexibility to changing water conditions and thus improving plant response to drought (Bárzana *et al.*, 2012).

## 1.8 Aims of the thesis

The first aim of my thesis was the isolation and propagation of AM fungal species originating from high water stress sites. The choice was a hyper arid desert occurring in Oman in Southern Arabia. Earlier investigations revealed the uniqueness of AM fungal communities occurring in these habitats (Al-Yahya'ei *et al.*, 2011). Within the theme of this thesis, several AM fungal strains were isolated from single-spore derived cultures and identified by morphological and molecular methods (chapter 2). In collaboration with Prof. J. Blaszkowski from the West Pomeranian University of Technology, Szczecinin Poland, three new species were so far described from the investigated habitats: *Diversispora omaniana*, *Septoglomus nakheelum* and *Rhizophagus arabicus* (Symanczik *et al.*, 2014a). In addition to the newly described AM fungal species, four other AM fungal species were discovered. They had been already described, but their occurrence in this Arabian Peninsula was reported for the first time (chapter 3; Symanczik et al., 2014b).

The second aim of my thesis was to investigate some functional aspects of the isolated AM fungal species. In the first experiment (chapter 4) we evaluated the interacting effects of water regime and the presence of a potentially invasive AM fungal species, *R. irrigularis*, on four AM fungal species representing an AM fungal native assemblage. For revealing the community structure of the AM fungi colonizing sorghum roots, I developed a real-time quantification approach to specifically target the different AM fungal species and to determine the transcript numbers of large ribosomal subunit (rLSU) genes of each individual AM fungus. Functionality of the AM symbiosis was considered in terms of plant growth and extraradical mycelium production. As mentioned in section 1.5.2, community composition is affected by environmental conditions as well as competition. The effect of different water regimes on community compositions was already assessed but never in terms of abundance of single AM fungal species colonizing a host root. Further, we investigated the effect of introduction of *R. irregularis* (as a model invasive species) on the same native assemblage under different water regimes.

In a further greenhouse experiment (chapter 5) we studied functional characteristics of *R. arabicus*, one of our newly discovered AM fungi, in comparison to *R. irregularis*, a well-studied and regularly used AM fungus under well-watered and drought conditions. We tested their ability to (i) promote drought tolerance of sorghum, (ii) produce extraradical hyphae close and apart from the root and (iii) their efficiency to extract nutrients from soil under the two water regimes. The AM fungi were selected on the basis of their close relatedness and their contrasting adaptations: *R. arabicus*, isolated from natural habitat of Oman and *R. irregulare* BEG 75, isolated from an agricultural soil in Switzerland. In additions, we identified and characterized AQP encoding genes in the fully-sequenced genome of sorghum and studied their regulation in mycorrhizal and non-mycorrhizal sorghum plants as an effect of water regime and the

association with the two AM fungal species (appendix). The transcript abundance of selected AQPs was measured by quantitative reverse-transcription PCR in roots of sorghum.

It has been shown that especially in arid and semiarid ecosystems, the use of AM fungal inocula is of particular advantage due to the extreme environmental conditions and the low mycorrhizal potential in those soils. For this reason, the additionisal application of AM fungi guarantees an improved colonization of the host plant with improved functionality of the AM symbiosis. Therefore, and in order to widen the scope of my PhD work to a more applicable aspects of science, the last part of my thesis are functional trials to evaluate the efficiency of the isolated AM fungi as inocula in Oman, their original habitat. The aim was to evaluate the efficiency of different AM fungi in promoting growth of Ghaf (*Prosopis cineraria*) and date palms (*Phoenix dactylifera*) seedlings, first under nursery conditions and later after transplantation into the field (chapter 6 and 7).

# 2 Three new species of arbuscular mycorrhizal fungi discovered at one location in a desert of Oman: *Diversispora omaniana, Septoglomus nakheelum* and *Rhizophagus arabicus*

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## 2.1 Abstract

Three new species of arbuscular mycorrhizal (AM) fungi - phylum Glomeromycota - were found in studies at one location in an arid sandy plain of South Arabia. Phylogenetic analyses of SSU-ITS-LSU rDNA sequences and, additionally, of ITS sequences exclusively showed that the three species belong to described genera in which they differ from the species known. Morphological characteristics of spores also clearly differentiate them from the morphologically and phylogenetically closely related described species. Consequently, we here describe them as *Diversispora omaniana*, *Septoglomus nakheelum* and *Rhizophagus arabicus* spp. nov. The discovery of three new species of AM fungi at one location in this area shows the potential to find a wide diversity and new specializations of AM fungal communities in such desert ecosystems. In addition, it may open new opportunities for the current endeavors of conservation, re-vegetation and sustainable agriculture.

## 2.2 Introduction

Arbuscular mycorrhizal (AM) fungi coexist in a symbiotic association with ca. 70-90% of land plants (Smith & Read, 2008). Traditionally, the taxonomy of these fungi has been based on morphology of spores. Families and genera were distinguished mainly by considering modes of spore formation, and species on the basis of spore color, size and subcellular structure, in particular phenotypic and histochemical characteristics of spore wall components (Walker, 1983; Blaszkowski, 2012; http://invam.caf.wvu.edu/). However, molecular phylogenetic analyses indicated that the mode of spore formation by, e.g. *Acaulospora laevis* Gerd. & Trappe (laterally on the neck of a sporiferous saccule) and the former *Entrophospora colombiana* Spain & N.C. Schenck (inside the neck) is not a genus-specific character (Kaonongbua *et al.*, 2010). Similarly species with typical glomoid spores (i.e. 1-walled spores arising identically to those of *Glomus macrocarpum* Tul & C. Tul., the type species of genus *Glomus* Tul. & C. Tul.), now are accommodated in twelve genera and constitute ca. 62% of all known species of Glomeromycota (Redecker *et al.*, 2013). The spore morphology of these species in most cases does not indicate unambiguously their generic affiliation. For example, *Pacispora* spp. also form glomoid spores, but they have a spore wall and an inner wall, called a germinal wall, that originates *de novo* after the full differentiation of the spore wall and has no physical contact with this wall (Oehl *et al.*, 2004; Walker *et al.*, 2004). Up to now, a clear assignment of the genus of a given spore is feasible only by using molecular phylogeny, based on specific sequences of the ribosomal DNA (rDNA).

Based on the morphological species concept, Glomeromycota is viewed as a species-poor group, with around 250 so far recognized species (www.AM fungal-phylogeny.com) from almost all main regions of the world (Blaszkowski, 2012; www.zor.zut.edu.pl/Glomeromycota/). Since such a small number of AM fungal species can associate with around 250 000 plant species, it seems to be self-evident that these fungi are not host specific. However, it is possible that some species are specialists and occur only in certain specific ecosystems and, thus only on the plants adapted to these ecosystems.With the advent of phylogenetic tools that allow identification of AM fungi colonizing plant roots independently from spore formation, some recent studies of certain ecosystems indeed identified DNA-sequence types previously unknown from culture collections, while others clearly represented widespread taxa that are found almost ubiquitously (see Öpik *et al.*, 2006 for review).

AM fungal community compositions seem to be shaped also by their habitats. Öpik et al. (2006) used 26 published studies on the molecular identification of AM fungi colonizing roots of different plants to compare the diversity and composition of AM fungal communities in different environments around the world. They found these communities to exhibit different compositions in broadly defined habitat types like tropical and temperate forests and habitats under anthropogenic influence. An unusual composition of

the AM fungal community may prevail at ecosystems under extremely stressful conditions, exemplified by the geothermal sites in Yellowstone National Park (USA) (Appoloni *et al.*, 2008). Sequences amplified from root samples also suggest that a vast majority of AM fungal species might still be undescribed (Fitter, 2005; Hijri *et al.*, 2006; Kovács *et al.*, 2007; Öpik *et al.*, 2009).

One of the areas of the world that has been neglected with respect to exploring AM fungi until recently is the Arabian Peninsula. Morphological and molecular analyses of the AM fungal communities in date palm plantations and the surrounding desert habitats in Southern Arabia revealed a high level of novelty (Al-Yahya'ei *et al.*, 2011). This novelty was attributed to the particularity of the studied ecosystem. Geological history and the influence of past climates of this area (Preusser *et al.*, 2002) have played a major role in shaping its unique environmental settings producing a simple and harsh ecosystem (Fisher & Membery, 1998; Glennie & Singhvi, 2002) that may be inhabited by unique forms of life.

Our goal was to complement our biodiversity explorations in Southern Arabia with the establishment of a Gene Bank specific for AM fungi from arid lands, a prerequisite for various planned projects of basic and applied research. We used morphological and molecular identification techniques to characterize the AM fungal species that we succeeded to isolate and propagate in a first round. This undertaking revealed spores of three undescribed morphotypes. Phylogenetic analysis of sequences of spore rDNA confirmed their uniqueness and placed them in three different genera of Glomeromycota sensu Redecker *et al.* (2013). These species are the first newly described AM fungi from the Arabian Peninsula and named here as *Diversispora omaniana*, *Septoglomus nakheelum* and *Rhizophagus arabicus* spp. nov.

## 2.3 Material and Methods

*Study sites.*

The sites studied occur at a single location (AlKamel) in the area between Al-Sharqiya Sands and Oman Mountains in Southern Arabia in the Sultanate of Oman (see Al-Yahya'ei *et al.*, 2011). It is a sandy plain area characterized by its hyper-aridity based on the aridity index defined by the United Nations Environmental Programme (UNEP, 2006). The annual rainfall does not exceed 100 mm (Fisher & Membery, 1998) and the high summer temperatures exceed 48°C ( Al-Yahya'ei, pers. observ., Glennie and Singhvi, 2002). The newly described species were found in two sites at this location. The first was an undisturbed site (22°14'11"N, 59°10'53"E) protected by a fence (since 1990) from human activity and grazing. The natural vegetation in the sampling area consisted mainly of three perennial plants, namely *Tetraena qatarense* Beier & Thulin., *Salvadora persica* Wall. and *Prosopis cineraria* (L.) Druce. The area between these plants was also sampled and here referred to as inter-plant area. It was sparsely

covered with dried up grasses which grow only after the rare raining events. The second site was on the margin of the oasis of Al-Kamel (22°12'56"N, 59°12'9"E), situated 3 km southward of the undisturbed site. It was cultivated mainly with date palms. Management followed local traditional farming practice since the establishment of the farm in 1992. The only source of fertilization was the manure produced onsite. Irrigation was performed by the traditional flooding system with water flowing from a natural source a few kilometers away through narrow channels called *Aflaj* (Al-Marshudi, 2001; UNESCO, 2006).Soils properties from the two sites studied are presented in Table 2.1. Soil analysis were performed on pooled samples from the root zones of the plants in each site.

Table 2.1 Soil properties of the two study sites

| | P[a] ($mg\ kg^{-1}$) | % N | pH $H_2O$ | pH KCl | % Organic matter | % Sand | % Silt | % Clay | Soil texture |
|---|---|---|---|---|---|---|---|---|---|
| Undisturbed site | 41.4 | <0.1 | 8.2 | 8.1 | 1.5 | 70.7 | 25.4 | 4.3 | Sandy loam |
| Date palm site | 71 | 0.18 | 8.4 | 7.9 | 3.7 | 67.2 | 24.8 | 8.1 | Sandy loam |

*Sampling, establishment and growth of trap and single-species cultures.*

Soil and root samples were collected in 2006. The methods used to establish trap cultures and the green house growing conditions have been described previously (Al-Yahya'ei *et al.*, 2011).

Spores examined in this study were isolates derived from single-spore cultures established essentially as described (Tchabi *et al.*, 2010). More than 600 essays to establish single-spore cultures were initiated by positioning a single spore close to a leek (*Allium porrum* L.) seed in a pipette tip of 1 ml volume (RC-LAB, Ascona, Switzerland). The growth substrate made of a mixture of sand and loess (4:1 v/v) was autoclaved at 120°C for 20 min. These cultures were kept for one month in a growth chamber (light: 16 hr of 700 µmol $m^{-2}$ $s^{-1}$ intensity (PPFD); temperature: 16-21°C; relative humidity: 70%). Pipette tips containing the seedlings were then transferred to pots (VEFI Europa, Poland) containing 130 ml autoclaved substrate consisting of Terragreen (American aluminium oxide, oil dry US special, type III R, <0.125 mm; Lobbe Umwelttechnik, Iserlohn, Germany), sand and loess at a ratio of 5:4:1 (w/w/w). The pipette tips were cut at the tip before transfer to give a chance to the roots and mycorrhizal mycelium to expand in the new substrate in the pots. Two seeds of *Linum usitatissimum* L. were planted additionally in each pot to increase root biomass and, consequently AM-spore formation. Plants were kept in a green house (light: 16 hr of 20 klux intensity; temperature: 19-23°C; relative humidity: 50%) for three months and watered when needed. AM fungal spores were formed only in 86 of the 600 single-spore essays. These were used as inocula for further propagation by culturing with a consortium of *A. porrum*, *Hieracium pilosella* L. and *Plantago lanceolata* L. as AM fungal host plants.

*Extraction of spores and staining of mycorrhiza*

Spores were extracted by wet sieving and sucrose density gradient centrifugation essentially as described by Daniels and Skipper (1982). For each single-spore derived culture, around 10 $cm^3$ of harvested substrate were well suspended in 20 ml of water in a 50 ml Falcon tube. A 25 ml sucrose solution (70% v/w) was injected to the bottom of the tube forming a density gradient that was centrifuged at 900× g for 2 min. The spores contained in the resulting supernatant were washed with tap water for 2 min on a 32-µm sieve and transferred with some water to Petri dishes. Spores were collected under stereomicroscope directly from the water by a pipette and either examined microscopically or used to extract DNA for molecular analysis.

AM root colonization was assessed on root pieces (1-cm segments) taken from the single AM-species cultures. The roots were cleaned with 10% KOH in a 90°C water bath for 5 min, rinsed with 2% lactic acid then incubated overnight at room temperature in 0.1% (w/v) of trypan blue.

*Microscopy survey*.

Morphological characteristics of spores and details of wall structure were determined by examining at least 100 spores mounted in water, lactic acid, polyvinyl alcohol/lactic acid/glycerol (PVLG) (Omar *et al.*, 1979) and a mixture of PVLG and Melzer's reagent (1:1, v/v). Spores at all developmental stages were crushed to varying degrees by applying pressure to the cover slip and then stored at $65^{o}$ C for 24 h to clear their contents from oil droplets. Thereafter they were examined under an Olympus BX 50 compound microscope equipped with Nomarski differential interference contrast optics. Microphotographs were recorded on a Sony 3CDD color video camera coupled to the microscope.

Terminology of spore structure is that suggested by Stürmer and Morton (1997) and Walker (1983). Spore color was examined under a dissecting microscope on fresh specimens immersed in water. Color names are from Kornerup and Wanscher (1983). Nomenclature of fungi is that of Schüßler and Walker (2010) and Redecker *et al*. (2013). The authors of the fungal names are those presented at the Index Fungorum website (http://www.indexfungorum.org/AuthorsOfFungalNames.htm). Voucher specimens were mounted in PVLG and a mixture of PVLG and Melzer's reagent (1:1, v/v) on slides and deposited in the Soil and Water Research Center, Ministry of Agriculture and Fisheries, Sultanate of Oman; Institute of Botany, University of Basel, Switzerland; Department of Plant Protection (DPP), West Pomeranian University of Technology, Szczecin, Poland; and in the herbarium at Oregon State University (OSC) in Corvallis, Oregon, USA.

*Molecular analyses and phylogeny.*

Crude DNA was extracted by crushing single spores with a pipette tip in a 0.2 ml PCR tube containing 2 µl of 0.25 M NaOH. The tubes were incubated in a T3 Thermocycler (Biometra GmbH, Goettingen, Germany) at 95°C for 2 min before 1 µl of 0.5 M Tris HCl (pH 8.0) and 2 µl of 0.25 M HCl were added and incubated again at 95°C for 2 min. In between incubations the tubes were kept on ice. The extracts were either directly used as templates for PCR or stored at -20°C. To obtain the partial SSU, ITS (ITS1, 5.8S and ITS2) and the partial LSU rDNA sequences, PCR was performed in a nested procedure with a protocol modified after Krüger *et al.* (2009) with the SSUmAf-LSUmAr and the SSUmCf-LSUmBr primer pairs for the first and second nested PCR respectively. The final concentration of the master mix contained 0.6 U Taq polymerase (GE Healthcare, Glattbrugg, Switzerland), 2 mM of $MgCl_2$, 0.2 µM of each primer, 0.125 mM of each desoxynucleotide and 5 µg of BSA (Bioconcept, Allschwil, Switzerland). Thermal cycling was done in the T3 Thermocycler with the following conditions for the first PCR: 3 min initial denaturation at 95°C, 35 cycles of 30 s denaturation at 95°C, 90 s annealing at 60° C, 2 min elongation at 72°C and 10 min at 72° C for final elongation. The same conditions were used for the nested PCR except that the annealing temperature was 63°C. The PCR products were visualized on 1.5% agarose gels with 1× TAE buffer and 1X Midori green for staining (NIPPON Genetics EUROPE GmbH, Dueren, Germany). The PCR products with the expected-size bands were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturers' protocols. PCR products were cloned into pGEM-T Vector system (Promega, Madison Wisconsin) and the clones were transformed into competent JM109 *Escherichia coli* cells (Promega, Madison Wisconsin) following the manufacturers' protocols. Ten positive clones derived from each single spore were confirmed with direct colony PCR technique using the universal M13F and M13R vector primers and purified with ExoSAP-IT (GE Healthcare, Glattbrugg, Switzerland). For sequencing of amplified clones the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California) and the ABI3500 were used. Sequencing primers for the respective samples were the mentioned universal vector primers. The electropherograms were processed and analyzed with ChromasPro Version 1.5 (www.technelysium.com.au).

The Glomeromycotan origin of the sequences was initially tested by BLAST (Zhang *et al.*, 2000) search. Phylogenetic analyses were performed with two sets of alignments. The first was done with the obtained full sequences that consisted of partial SSU (ca. 240 bp long), the whole ITS region (ITS1-5.8S-ITS2) (ca. 620 bp) and partial LSU (ca. 800-900 bp). This alignment was used to build the phylogenetic tree in Fig. 2.1. The second alignment was conducted with ITS sequences only and was used to build the phylogenetic tree in Fig. 2.2. The analyses with the ITS sequences were performed to reveal phylogenetic

relationship of our *R. arabicus* to *R. custos*, a species found morphologically very similar when observed under low microscope magnification. In GenBank, *R. custos* is represented by relatively short (ca. 500 bp long) ITS sequences only that could not be aligned with the long SSU-ITS-LSU sequences. To determine the generic affiliation of our new species we performed pilot phylogenetic analyses of all their sequences with those representing all recognized genera of *Glomeromycota* with glomoid spores published by Krüger *et al*. (2012). The final alignment comprised four representative sequences for each of the three newly described species (each two sequences representing a different culture of the species), two to four sequences (based on their availability in GenBank) for each of the phylogenetic or morphological closely related species to our newly described species and one (if available) sequence of the generic-related species.

The sequences were aligned with Clustal W (Thompson *et al.*, 1994) with default parameters. Maximum likelihood (ML) and Bayesian (BI) analyses were performed with PHYML (Guindon & Gascuel, 2003) and MrBayes 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) respectively. Before the analyses, the best-fit substitution models for the alignments were estimated by the Akaike information criterion (AIC) using Topali v. 2.5 (Milne *et al.*, 2004). *Ambispora fennica* C. Walker, Vestberg & A. Schüßler was outgroup in analyses of SSU-ITS-LSU and ITS sequences. In the ML and BI analyses of SSU-ITS-LSU and ITS sequences, the model employed was GTR + G. In the ML analyses the transition/transversion ratio for DNA models and the gamma distribution parameter were estimated. Six substitution rate categories were set. Topology and branch lengths and rate parameters were optimized. Support of branches in the ML analyses was estimated in a bootstrap analysis with 1000 replicates. In the BI analyses the Markov chain was run for 5000000 generations, sampling in every 500 steps, and with a burn-in at 3000. The details of the analyses are available on request. Phylogenetic trees were visualized and edited in MEGA5 (Tamura *et al.*, 2011).

## 2.4 Results

Morphological analyses of spores and mycorrhizae as well as phylogenetic analyses (based on SSU-ITS-LSU rDNA sequences and, separately, on ITS sequences only) of three AM fungi isolated from one location in a desert of Southern Arabia revealed that they are undescribed species affiliated to three genera of Glomeromycota *sensu* Redecker *et al*. (2013). The fungi are described below as *Diversispora omaniana*, *Septoglomus nakheelum* and *Rhizophagus arabicus* spp. nov.

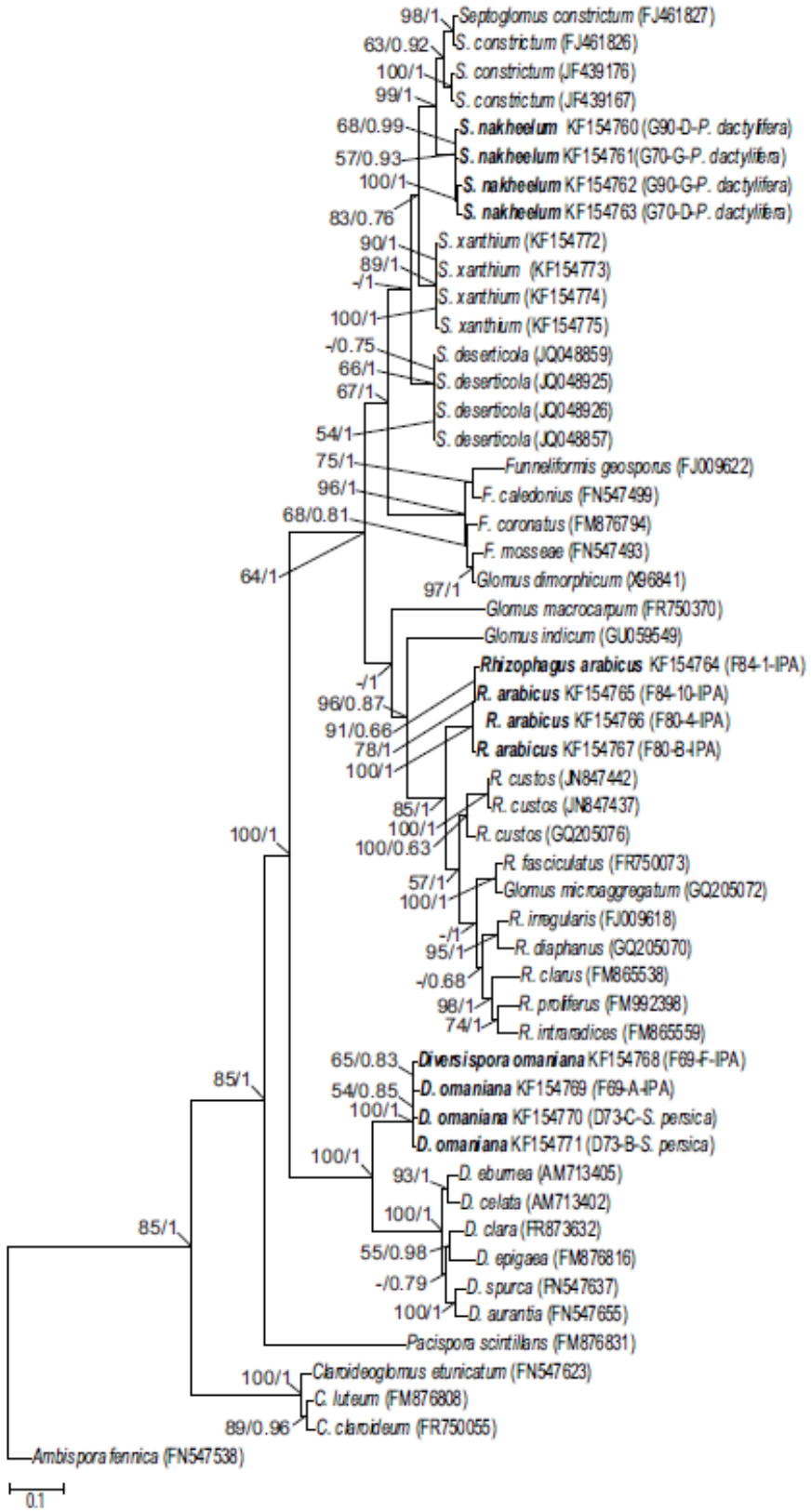


Fig. 2.1 Maximum likelihood (ML) tree showing the phylogenetic positions of the three new species among 28 known species of AM fungi. The tree was inferred from the analysis of SSU-ITS (ITS1, 5.8S and ITS2)-LSU rDNA sequences with *Ambispora fennica* as outgroup.

Our newly described species are shown in boldface followed by the GenBank accession number. The labels between the brackets indicate the isolate, clone code and either the likely host plant or the inter-plant area (IPA) from where the isolate was obtained. ML bootstrap values ≥50% and the Bayesian posterior probabilities ≥0.50 are shown near the branches respectively. Bar indicates 0.05 expected change per site per branch.

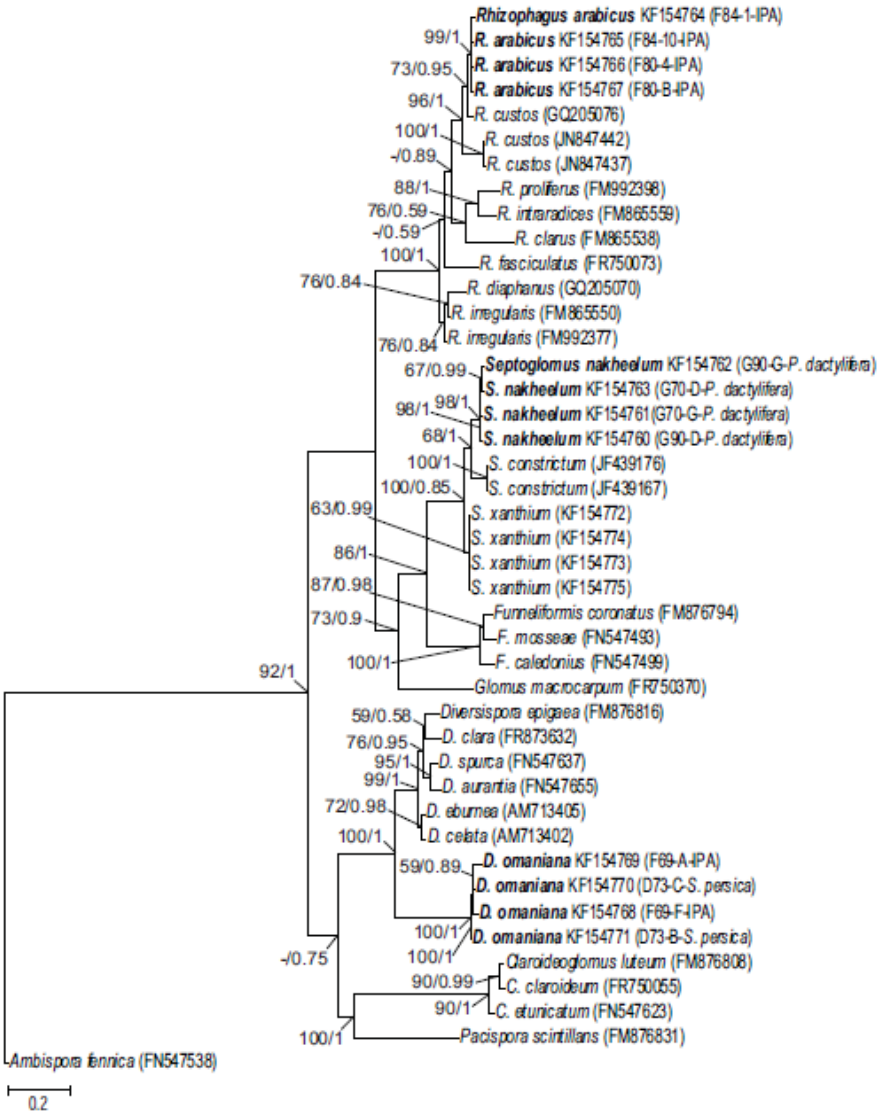


Fig. 2.2 Maximum likelihood (ML) tree showing the phylogenetic positions of the three new species among 23 known species of AM fungi. The tree was inferred from the analysis of ITS sequences only with *Ambispora fennica* as outgroup. Our newly described species are shown in boldface followed by the GenBank accession number. The labels between the brackets indicate the isolate, clone code and either the likely host plant or the inter-plant area (IPA) from where the isolate was obtained. ML bootstrap values ≥50% and the Bayesian posterior probabilities ≥0.50 are shown near the branches respectively. Bar indicates 0.05 expected change per site per branch.

## 2.5 Taxonomy

***Diversispora omaniana*** Symanczik, Błaszk. & Al-Yahya'ei, sp. nov. Fig. 2.1-2.12
MycoBank MB 804358

*Sporocarps* unknown. Spores formed singly in soil; develop blastically at the tip of sporogenous hyphae continuous with mycorrhizal extraradical hyphae (Fig. 2.3, 2.5, 2.9, 2.10). *Spores* hyaline to brownish yellow (5C8); globose to subglobose; (85-)136(-170) µm diam; rarely egg-shaped; 110-160 × 130-180 µm; with one subtending hypha (Fig. 2.3-2.5, 2.9, 2.10). *Spore wall* composed of three layers (layers 1-3; Fig. 2.5-2.11). Layer 1, forming the spore surface, evanescent, roughened, hyaline, (1.0-)1.9(-2.8) µm thick when intact, usually more or less deteriorated in mature spores, frequently partly or completely sloughed in older specimens (Fig. 2.4-2.10). Layer 2 permanent, pliable, uniform (not composed of sublayers), smooth, hyaline to pastel yellow (2A4), (1.5-)2.4(-3.8) µm thick; usually tearing with difficulty during crushing of spores (Fig. 2.4-2.10). Layer 3 laminate, smooth, hyaline, (4.8-)8.9(-22.0) µm thick, frequently stratifying into groups of or single laminae in crushed spores (Fig. 2.4-2.10). In Melzer's reagent layer 1 always remains nonreactive, layer 2 usually darkens to pale yellow (3A3), and layer 3 usually stains pinkish (9A2) to dull red (11C3; Fig. 2.5-2.10). *Subtending hypha* hyaline to pastel yellow (2A4); straight or recurved, cylindrical to funnel-shaped, rarely constricted at the spore base; (10.8-)14.3(-23.5) µm wide at the spore base (Fig. 2.3, 2.5, 2.9, 2.10). *Wall of subtending hypha* hyaline to pastel yellow (2A4); (2.3-)4.2(-5.5) µm thick at the spore base, composed of three layers continuous with spore wall layers 1-3 (Fig. 2.9, 2.10). *Pore* (3.0-)7.1(-14.3) µm diam, open or occluded by a curved septum, 1.0-1.3 µm thick, continuous with some innermost laminae of spore wall layer 3; septum positioned up to 15.7 µm below the spore base (Fig. 2.5, 2.9, 2.10). Spore contents of a hyaline to brownish yellow (5C8), sticky, opaque substance (Fig. 2.3-2.10). *Germination* unknown.

*Mycorrhizal associations*. In the field *D. omaniana* was associated with the rhizospheres of *S. persica* and *T. qatarense.* In addition it was recovered from an inter-plant area that was sparsely covered with dried up grasses growing after the rare raining events. In single-species pot cultures with *A. porrum* as host plant, *D. omaniana* formed a mycorrhiza with arbuscules and intra- and extra-radical hyphae (Fig. 2.11, 2.12). No vesicles were found. Arbuscules were infrequent and widely dispersed along the root fragments examined. They consisted of a short trunk developed from a parent hypha and numerous branches with fine tips (Fig. 2.11). Intraradical hyphae grew parallel to the longitudinal root axis, were straight to slightly curved, (2.5-)3.8(-7.8) µm wide, and were frequently filled with a dense, granular substance. They rarely formed coils (Fig. 2.12). Coils were ellipsoidal; 12.5-17.0 × 18.5-20.5 µm; when seen in a

plan view; and widely dispersed along the roots fragments. Extraradical hyphae occurred very rarely and were (1.8-)2.5(-3.5) µm wide. In 0.1% trypan blue arbuscules stained light lilac (16A5) to deep violet (16E8), intraradical hyphae violet white (16A2) to lilac (16B5), coils violet white (17A2) to pastel violet (164) and extraradical hyphae violet white (16A2) to pale violet (16A3; Fig. 2.11, 2.12).

*Phylogenetic position.* Phylogenetic analysis of the SSU-ITS-LSU sequences (Fig. 2.1) and the ITS sequences (Fig. 2.2) both placed *D. omaniana* in a clade sister to that comprising all *Diversispora* spp. of so far known molecular phylogenies.

*Specimens examined.* POLAND, Szczecin, under pot-cultured *A. porrum*, 10 March 2009, *Błaszkowski, J., 3222* (HOLOTYPE, DPP); *Błaszkowski, J., 3221*, 3223-*3243* (ISTOTYPES, DPP) and two slides at OSC.

*Etymology*. Latin, *omaniana*, referring to Oman on which the fungus was for the first time found.

*Distribution and habitat*. Three cultures of *D. omaniana* were successfully established from single spores. They were isolated from three different trap cultures established with *S. bicolor* (L.) Moench as host plant and a pooled sample of four soil subsamples coming from the rhizospheres of *S. persica* and *T. qatarense* and from an inter-plant area in undisturbed natural field (22°14'11"N, 59°10'53"E) at Al-Kamel in Al-Sharqyia region of Oman. Sampling took place in August 2006. Chemical and physical properties of the habitat soil are presented in Table 1.

*Commentary*. *Diversispora omaniana* is distinguished by its spore morphological characteristics and molecular separateness. The spore color mainly comes from the pigmentation of the spore contents, and not from the color of the spore wall (Fig. 3-10). The only spore wall component that becomes pigmented with age (layer 2) is much lighter [up to pastel yellow (2A4); Fig. 4] than the color of intact spores [up to brownish yellow (5C8); Fig. 3]. Spore wall layer 2 is highly pliable and thereby spores usually are difficult to crush. The laminate spore wall layer 3 frequently stratifies and swells in spores crushed in PVLG-based mountants (Fig. 2.5, 2.6, 2.9, 2.10) and usually clearly stains in Melzer's reagent (Fig. 2.6-2.8). However the intensity of this reaction frequently decreases or disappears with time, especially after heating of spores in an incubator (Fig. 2.5, 2.7, 2.9, 2.10).

In young spores slightly crushed in PVLG, spore wall layer 3 usually does not stratify and tightly adheres to the lower surface of spore wall layer 2, which usually is hyaline. Then, both layers make an impression to be one structure. However with age layer 2 turns pale yellowish white (2A3) to pastel yellow (2A4) and becomes better visible, because layer 3 remains colorless. Moreover in mature specimens layer 2

always remains intact, and layer 3 frequently stratifies and swells in spores even slightly crushed in PVLG. Most importantly only layer 3 shows dextrinoid properties in Melzer's reagent (Fig. 2.6-2.8).

Morphologically *D. omaniana* resembles only *D. trimurales* (Koske & Halvorson, 1989) C. Walker & A. Schüßler. Spores of both species are similar in color and have a spore wall consisting of three layers of identical positions in the wall and of similar phenotypic characteristics (Blaszkowski, 2012). The spore size range of both fungi produced in pot cultures also is similar.

However the color of *D. omaniana* spores mainly comes from their colored, sticky, opaque contents (Fig. 2.3-2.10), whereas that of *D. trimurales* spores derives only from pigments accumulated in spore wall layers 1 and 2 (Koske and Halvorson, 1989; Blaszkowski, 2012). The content of mature *D. trimurales* spores always is oily, transparent and colorless.

Spore wall layer 1 of *D. omaniana* usually is much thinner and less persistent than that of *D. trimurales*, which additionally is ornamented with thickening or blister-like outgrowths when intact (Blaszkowski, 2012; vs. no ornamentation in *D. omaniana*).

The laminate spore wall layer 3 of *D. omaniana* and *D. trimurales* consists of hyaline laminae easily separating from each other (Blaszkowski, 2012), but this layer stains in Melzer's reagent only in *D. omaniana* (Fig. 2.6-2.8).

Finally the mean width of the subtending hypha of *D. omaniana* spores is almost 2-fold higher than that of *D. trimurales* spores and its pore is much wider (Blaszkowski, 2012).

Of the seven other species of genus *Diversispora* with so far known phylogenies (Schüssler & Walker, 2010; Estrada *et al.*, 2011; Oehl *et al.*, 2011), *D. aurantia* (Błaszk., Blanke, Renker & Buscot) C. Walker & A. Schüßler, *D. celata* C. Walker, Gamper & A. Schüßler, *D. clara* Oehl et al. and *D. epigaea* (B.A. Daniels & Trappe) C. Walker & A. Schüßler form spores with a 3-layered spore wall, but layer 3 is flexible to semiflexible (Blaszkowski *et al.*, 2004; Gamper *et al.*, 2009; Estrada *et al.*, 2011; Schüssler *et al.*, 2011; vs. laminate in *D. omaniana*; Figs. 2.3-2.10). In *D. eburnea* (L.J. Kenn., J.C. Stutz & J.B. Morton) C. Walker & A. Schüßler, *D. insculpta* (Błaszk.) Oehl, G.A. Silva & Sieverd. and *D. spurca* (C.M. Pfeiff., C. Walker & Bloss) C. Walker & A. Schüßler, type species of the genus, the spore wall comprises only two layers (Kennedy *et al.*, 1999; Blaszkowski, 2012), lacking spore wall layer 2 of the species described here.

Apart from the morphological differences between *D. omaniana* and the *Diversispora* spp. listed above, the former fungus also distinguishes its phylogeny (Fig. 2.1, 2.2).

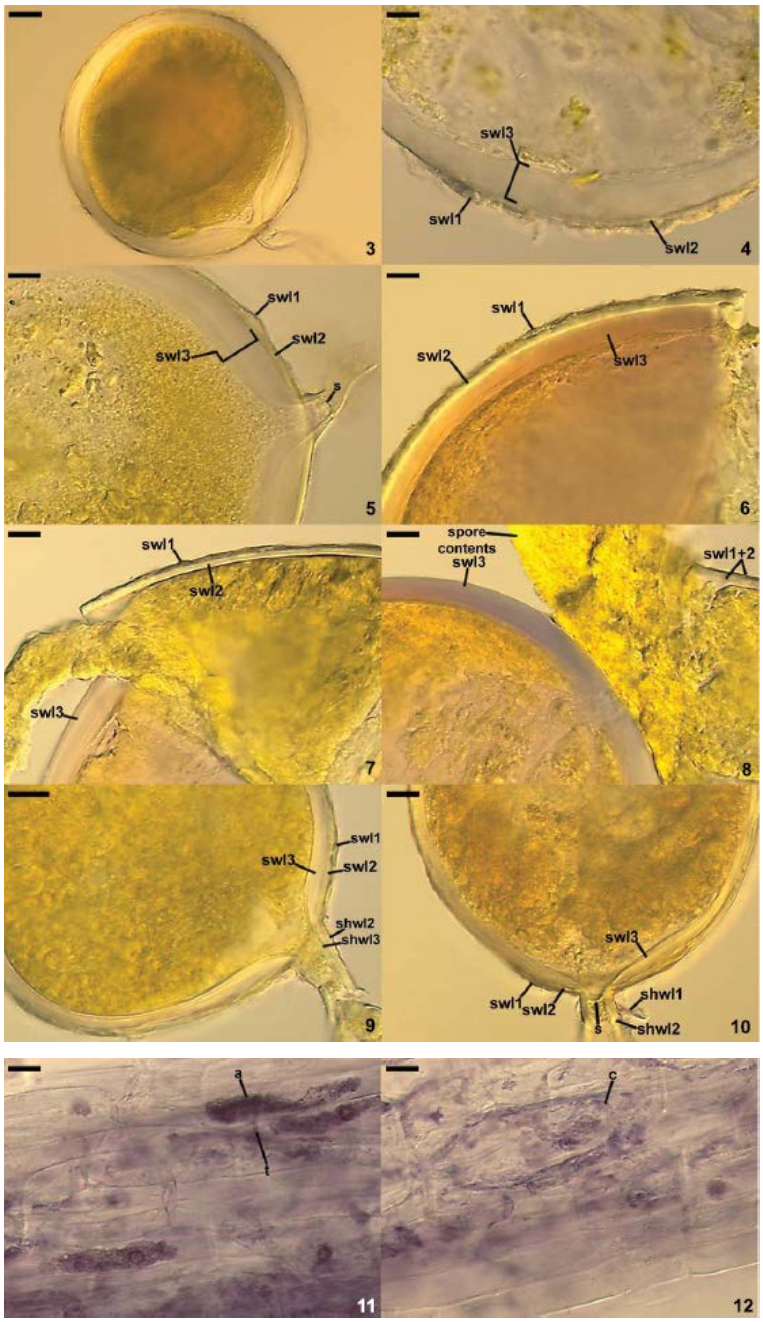


Fig. 2.3-2.12. *Diversispora omaniana*. 3. Intact spore with subtending hypha; note the darkly-colored spore contents and the almost colorless spore wall. 4. Spore wall layers (swl) 1-3; swl1 is highly deteriorated and partly sloughed. 5. Spore wall layers (swl) 1-3 and septum (s) in the lumen of the subtending hypha; swl3 is swollen. 6-8. Spore wall layers (swl) 1-3; note swl3 stained in Melzer's reagent and the sticky, colored spore contents.9. Spore wall layers (swl) 1-3 and subtending hyphal wall layers (shwl) 2 and 3; shwl1 is completely sloughed; note the open lumen of the subtending hypha. 10. Spore wall layers (swl) 1-3 continuous with subtending hyphal wall layers (shwl) 1-3; some innermost laminae of swl3 form a curved septum (s) in the lumen of the subtending hypha.3. Spore in lactic acid. 4. Spore crushed in PVLG. 5-10. Spores in PVLG+Melzer's reagent. 3-10. Differential interference microscopy. Scale bars: 3= 20 μm, 4-10 = 10 μm.
Mycorrhizae of *Diversispora omaniana* in roots of *Allium porrum* stained in 0.1% trypan blue. 11. Arbuscule (a) with trunk (t). 12. Coil (c). 11, 12. In PVLG. 11, 12. Differential interference microscopy. Scale bars: 11, 12 = 10 μm.

***Septoglomus nakheelum*** Al-Yahya'ei, Symanczik & Błaszk., sp. nov. Fig. 2.1, 2.2, 2.13-2.20
MycoBank MB 804359

*Sporocarps* unknown. Spores formed in loose clusters or singly in soil (Fig. 2.13); develop blastically at the tip of sporogenous hyphae either branched from a parent hypha continuous with a mycorrhizal extraradical hypha (spores in clusters) or directly developed from mycorrhizal extraradical hyphae (single spores). *Clusters* 58-142 × 111-269 µm with 2-4 spores (Fig. 2.13). *Spores* pale orange (5B3) to brown (6E8); globose to subglobose; (25-)52(-70) µm diam; sometimes ovoid to irregular; 45-140 × 80-160 µm; with one subtending hypha (Fig. 2.13-2.18). *Spore wall* consists of two layers (Fig. 2.14-2.17). Layer 1, forming the spore surface, semi-permanent, light yellow (A4-5), (0.8-)1.4(-2.0) µm thick, very slowly decomposing with time, always only slightly deteriorated even in older specimens; in young and freshly matured spores the upper surface of this layer is covered frequently with small, local thickenings, (0.8-)1.1(-1.5) µm high, rarely is smooth (Fig. 2.14-2.17). Layer 2 laminate, smooth, greyish yellow (4B6) to brown (6E8), (1.0-)1.7(-3.5) µm thick (Fig. 2.14-2.17). Layers 1 and 2 do not stain in Melzer's reagent (Fig. 15). *Subtending hypha* pale orange (5B3) to brown (6E8); straight or recurved, flared to funnel-shaped, sometimes slightly constricted at the spore base; (4.8-)7.2(-8.2) µm wide at the spore base (Fig. 17, 18). *Wall of subtending hypha* pale orange (5B3) to brown (6E8); (1.8-)2.7(-4.0) µm thick at the spore base; continuous with spore wall layers 1 and 2; subtending hyphal wall layers 1 and 2 (1.0-)1.2(-1.4) µm thick and (1.0-)1.7(-2.8) µm thick, respectively, at the spore base; layers 1 and 2 usually extend far below the spore base in mature spores (Fig. 2.17, 2.18). *Pore* (0.8-)2.6(-5.0) µm diam, open (Fig. 2.17) or occluded by a curved septum continuous with some innermost laminae of spore wall layer 2 (Fig. 2.18); septum positioned up to 6.0 µm below the spore base. *Germination* unknown.

*Mycorrhizal associations.* In the field *S. nakheelum* was associated with the rhizosphere of *Phoenix dactylifera* L.

In single-species cultures with *A. porrum* as host plant *S. nakheelum* formed mycorrhiza with arbuscules and intra- and extraradical hyphae (Fig. 2.19, 2.20). No vesicles were found. Arbuscules were very numerous and evenly distributed along the root fragments examined. They consisted of a short trunk grown from a parent hypha and numerous branches with fine tips (Fig. 2.19, 2.20). Intraradical hyphae grew along the root axis, were (1.3-)3.5(-7.0) µm wide, straight or slightly recurved and rarely formed Y-shaped branches (Fig. 2.20). They were frequently filled with a dense, fine-grained substance and occasionally had short, hairy processes (Fig. 2.20). No H-shaped branches and coils were found. Extraradical hyphae were (1.2-)3.6(-6.5) µm wide and occurred infrequently. In 0.1% trypan blue

arbuscules stained light blue (21A6) to blue (21C8), intraradical hyphae light blue (20A6) to blue (21B8) and extraradical hyphae pale blue (21A3) to blue (21B8; Fig. 2.19, 2.20).

*Phylogenetic position.* Phylogenetic analyses of the SSU-ITS-LSU (Fig. 2.1) and ITS (Fig. 2.2) sequences placed *S. nakheelum* in a monophyletic group sister to *S. constrictum* (Trappe) Sieverd., G.A. Silva & Oehl.

*Specimens examined.* POLAND, Szczecin, under pot-cultured *A. porrum*, 10 March 2009, *Błaszkowski, J., 3282* (HOLOTYPE, DPP); *Błaszkowski, J., 3281*, 3283-*3306* (ISTOTYPES, DPP) and two slides at OSC.

*Etymology*. *Latinized, nakheelum*, referring to Nakheel (plural), the Arabic name for the date palms.

*Distribution and habitat.* Three cultures of *S. nakheelum* were successfully established from single spores. The spores were isolated from one trap culture established with a consortium of *A. porrum*, *H. pilosella* and *P. lanceolata* and inoculation with a pooled sample of four soil subsamples and root fragments collected under four *P. dactylifera* trees from a plantation at Al-Kamel in Al-Sharqyia region of Oman (22°14'11"N, 59°10'53"E). Sampling took place in August 2006. Chemical and physical properties of the habitat soils are presented in Table 1.

*Commentary.* The distinctive morphological structures of *S. nakheelum* are its dark-coloured, small spores and their layer 1 in the 2-layered spore wall, which is coloured and generally only slightly deteriorates with age (Fig. 2.13-2.18).

Morphologically *S. nakheelum* spores resemble most those of *S. deserticola* (Trappe, Bloss & J.A. Menge) G.A. Silva, Oehl & Sieverd. and *G. invermaium* I.R. Hall. Phylogenetically *S. nakheelum* is most closely related to *S. constrictum* and *S. xanthium* (Błaszk., Blanke, Renker & Buscot) G.A. Silva, Oehl & Sieverd. (Fig. 2.1, 2.2). Except for *S. xanthium*, all the other species form spores with a 2-layered spore wall in which layer 2 is laminate. However the species with a 2-layered spore wall differ clearly from *S. nakheelum* in color (*S. constrictum* with dark brown to black color) and size (*S. deserticola* 80-120 µm diam) of spores, in persistency (*S. deserticola*, *G. invermaium* with evanescent swl1), thickness (*S. constrictum* up 20 µm thick) and color (*S. constrictum*, *S. deserticola*, *G. invermaium* with hyaline swl1) of spore wall layer 1 and in width (all three species) and shape (*S. constrictum* constricted subtending hypha) of the subtending hypha (Hall, 1977; Trappe, 1977; Błaszkowski *et al.*, 2010; Blaszkowski, 2012). The most evident feature separating *S. nakheelum* from *S. xanthium* is the number of spore wall layers [two in *S. nakheelum* (Fig. 2.14-2.17) vs. three in *S. xanthium*; Błaszkowski 2012]. *Septoglomus nakheelum* does not differentiate the rigid, smooth, hyaline spore wall layer 2 of *S. xanthium*. In addition *S. xanthium* spores usually tightly adhere to roots and frequently form inside roots (vs. usually occur at

some distance from roots and do not form intraradical spores in *S. nakheelum*), are slightly lighter in color and have a more regular in shape (cylindrical to flared) subtending hypha.

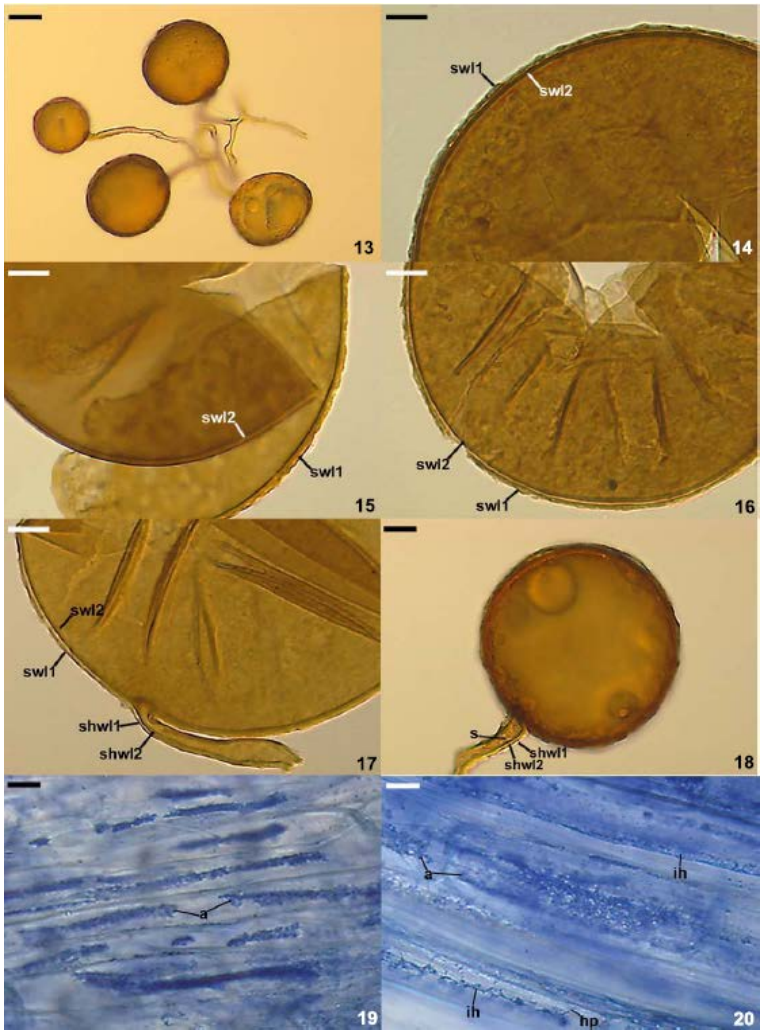


Fig. 2.13-2.20. *Septoglomus nakheelum*. 13. Intact spores in a loose cluster. 14-16. Spore wall layers (swl) 1 and 2; note the small thickenings of swl1 on its upper surface. 17. Spore wall layers (swl) 1 and 2 continuous with subtending hyphal wall layers (shwl) 1 and 2; note the open lumen of the subtending hypha. 18. Subtending hyphal wall layers (shwl) 1 and 2 and the lumen of the subtending hypha closed by a curved septum (s). 13, 14, 16-18. Spores in PVLG. 15. Spore in PVLG+Melzer's reagent. 13-18. Differential interference microscopy. Scale bars: 13 = 20 µm, 14-18 = 10 µm. 19-20
Mycorrhizae of *Septoglomus nakheelum* in roots of *Allium porrum* stained in 0.1% trypan blue. 19. Numerous arbuscules (a) evenly distributed in the root fragment. 20. Arbuscules and intraradical hyphae (ih) filled with dense, fine-grained substance and with hairy processes (hp). 19, 20. In PVLG. 19, 20. Differential interference microscopy. Scale bars: 19, 20 = 10 µm.

***Rhizophagus arabicus*** Błaszk., Symanczik & Al-Yahya'ei, sp. nov. Fig. 2.1, 2.2, 2.21-2.32
MycoBank MB 804360

*Sporocarps* unknown. Spores formed in loose clusters, singly in soil and inside roots (Fig. 2.21, 2.22, 2.25); extraradical spores develop blastically at the tip of sporogenous hyphae either branched from a parent hypha continuous with a mycorrhizal extraradical hypha (spores in clusters) or directly developed from mycorrhizal extraradical hyphae (single spores). *Clusters* 360-780 × 460-1000 µm with 2-49 spores (Fig. 2.21). *Spores* hyaline when young, pale yellow (3A3) to greyish yellow (3B6) at maturity; globose to subglobose; (33-)65(-105) µm diam; sometimes ovoid to irregular; 30-85 × 50-125 µm; with one subtending hypha (Fig. 2.21-2.28). *Spore wall* consists of two layers (Fig. 2.21-2.28). Layer 1, forming the spore surface, mucilaginous, short-lived, hyaline, (0.6-)1.6(-2.5) µm thick, usually completely sloughed in mature specimens; when intact its upper surface frequently is rough (Fig. 2.23-2.25, 2.27, 2.28). Layer 2 laminate, smooth, pale yellow (3A3) to greyish yellow (3B6), (1.8-)2.6(-5.5) µm thick, consisting of pliable laminae, each (0.5-)1.0(-1.4) µm thick, easily separating from each other even in slightly crushed spores; in intact spores mounted in PVLG the outermost lamina with the adherent spore wall layer 1 sometimes balloons and separates up to 10 µm from the other laminae of spore wall layer 2 (Fig. 2.23-2.28). In Melzer's reagent layers 1 and 2 usually stain pinkish white (11A2) to dark red (11C7) and pale orange (6A3) to pale red (11A3), respectively, sometimes are nonreactive (Fig. 2.21, 2.22, 2.24-2.27). *Subtending hypha* pale yellow (3A3) to greyish yellow (3B6); straight or recurved, flared to slightly funnel-shaped, occasionally slightly constricted at the spore base; (5.0-)9.2(-19.6) µm wide at the spore base (Fig. 2.25-2.28). *Wall of subtending hypha* pale yellow (3A3) to greyish yellow (3B6); (1.2-)2.2(-2.8) µm thick at the spore base; continuous with spore wall layers 1 and 2; layer 1 usually completely sloughed in mature spores (Fig. 2.28). *Pore* (1.5-)2.9(-6.8) µm diam, open (Fig. 2.28). *Germination* unknown.

*Mycorrhizal associations*. In the field *R. arabicus* occurred in the inter-plant area sparsely covered with dried up grasses growing after the rare raining events.

In single-species *R. arabicus* cultures with *A. porrum* as host, a mycorrhiza with arbuscules, vesicles and intra- and extraradical hyphae was formed (Fig. 2.29-2.32). Arbuscules occurred rarely, were widely dispersed along the root fragments examined and generally difficult to see because of the faint staining of their delicate trunk with fine tips (Fig. 2.20, 2.32). Vesicles were not numerous, ellipsoidal to oblong; 11.8-26.3 × 48.0-83.3 µm; when observed in a plan view (Fig. 2.30). Intraradical hyphae grew along the root axis, were (1.4-)4.5(-9.8) µm wide, straight or slightly recurved and occasionally formed Y-shaped branches and coils (Fig. 2.29, 2.31, 2.32). The coils were ellipsoidal to oblong; 20.2-36.5 × 43.0-84.0 µm;

when seen in a plan view (Fig. 2.32). Extraradical hyphae were (2.3-)4.0(-5.2) µm wide and occurred not abundantly. In 0.1% trypan blue arbuscules stained violet white (15A2) to lilac (16B3), vesicles pale violet (17A3) to deep violet (16D8), intraradical hyphae violet white (15A2) to greyish violet (16C6), coils violet white (15A2) to pastel violet (16A4) and extraradical hyphae pale violet (15A3) to reddish violet (16C7; Fig. 2.29-2.32).

*Phylogenetic position.* Phylogenetic analyses of the SSU-ITS-LSU (Fig. 2.1) and ITS Fig. 2.2) sequences placed *R. arabicus* in a monophyletic group sister to *R. custos*.

*Specimens examined.* POLAND, Szczecin, under pot-cultured *A. porrum*, 10 March 2009, *Błaszkowski, J., 3250* (HOLOTYPE, DPP); *Błaszkowski, J., 3244-3249, 3251-3256* (ISTOTYPES, DPP) and two slides at OSC.

*Etymology*. Latin, *arabicus*, referring to the Arabian Peninsula on which the fungus was for the first time found.

*Distribution and habitat.* Two cultures of *R. arabicus* were successfully established from single spores. These spores were isolated from a trap culture established with *Sorghum bicolor* as host and a pooled sample of four soil subsamples collected at the inter-plant area in an undisturbed natural field (22°14'11"N, 59°10'53"E) at Al-Kamel in Al-Sharqyia region of Oman. The inter-plant area was sparsely covered with dried up grasses which grow only after the rare raining events. Sampling took place in August 2006. Chemical and physical properties of the habitat soil are presented in Table 2.1.

*Commentary*. The morphological and histochemical characteristics distinguishing *R. arabicus* are its small spores with a simple, 2-layered wall in which the laminate layer 2 consists of pliable, difficult to tear, thin sublayers easily separating from each other and the reactivity of its both spore wall layers in Melzer's reagent (Fig. 2.21-2.28). However the staining reaction in Melzer's sometimes does not appear at all or appears but diminishes with time, especially after heating of spores in an incubator (Fig. 2.25, 2.27, 2.28). These characters render *R. arabicus* unique in the Glomeromycota.

Except for *R. custos* (Cano & Dalpé) C. Walker & A. Schüßler, all the other known species of genus *Rhizophagus* have a spore wall composed of three layers, of which either only layer 1 [all but *R. irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler] or layer 3 (*R. irregularis*) or all three layers [*R. fasciculatus* (Thaxt.) C. Walker & A. Schüßler stain in Melzer's reagent (Blaszkowski *et al.*, 2008; Blaszkowski, 2012). The spore wall of *R. custos*, the species most closely related

phylogenetically to *R. arabicus* (Fig. 2), comprises four layers with layers 1 and 4 staining in this reagent (Cano and Bago, 2009; Blaszkowski, 2012). In addition spores of *R. clarus*, *R. custos* and *R. manihotis* usually are much larger, *R. clarum* and *R. manihotis* produce only single spores (vs. spores in loose clusters and single in *R. arabicus*; Fig. 2.21, 2.25) and spores of *R. diaphanus* and *R. proliferus* remain hyaline throughout their entire life cycle (Morton and Walker, 1984; Blaszkowski, 2012*a*; Declerck *et al.*, 2000; vs. always being coloured in *R. arabicus*).

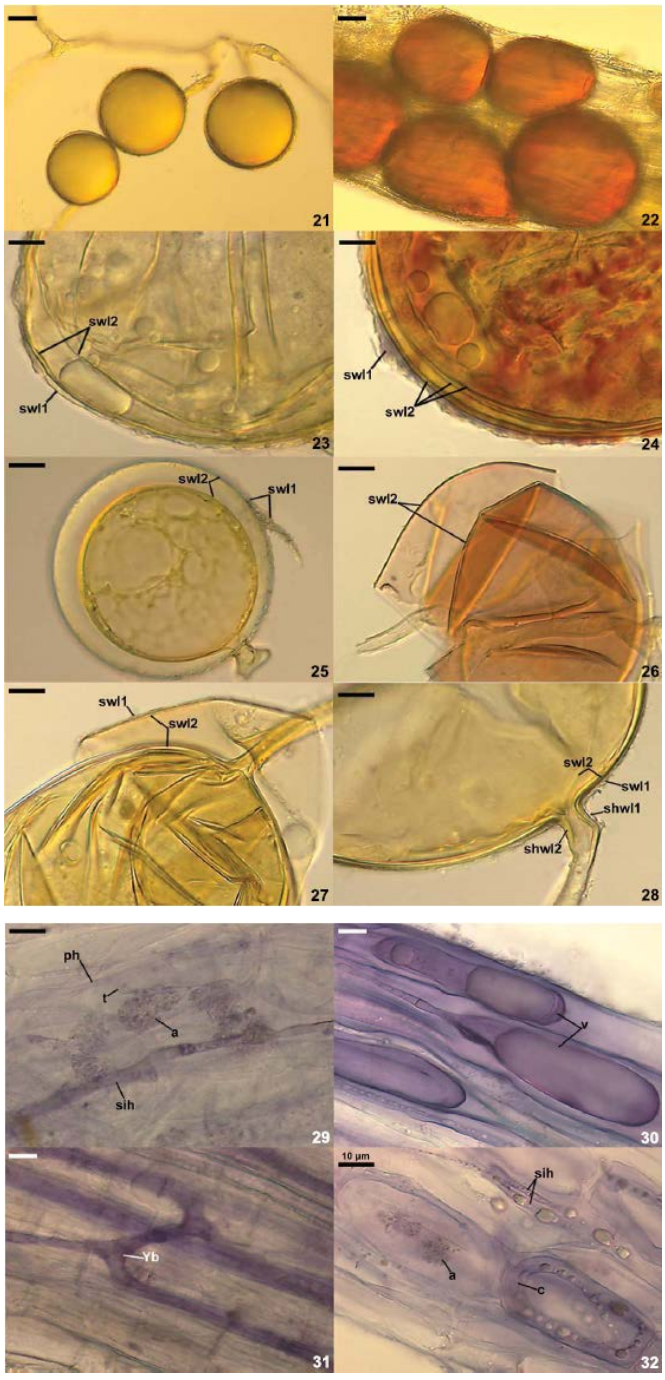


Fig. 21-32. *Rhizophagus arabicus*. 21. Intact spores in loose cluster. 22. Intraradical spores. 23-27. Spore wall layers (swl) 1 and 2; note the outermost lamina of swl2 that separated together with swl1 from the other laminae of the laminate swl2. 28. Subtending hyphal wall layers (shwl) 1 and 2 continuous with spore wall layers (swl) 1 and 2; note the open lumen of the subtending hypha. 23, 28. Spores in PVLG. 22, 23, 24-27. Spores in PVLG+Melzer's reagent. 21-28. Differential interference microscopy. Scale bars: 21, 22 = 20 µm, 23-28 = 10 µm.

Mycorrhizae of *Rhizophagus arabicus* in roots of *Allium porrum* stained in 0.1% trypan blue. 29. Arbuscule (a) with trunk (t) developed from parent hypha (ph) and straight intraradical hypha (sih). 30. Vesicles (v). 31. Y-shaped branch (Yb) of intraradical hypha. 32. Arbuscule (a), coil (c) and straight intraradical hypha (sih). 29-32. In PVLG. 29-32. Differential interference microscopy. Scale bars: 29-32 = 10 µm.

## 2.6 Discussion

Here we report three new species of AM fungi, *D. omaniana*, *S. nakheelum* and *R. arabicus* and thus expand the currently known world-wide diversity and biogeography of this group of fungi. To our knowledge, these three species of AM fungi are the first ones ever to be newly discovered in the Arabian Peninsula. All three were found in a single geographical location characterized by extreme aridity. While one of the new species (*S. nakheelum*) was isolated from a date palm plantation subjected to irrigation, remarkably, the other two came from an undisturbed hyper-arid ecosystem, characterized by very low plant richness and abundance (Ghazanfar & Fisher, 1998; Pickering & Patzelt, 2008).

Our explorations focused only on a single geographical location and one sampling time. Therefore it is likely that our three new species represent only a small part of a larger, still hidden AM fungal biodiversity in these arid lands. It is known that many AM fungi display seasonal patterns of sporulation or may sporulate only rarely or not at all (Gemma *et al.*, 1989; Stürmer & Bellei, 1994; Stutz & Morton, 1996). Finally, we restricted ourselves to characterize AM fungi that could be cultured with leek as a host plant, and were derived from single-spore cultures. Indeed, out of more than 600 single-spore culture essays only 89 (ca. 15%) were successfully established. Based on this we suspect that many more AM fungal species may yet to be discovered in our study sites.

Our findings are relevant with regard to the raising interest in conserving all forms of life (World Conservation Union, 1980; Kareiva & Marvier, 2003). Two of the newly described species (*D. omaniana* and *R. arabicus*) were found exclusively in the harsh, undisturbed habitats and never under the date palms in the nearby experimental plantation, nor were they recovered in the respective trap cultures by which 16 soil samples were analyzed. Similarly, *G. macrocarpum* and *Ambispora gerdemannii* (S.L. Rose, B.A. Daniels & Trappe) C. Walker, Vestberg & A. Schüßler as well as other species were found only in the undisturbed habitats and not in the experimental date palm plantation (Al-Yahya'ei *et al.*, 2011). These species might be sensitive to the habitat alteration imposed by the plantation.

Currently we are subjecting the three newly discovered species to different investigations aiming to better understand their symbiotic functioning and capabilities for survival under extreme conditions of drought and heat. In addition we are expanding our explorations to other habitats in Southern Arabia to uncover a hidden part of ecosystem biodiversity that deserves to be revealed, appreciated and protected.

## 2.7 Aknowledgements

We thank Paola Bonfante (University of Torino) for her supportive role during the initiation of the AM fungal diversity studies in Southern Arabia; Ahmad Al-Bakri, the Director General of Agricultural and Livestock Research of Oman's Ministry of Agriculture and Fisheries for providing formal and logistical support; Hamood Al-Hashmi for his assistance during the sampling trips; Annette Patzelt (Oman Botanic Garden) for the plant identification. The study was financed by complementary grants from Oman's Ministry of Agriculture and Fisheries, the University of Basel, the National Centre of Science (grants no. N N304 061739 and DEC-2012/05/B/NZ8/00498) and the Swiss National Science Foundation which are gratefully acknowledged.

# 3 Isolation and identification of desert habituated arbuscular mycorrhizal fungi newly reported from the Arabian Peninsula

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## 3.1 Abstract

Arbuscular mycorrhizal fungi (AMF) are known to facilitate growth and vigour of many plants particularly in arid ecosystems. In a survey of AMF in a date palm plantation and two natural sites of a desert in Oman, we generated many single spore-derived cultures of AMF. We identified a number of these isolates based on spore morphotyping and molecular phylogenetic analysis using the sequence of the LSU-rDNA. Here, we present the characteristics of four species of AMF recovered, namely *Claroideoglomus drummondii*, *Diversispora aurantia*, *Diversispora spurca* and *Funneliformis africanum*. These four species have been described previously, but for the Arabian Peninsula they are reported here for the first time. Our endeavor of isolation and characterization of some AMF habituated to arid sites of Arabia represents a first step towards application for environmental conservation and sustainable agriculture in this region.

## 3.2 Introduction

Arbuscular mycorrhizal fungi (AMF) are well known to convey multiple benefits for plant growth and health, above all in stressful environments (Newsham *et al.*, 1995; Smith & Read, 2008). Under arid conditions, for example, mycorrhizal plants were found to maintain higher drought tolerance (Augé, 2001) and to have better access to phosphorus than non-mycorrhizal ones (Neumann & George, 2004). AMF may also enhance soil aggregate stability (Rillig & Mummey, 2006), a feature of particular relevance for the sandy soils prone to erosion.

Under the extreme conditions of desert ecosystems, AMF are believed to play an important role for the prospering of vegetation. Inoculation with AMF, for example, was found to improve water and nutrient uptake of desert succulents (Cui & Nobel, 2006), promote the desert tree *Prosopis cineraria* and thereby restore soil fertility by increasing soil organic carbon (Mathur & Vyas, 1995), enhance growth of the desert ephemeral plant *Plantago minuta* (Zhang *et al.*, 2011)and the productivity of *Plantago ovata*, a cash crop in arid and semiarid regions (Mathur *et al.*, 2006) and, furthermore, to have the potential to contribute to conservation efforts for endangered tree species (Panwar *et al.*, 2002; Shen & Wang, 2011).

The diversity of AMF has been investigated in many desert ecosystems of the world (Kiran *et al.*, 1989; Jacobson, 1997; Stutz *et al.*, 2000; Blaszkowski & Czerniawska, 2006; Uhlmann *et al.*, 2006; Shi *et al.*, 2007; Blaszkowski, 2012). One of the areas that have been neglected with respect to AMF until recently is the Arabian Peninsula. Our morphological and molecular analyses of this area revealed unique communities of AMF (Al-Yahya'ei *et al.*, 2011). This uniqueness may be attributed to the particularity of the studied ecosystems. Geological history and the influence of past climates of this area (Preusser *et al.*, 2002) have played a major role in shaping its unique and harsh environmental settings (Fisher & Membery, 1998; Glennie & Singhvi, 2002).

In the current study we combined further biodiversity explorations with the establishment of a single-species culture collection of AMF typical of this region. Such a collection is crucially needed as a basis for various basic and applied research. Isolation, characterization and propagation of AMF from these sites can open the door to apply them as a biotechnological tool (Gianinazzi-Pearson, 2002) suitable to promote sustainable agriculture and environmental conservation. As recently summarized (Antunes *et al.*, 2011), strains of AMF isolated from such sites are likely well-adapted ecotypes, and they may have unique physiological capabilities to cope with the extreme conditions prevailing in the desert, such as long drought seasons leading to desiccation, extreme heat during summer and low availability of soil nutrients. Therefore, these isolates might exhibit optimal performance in their original climate (Caravaca *et al.*, 2003; Calvente *et al.*, 2004; Marulanda *et al.*, 2007).

Here, we used morphological and molecular identification techniques to characterize a part of the successfully cultured single-spore derived isolates of AMF, namely those which have obvious affinities to well-described species.

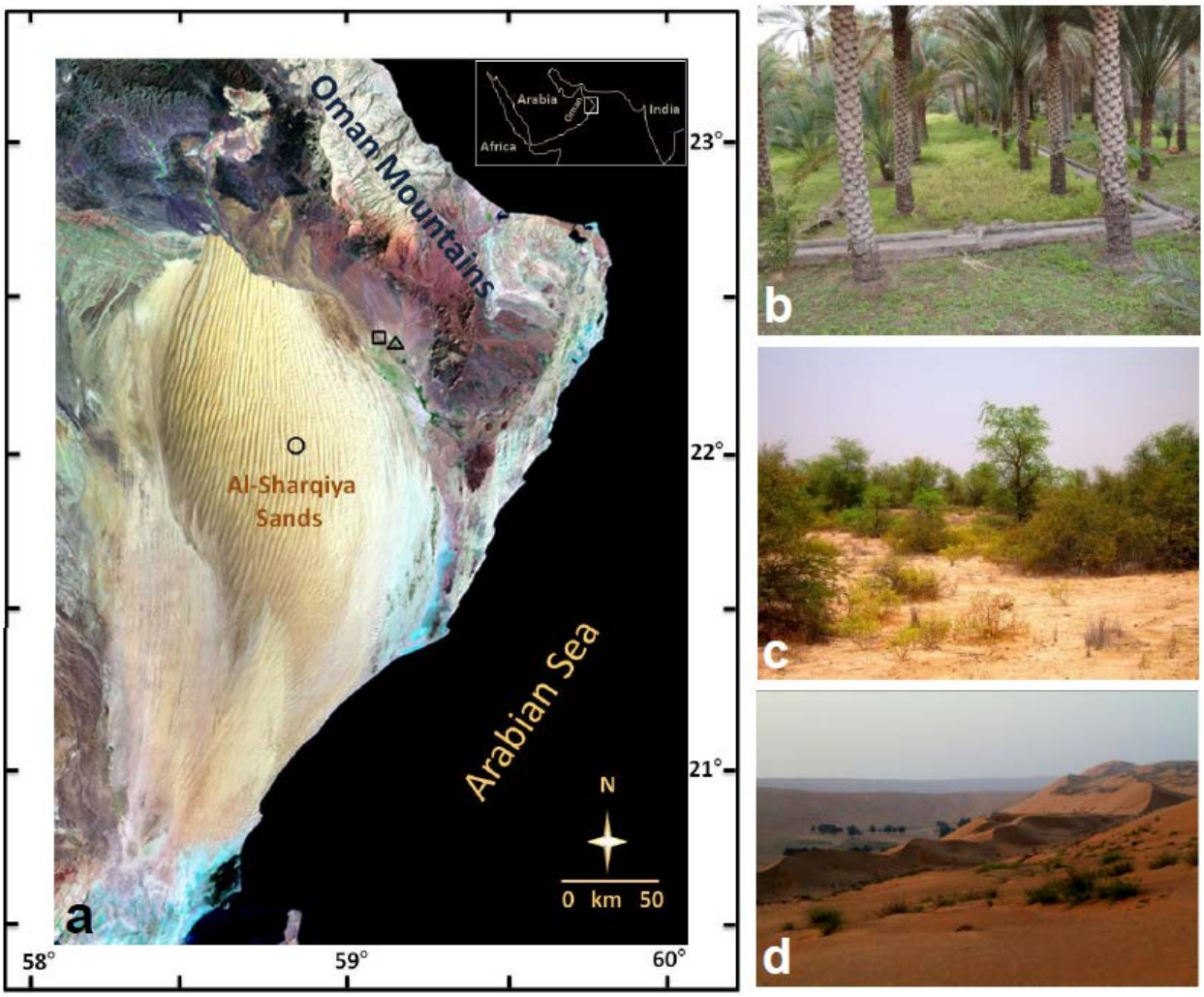


Fig. 3.1 The geography of the area and the explored sites. (a) Satellite image (Landsat TM RGB 742 courtesy of the U.S. Geological Survey) of Southern Arabia showing main geographical features of the area. Symbols correspond to sites depicted in b-d. (b) Traditional date palm plantation (for site, see triangle on Fig.3.1a). (c) Natural undisturbed site (square on Fig. 3.1a) with *Tetraena qatarensis*, *Salvadora persica* and *Prosopis cineraria* and interspersed patches of dry grasses. (d) Typical sand dune site in the region of Al-Sharqiya Sands (circle on Fig. 3.1a) where *Heliotropium kotschyi* occurs.

## 3.3 Materials and Methods

*Study area*

The area studied (Fig. 3.1a) occur in the northern territory of the Sultanate of Oman. The area is characterized by its hyper-aridity (UNEP, 2006) with an annual rainfall which does not exceed 100 mm (Fisher & Membery, 1998) and high summer temperatures exceeding 48°C (Glennie & Singhvi, 2002). The four species of AMF described here were recovered from one or more of three distinct sites (i) a date palm (*Phoenix dactylifera*) plantation in the oasis of Al-Kamel (22°12'56"N, 59°12'9"E) (Fig. 3.1b), (ii) a natural, undisturbed site (22°14'11"N, 59°10'53"E) (Fig. 3.1c) with *Tetraena qatarensis* Beier & Thulin.(previously *Zygophyllum qatarense*), *Salvadora persica* Wall., *Prosopis cineraria* (L.) Druce, and, in the area between these plants which we referred to as inter-plant area (IPA) where dispersed patches of dry grass were apparently growing after rainfall and (iii) a sand dune site (21°52'39"N, 58°52'44"E) in the Al-Sharqiya Sands (Fig.3.1d) with scattered *Heliotropium kotschyi* Gürke. More detailed information about the characteristics of the three sites is given in Al-Yahya'ei *et al*.(2011).

*Sampling, establishment and growth of trap and single-species cultures*

Four replicate plots of ca. 200 $m^2$ were randomly chosen at each site. From each plot, four plants of each species were selected for obtaining a pooled sample per plant species. The collection of soil and root samples and the procedure to establish the initial trap cultures have been described previously (Al-Yahya'ei *et al*., 2011).

The spores used for the morphological characterization were obtained from single-spore derived cultures established by the previously described pipette-tip procedure (Tchabi *et al.*, 2010), starting with single spores isolated from the initial trap cultures. The cultures were maintained for six months using a consortium of the following host plants for AMF: *Allium porrum* L., *Hieracium pilosella* L. and *Plantago lanceolata* L.

*Spore extractions*

Spores were extracted by wet sieving and sucrose density gradient centrifugation, using a modification of the method of Daniels and Skipper (1982). For each of the single-spore derived cultures, around 10 ml of harvested substrate were well suspended in 20 ml of water in a 50 ml Falcon tube. A 25 ml sucrose solution (70% v/w) was injected to the bottom of the tube forming a stepped density gradient that was centrifuged at 900x g for 2 min. Spores of AMF were collected from the interface of sucrose solution, washed with tap water on a 32-µm sieve for 2 min, and transferred to Petri dishes. Spores were then

picked individually under a stereomicroscope and either examined morphologically or used to extract DNA for molecular analysis.

*Microscopy identification*

Morphological characteristics of spores including their wall structure were determined based on examination of at least 100 spores mounted either in water, lactic acid, polyvinyl alcohol/lactic acid/glycerol (PVLG) (Omar *et al.*, 1979) or in a mixture (1:1, v/v) of PVLG and Melzer's reagent (water, iodine, potassium iodide). Spores at all developmental stages were crushed to varying degrees by applying pressure to the cover slip and then stored at 65$^{o}$ C for 24 h to clear their contents from oil droplets. They were then examined under an Olympus BX 50 compound microscope equipped with Nomarski differential interference contrast optics. Microphotographs were recorded on a Sony 3CDD color video camera coupled to the microscope.

Voucher specimens were mounted in PVLG and a mixture of PVLG and Melzer's reagent (1:1, v/v) on slides and deposited in the Soil and Water Research Center, Ministry of Agriculture and Fisheries, Sultanate of Oman; Institute of Botany, University of Basel, Switzerland; Department of Plant Protection (DPP), West Pomeranian University of Technology, Szczecin, Poland; and in the herbarium at Oregon State University (OSC) in Corvallis, Oregon, USA.

*Molecular analyses and phylogeny*

DNA was extracted by crushing single spores with a pipette tip in a 0.2 ml PCR tube containing 2 µl of 0.25 M NaOH. The tubes were incubated in a T3 Thermocycler (Biometra GmbH, Goettingen, Germany) at 95°C for 2 min before 1 µl of 0.5 M Tris HCl (pH 8.0) and 2 µl of 0.25 M HCl were added and incubated again at 95°C for 2 min. In between incubations the tubes were kept on ice. The extracts were either directly used as templates for polymerase chain reaction (PCR) or stored at -20°C. To obtain the LSU rDNA sequences, PCR was performed in a nested procedure with the SSUmAf-LSUmAr and the SSUmCf-LSUmBr primer pairs for the first and second nested PCR, respectively (Krüger *et al.*, 2009). The final concentration of the master mix contained 0.6 U Taq polymerase (GE Healthcare, Glattbrugg, Switzerland), 2 mM $MgCl_2$, 0.2 µM of each primer, 0.125 mM of each deoxynucleotide and 5 µg BSA (Bioconcept, Allschwil, Switzerland). Thermal cycling was done in the T3 Thermocycler with the following conditions for the first PCR: 3 min initial denaturation at 95°C; 35 cycles of 30 s denaturation at 95°C, 90 s annealing at 60° C, 2 min elongation at 72°C and 10 min at 72° C for final elongation. The same conditions were used for the nested PCR except that the annealing temperature was 63°C. The PCR products were visualized on 1.5% agarose gels with 1x TAE buffer and 1x Midori green for staining (NIPPON Genetics EUROPE GmbH, Dueren, Germany). The PCR products with the expected-size bands

of 1500 bp were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturers' protocols. PCR products were cloned into pGEM-T Vector system (Promega, Madison Wisconsin) and the clones were transformed into competent JM109 *Escherichia coli* cells (Promega, Madison Wisconsin) following the manufacturers' protocols. Ten positive clones from each single spore were confirmed with direct colony PCR technique using the universal M13F and M13R vector primers and purified with ExoSAP-IT (GE Healthcare, Glattbrugg, Switzerland). For sequencing of amplified clones the BigDye Terminator Cycle Sequencing Kit and the ABI3500 were used (Applied Biosystems, Foster City, California). Sequencing primers for the respective samples were the universal vector primers mentioned above in addition to ITS4 and ITS3 primers (White et al. 1990). The electropherograms were processed and analyzed with ChromasPro Version 1.5 (www.technelysium.com.au).

The Glomeromycotan origin of the sequences was initially tested by BLAST search. Sequences from the present study were aligned in PAUP*4b10 (Swofford, 2001) to those sequences from GenBank which showed high similarity to them, in addition to all available sequences for other species in their genera. The phylogenetic tree was inferred using maximum likelihood criteria as implemented in PAUP*. Sequences generated in this study were registered in GenBank under the accession numbers JQ287625 to JQ287641. The taxonomic nomenclature recently proposed by Redecker *et al.* (2013) was used for the species description of AM FUNGAL.

## 3.4 Results and discussion

*Taxonomy*

Morphological and molecular phylogenetic analyses (Fig. 3.2 and 3.3, respectively) consistently confirmed the affiliations of the fungal strains recovered in one or more of the three desert sites studied, and presented here, to four species of AMF. They were identified as *Claroideoglomus drummondii*, *Diversispora aurantia*, *Diversispora spurca* and *Funneliformis africanum*, clearly differing from each other and from other known species of AMF with glomoid spores and closely related in both morphology and molecular phylogeny.

Our two single spore-isolates of *Claroideoglomus drummondii* were clearly recognized morphologically by their yellow-coloured, small spores and the innermost spore wall layer 3, which usually stains intensively in Melzer's reagent (Fig. 3.2a,b) (Błaszkowski *et al.*, 2006). *C. walkeri*, the closest molecular phylogenetic relative of *C. drummondii*, produces white or much lighter spores (Błaszkowski *et al.*, 2006). Sequences from spores from two cultures (F41 and F72) of our collection of *C. drummondii*

clustered as a sister group to both *C. drummondii* (96% sequence similarity) and *C. walkeri* (94% sequence similarity) with a clearly shorter distance to *C. drummondii*.

Two of our single spore isolates belonged to *Diversispora aurantia*. The most distinct morphological feature of this species is the deep orange to golden yellow colour of its spores, their smooth surface, and their relatively thick, coriaceous *sensu* Walker (1986), innermost spore wall layer 3 (Fig. 3.2c,d) (Blaszkowski *et al.*, 2004). These render the spores of *D. aurantia* unique among species of AMF with glomoid coloured spores of a 3-layered spore wall, of which layer 3 is flexible to semi-flexible (Blaszkowski, 2012). Sequences from two cultures (G5 and I76) of *D. aurantia* cluster together with a sequence of this species (96% sequence similarity) (Fig. 3.3). *Diversispora spurca* spores are clearly distinct morphologically because their spore wall layer 1, which is loosely associated with a laminate spore wall layer 2, is continuous with subtending hyphal wall layer 1, being the main structural component of the subtending hyphal wall (Fig. 3.2e,f) (Pfeiffer *et al.*, 1996; Kennedy *et al.*, 1999; Blaszkowski, 2003). Subtending hyphal wall layer 2, continuous with spore wall layer 2, is present only closely at the spore base and hence it barely touches layer 1 of the subtending hypha. Consequently, in crushed spores the subtending hypha usually detaches along with spore wall layer 1 from the structural spore wall layer 2, forming the main extraradical body of the fungus (Fig. 3.2f). This phenomenon does not occur in any of the two other known *Diversispora* spp. and other described species of AMF with glomoid spores (Blaszkowski, 2012). Six different isolates (G64, E58, G1, K46, E84 and C65) showed these morphological characteristics. As it is shown in the phylogenetic tree (Fig. 3.3), they were all closest to *D. spurca* based on ribosomal LSU-based phylogeny (95% sequence similarity).

Two of our isolates belonged to *Funnelliformis africanum*. Among so far recognized species forming coloured spores with two spore wall layers, *F. africanum* is unique, because only its structural laminate spore wall layer 2 is thinner than the sloughing spore wall layer 1, forming the spore surface (Fig. 3.2g, h) (Błaszkowski *et al.*, 2010). Its closest phylogenetic relative, as indicated in our analyses of sequences of the LSU rDNA region (Fig. 3.3), *F. xanthium*, differs clearly in spore colour and size (much lighter and larger, respectively, in *F. africanum*), the number of spore wall layers (2 vs. 3 in *F. xanthium*), their phenotypic characters (e.g., layer 1, forming the spore surface, is much thicker and less persistent than that of *F. xanthium*), and features of the spore subtending hypha (Blaszkowski *et al.*, 2004, 2010). However, as results from phylogenetic analyses of sequences of the SSU region of rDNA (Błaszkowski et al., 2010 and our data not shown), *F. africanum* is closest to *F. constrictum*, which also differs much in spore colour, the phenotypic characters of spore wall components, and features of the spore subtending hypha (Trappe, 1977; Blaszkowski, 2003, 2012).

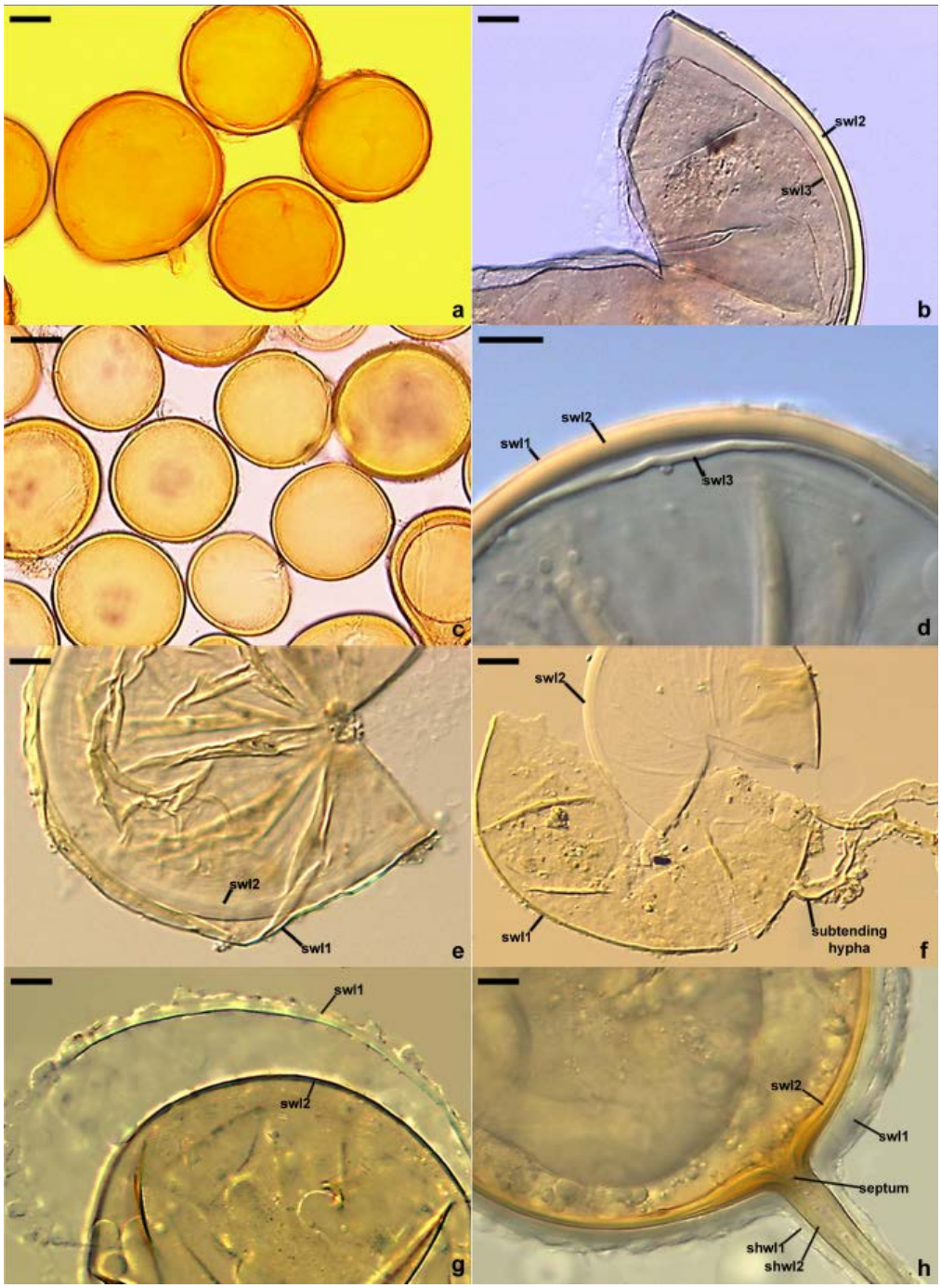


Fig. 3.2 Morphological characteristics of the spores. (a, b) *Claroideoglomus drummondii*. (a) Intact spores. (b) Laminate spore wall layer 2 (swl2) and flexible swl3 stained intensively in Melzer's reagent. Swl1 is completely sloughed in this spore. (c, d) *Diversispora aurantia*. (c) Intact orange spores. (d) Swl 1-3; note the thick, coriaceous swl3 separated from laminate swl2. (e, f) *Diversispora spurca*. (e) Crushed spore with clearly separated swl 1 and 2. (f) Swl 1 continuous with the main subtending hyphal wall layer 1 completely separated from swl2 in crushed spore. (g, h) *Funneliformis africanum*. (g) Separated swl 1 and 2 in crushed spore. (h) Swl1 and 2 continuous with subtending hyphal wall layers (shwl) 1 and 2 and septum in the lumen of subtending hypha; (g, h) note the much thicker swl1 than swl2. (a, c) Spores in lactic acid. (d, g, h) Spores in PVLG. (e, f) Spores in PVLG+Melzer's reagent. (a-h) differential interference microscopy. Scale bars: a, f = 20 μm, c = 50 μm, b, d, e, g, h = 10 μm.

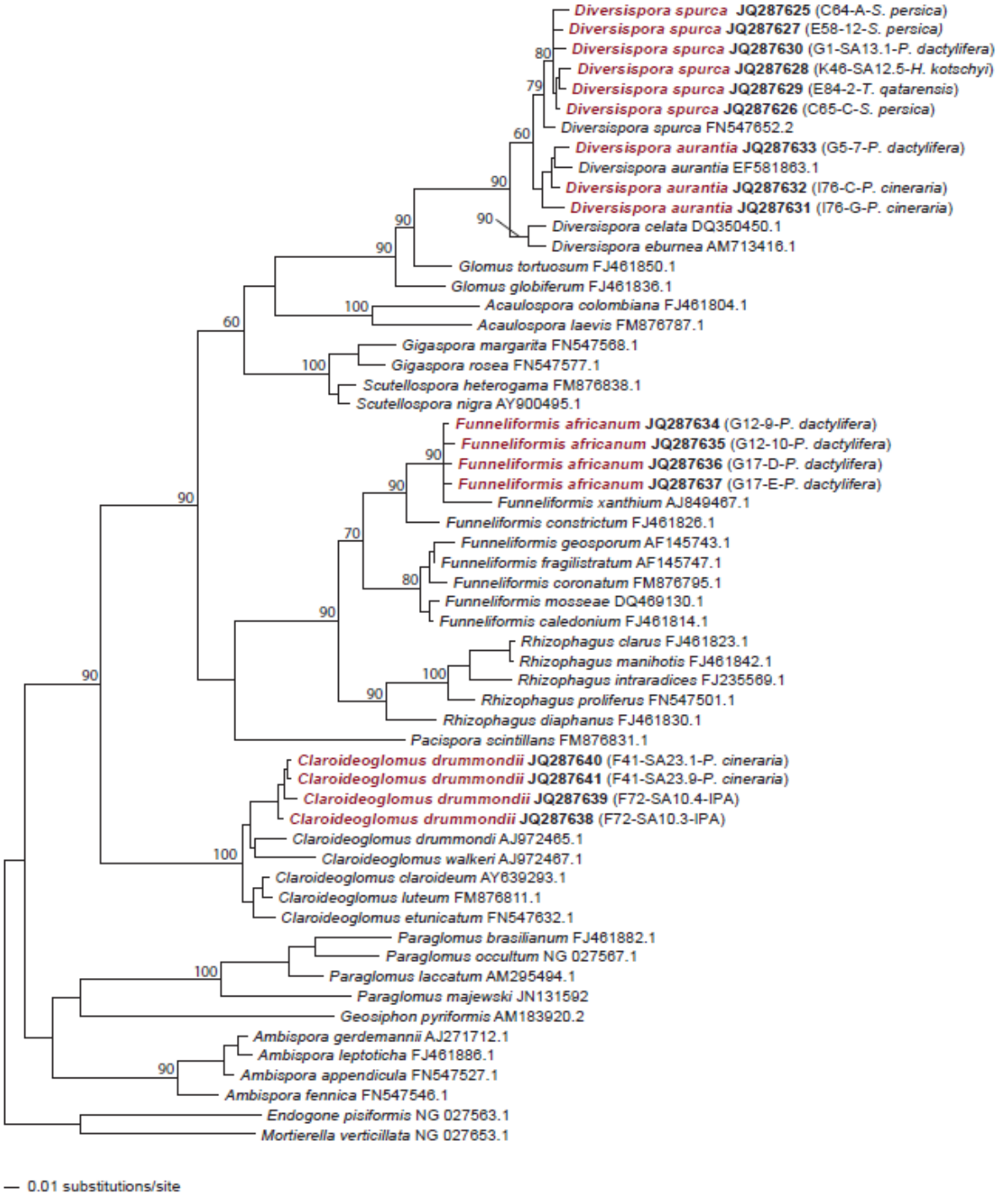


Fig. 3.3 Phylogenetic tree (maximum likelihood) was inferred from the partial LSU rDNA region (628 dataset characters), showing the positions of the four isolated species of AMF. The sequences obtained from these species are shown in coloured boldface followed by the GenBank accession number. The labels between the brackets indicate the isolate, clone code and either the likely host plant or the interspersed patch of dry grasses (IPA) from where the isolate was obtained. Values associated with branches are bootstrap values of maximum likelihood (100 replicates) as percentages. Only bootstraps above 50% are included.

*Global and local distribution*

Based on the previous literature review, all the species described here, except for *D. aurantia*, have a worldwide distribution. They were recorded from different sites in Asia, Europe, and the Americas (Trappe, 1977; Pfeiffer *et al.*, 1996; Kennedy *et al.*, 1999; Blaszkowski, 2003, 2012; Blaszkowski and Czerniawska, 2006; Błaszkowski *et al.*, 2010). The occurrence of *D. aurantia* is likely restricted to regions with warm climate. Of the *ca*. 6000 examined rhizosphere soils coming from different regions of the world, this species has never been found in its northern parts (Blaszkowski *et al.*, 2004; Blaszkowski, 2012*;* Błaszkowski, unpublished data). Pirozynski (1968) concluded that temperature is the major climatic factor determining the distribution and occurrence of fungi in general.

In this study, *D. aurantia* was recovered at two sites, the date palm plantation (*P. dactylifera*), and the natural undisturbed site (*P. cineraria*) while *F. africanum* and *C. drummondii* were recovered only at a single site, the date palm plantation and the undisturbed natural site (*P. cineraria* and IPA), respectively.

*D. spurca* was, interestingly, recovered at all the three contrasting sites and was associated with the five different plants in these sites, makes it a possibly generalist species in the area. This might be due to its ability to survive and function under contrasting soil conditions and different host plants in addition to its apparent ability to cope with the agricultural practices in the date palm plantation.

*Importance of the isolated species*

AMF are potential biotechnological tools (Azcón-Aguilar & Barea, 1997; Gianinazzi-Pearson, 2002; Koltai & Kapulnik, 2010) to be used as biofertilizers for sustainable agriculture (Siddiqui *et al.*, 2008) and in re-vegetation programs, especially for endangered plants (Bothe *et al.*, 2010) and desertified ecosystems (Requena *et al.*, 2001). A high potential for success was obtained when AMF were introduced to sites resembling to the site of their origin (Caravaca *et al.*, 2003; Calvente *et al.*, 2004; Marulanda *et al.*, 2007; Shen & Wang, 2011).

It has been suggested that AMF of the same species originating from contrasting climatic conditions are functionally diverse suggesting ecotypic differentiation (Antunes *et al.*, 2011) and that fungi exhibit optimal performance within the range of temperature resembling to their original climate (López-Gutiérrez *et al.*, 2008). Such a population differentiation has also been found to result from salinity stress (Carvalho *et al.*, 2004) and edaphic nutrient availability (Johnson *et al.*, 2010). It is reasonable then to propose that our isolated species belong to populations exhibiting unique traits and physiological properties adapted to withstand the extreme desert conditions such as desiccation in long drought seasons, hot summer temperatures and low soil nutrients. Therefore, these species represent good candidates to be used as biofertilizers to enhance crop productivity and the fitness and survival of native plants in soil re-vegetation and reclamation measures under the environmental conditions prevailing in the Arabian

Peninsula and, perhaps, other similar regions. However, functional studies under different arid land conditions and stresses are still needed to verify the physiological adaptability of our species to such conditions.

A prerequisite for the application of AMF as biofertilizers in agriculture and landscaping (Koltai & Kapulnik, 2010) is the availability of mass production procedures for the selected strains of AMF and of suitable, preferentially locally available carriers (Sreenivasa & Bagyaraj, 1988; Barea *et al.*, 1993; Kapulnik *et al.*, 1994; Douds *et al.*, 2006). Moreover, a strict quality control is essential including methods for ascertaining the absence of infestations with soil-borne pathogens and, preferentially, for assessing the performance of the inoculated AMF in the field.

In Oman, where we identified this collection of potentially desert-adapted strains of AMF, there is a great effort to preserve the country's natural vegetation (Patzelt *et al.*, 2008; Pickering and Patzelt, 2008; http://www.oman-botanic-garden.org). Mycorrhizal biotechnology might be a crucial factor to overcome difficulties met in propagation and maintenance of some indigenous plants (Patzelt *et al.*, 2008).

## 3.5 Conclusions

The four described species in this paper are recorded for the first time in the whole Arabian Peninsula and, thus, expand the species number currently known to occur in this arid region (Khaliel, 1989; Al-Yahya'ei *et al.*, 2011). Since some of our isolates come from extremely arid environments, they may be an asset to biodiversity conservation in desert areas, particularly in the Arabian Peninsula. They may also open the door for basic research regarding functional characterization, as well as for applied research in view of their use as biofertilizers to promote sustainable agriculture under arid conditions.

## 3.6 Acknowledgments

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# 4 Impact of water regimes on an experimental community of four desert arbuscular mycorrhizal fungal (AMF) species, as affected by the introduction of a non-native AMF species

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## 4.1 Abstract

Field studies have revealed the impact of changing water regimes on the structure of arbuscular mycorrhizal fungal (AMF) communities, but it is not known what happens to the abundance of individual AMF species within the community when the water conditions in the rhizosphere change. The behavior of four AMF species isolated from the Arabian desert (*Diversispora aurantia, Diversispora omaniana, Septoglomus africanum* and an undescribed *Paraglomus* species) was investigated when assembled in microcosms containing Sorghum bicolor as host plant, and treated with various water regimes. Furthermore, the impact of invasion of these assemblages by *Rhizophagus irregularis,* an AMF species widely used in commercial inocula, was studied. The abundance of each AMF species in sorghum roots was measured by determining the transcript numbers of their large ribosomal subunit (rLSU) by real-time PCR, using cDNA and species-specific primers. Plant biomass and length of AMF extraradical hyphae were also measured. The abundance of each AMF species within the sorghum roots was influenced by both the water regime and the introduction of R. irregularis. Under dry conditions, the introduction of *R. irregularis* reduced the total abundance of all native AMF species in roots and also led to a reduction in the amount of extraradical mycelium, as well as to a partial decrease in plant biomass. The results indicate that both water regime and the introduction of an invasive AMF species can strongly alter the structure of an AMF native assemblage with a consequent impact on the entire symbiotic mycorrhizal relationship.

## 4.2 Introduction

Water availability, and hence water regimes, in the soil are strong determinants for the community composition of microorganisms inhabiting the rhizospere. Soil harbors a considerable fraction of global biodiversity (Decaëns 2010) and soil microbial communities support a wide range of key ecosystem functions (Eisenhauer et al. 2012). Studies have demonstrated detrimental effects of drought on soil biota, with a consequent reduction in below-ground functioning (Kardol et al. 2010; Blankinship et al. 2011; Landesman et al. 2011). One of the widespread constituents of soil communities are the symbiotic arbuscular mycorrhizal fungi (AMF) which associate in a mutualistic symbiosis with 80% of all land plants (Parniske 2008). They have been shown to positively influence plant nutrition (Smith and Read 2008), plant productivity (Klironomos et al. 2000) and improve their host plants tolerance to biotic and abiotic stresses (Azcón-Aguilar and Barea 1996; Augé 2001; Hildebrandt et al. 2007; Pozo et al. 2010; Porcel et al. 2011).

The effect of drought on AMF communities has frequently been studied under field conditions. Changes in the community structure were revealed by assessment of AMF structures inside plant roots (Apple et al. 2005), by quantifying the amount of extraradical mycelium in the soil (Clark et al. 2009) and by molecular approaches (Querejeta et al. 2009; Sánchez-Castro et al. 2012). However, there is a lack in understanding how the soil water regime, as a single factor, affects individual AMF species in AMF communities. This can be studied best in model experiments under controlled conditions, in which the abundance of each AMF species can be traced individually using molecular markers.

Biotic exchange could threaten biodiversity (Sala 2000). The deliberate or accidental introduction of a species to an ecosystem can lead to the displacement or a change in the structure of native species communities. Accidental introductions can happen by a wide range of human activities; they have increased dramatically in the run of globalization (Mooney and Hobbs 2000; Hendrix et al. 2006). Deliberate introductions where an exotic species is set into a new habitat, for example to fight against a pest, have already led to a negative impact on biodiversity (Hall and Mills 2000; Lowe et al. 2000). Further, it has been shown that invasive belowground organisms can greatly alter aboveground and belowground ecosystem properties (Bohlen and Scheu, 2004).

Currently, the use of commercial AMF inoculants is growing, and certain AMF species (mainly Rhizophagus irregularis) are traded globally as biofertilizers and used in agriculture and revegetation

programs (Gianinazzi et al. 2002), even if they are not native at the site of application. The question of how the introduced AMF species would alter the native AMF community is still poorly understood and it has only been addressed in a few greenhouse studies. Recent studies demonstrated a decrease in the diversity of the native AMF community after inoculation, as revealed by terminal restriction fragment length polymorphism (T-RFLP) techniques (Mummey et al. 2009; Koch et al. 2010). However, no study evaluated the effect of the worldwide commercially used AMF species R. irregularis on the total abundance of AMF species inhabiting roots within native AMF communities.

A controlled experiment was therefore conducted under greenhouse conditions to evaluate the interacting effects of water regime and the presence of the potentially invasive AMF species R. irregularis, on an assemblage of AMF species previously isolated from natural and agricultural sites in Southern Arabia (Al-Yahya'ei et al. 2011; Symanczik et al. 2014a; Symanczik et al. 2014b). The AMF community colonizing roots of Sorghum bicolor was assessed by determining the transcript numbers of large ribosomal subunit (rLSU) genes of each individual fungus, using real-time PCR and species-specific primers. Using transcript numbers is considered more suitable for the comparison of AMF species differing in gene copy numbers, because the total expression of functional genes (the total amount of transcripts) can be expected to be similar between species to maintain their physiological functions (Gamper et al. 2008). The results show that both water regime and the introduction of a non-native AMF species can strongly alter the structure of a native AMF assemblage. These findings highlight the fragility of ecosystems and suggest that both factors can drastically influence the symbiotic mycorrhizal relationship. These changes could have great impacts on whole ecosystem functioning, especially in arid environments where the occurring AMF species are adapted to the environmental conditions.

## 4.3 Materials and Methods

*Plant growth conditions and experimental set-up*

Experiments were performed with sorghum (*Sorghum bicolor* (L.) Moench), cv Pant-5. Seeds were surface sterilized (10 min in 2.5 % KClO) and then rinsed with sterile water several times and soaked in sterile water overnight. Seeds were germinated on sterile moist sand at 25 °C for 3 days in darkness. The four fungal strains isolated from a hyper-arid sand plain in Oman (*Diversispora aurantia*, *Diversispora omaniana*, *Septoglomus africanum* and an undescribed *Paraglomus* sp.) and additionally *Rhizophagus irregularis* BEG-75 (Botanical Institute, Basel, Switzerland) were propagated as previously described (Symanczik *et al*., in press a/b). To establish AM symbiosis, three pre-germinated seeds were individually inoculated in 1-L pots filled with 1100g of autoclaved (120 °C, 20 min) mixture of sand (quartz sand,

0.125-0.25mm; Kaltenhouse, Alsace, France), Terragreen (American aluminum oxide, oil-dry U.S. special, type III R, 0.125 mm; Lobbe Umwelttechnik, Iserlohm, Germany), and Loess from a local site (8:2:1, w/w/w). Two fungal treatments were applied: the AMF-native-assemblage (*D. aurantia, D. omaniana, S. africanum* and *Paraglomus* sp.) and the AMF-invaded-assemblage (*D.aurantia, D. omaniana, S. africanum, Paraglomus* sp. and in addition *R. irregularis*). Hundred spores of each AMF species were used to prepare each mix, i.e. a total of 400 spores for the AMF-native-assemblage and 500 spores for the AMF-invaded-assemblage. Each pot received 5 ml of filtered washings of AMF inoculum to correct for possible differences in microbial communities (Koide & Elliott, 1989) . This filtrate was prepared by wet sieving 100 g of each inoculum through a 32-mm sieve and a paper filter (FS 14 1/2; Schleicher & Schuell), yielding a final volume of 1 L.

During five weeks, plants were watered twice a week with distilled water. Then, three water regimes were applied namely "WW" (well-watered condition, 80-100% field capacity "FC"), "DS" (drought-stressed condition, 35-55% FC) and "DC" (drying cycles, 35-100% FC). Soil water content was monitored and adjusted by weighing the pots periodically twice per week. In addition, the pots received 10 mL of a phosphorus-free Hoagland solution (Gamborg & Wetter, 1975) weekly. All plants were grown in the greenhouse under controlled conditions (light: 16 h of $\geq$700µmol $m^{-2}$ $s^{-1}$intensity (PPFD); temperature: 20-25°C; relative humidity: 65%). The experiment was set up in a randomized block design where each treatment was replicated six times.

*S. bicolor* plants were harvested after 16 weeks of growth. Three subsamples of about 100mg of chopped fresh root pieces were snap-frozen and stored at -80°C for further quantification.

*Quantification of AMF root colonization by real-time PCR, using species-specific primers for rLSU*

The abundance of each fungal species in sorghum roots was measured within the two AMF assemblages by quantitative PCR on cDNA, using primers targeting species-specific motifs in the rLSU genes. RNA extraction, cDNA synthesis and qPCR analysis were performed as described by Courty et al. (2009), and as specified in Online Resource 1. Selected primers (Microsynth AG, Balgach, Switzerland) were specific and yielded amplification products only with the targeted species, and not with any of the other four species.

*Hyphal length density*

Hyphal length density (HLD) was determined by the modified grid-line intersection method (Jakobsen *et al.*, 1992), using 10 g of the growth substrate. After sieving successively through a 400 and a 32 µm mesh, the material was collected and transferred into 50 mL of distilled water and homogenized for 10 s at full speed in a blender. The suspension was transferred into a beaker, diluted to 500 mL and stirred for 1 min before five subsamples were taken every 10 s and loaded onto the Filtration apparatus (MF-Membranfilter 1.2µm; Millipore).

*Statistical analyses*

Data were analyzed using either one-way ANOVA (to compare transcript numbers of rLSU gene of individual AMF species among water regimes), two-way ANOVA (with the factors AMF assemblage and water regime for HLD and biomass) followed by Tukey's honest difference test with a significance level of $\alpha = 0.05$ or an independent-samples t-test. Transcript numbers of rLSU genes of the AMF species were ln(x)-transformed before statistical analyses. Correlations were calculated using Pearsons correlation. Analyses were performed using JMP software version 5.0.1 (SAS, North Carolina, USA)..

Table 4.1 Transcript numbers of large ribosomal subunit genes (rLSU per ng RNA) of the arbuscular mycorrhizal fungal (AMF) species within the native AMF and *Rhizophagus irregularis*-invaded assemblages exposed to three water regimes.

| AMF assemblage | Water regime | AMF species abundance | | | | |
|---|---|---|---|---|---|---|
| | | *Septoglomus africanum* | *Diversispora aurantia* | *Diversispora omaniana* | *Paraglomus* sp. | *Rhizophagus irregularis* |
| AMF native assemblage | WW | 196 a | 40 a | 714 a | 376 a | np |
| | DC | 163 a | 1 b | 210 b | 58 b | np |
| | DS | 308 a | 4 b | 122 b | 176 a | np |
| | $F_{ANOVA}$ | 0.16ns | 22.69*** | 12.82** | 11.3** | |
| AMF invaded assemblage | WW | 77 a | 122 a | 225 a | 548 a | 2747 b |
| | DC | 34 a | 2 b | 43 a | 101 b | 1763 b |
| | DS | 79 a | 1 c | 6 b | 56 b | 6948 a |
| | $F_{ANOVA}$ | 0.16ns | 121.49*** | 15.82*** | 37.06*** | 13.83*** |

The AMF-native-assemblage includes the species *Septoglomus africanum*, *Diversispora aurantia*, *Diversispora omaniana* and *Paraglomus* sp., the AMF-invaded-assemblage includes the same four species and additionally *Rhizophagus irregularis*. The water regimes analysed were well-watered (WW), drying cycles (DC) and drought-stressed (DS). Letters following the means of transcript numbers of large ribosomal subunit genes (n=6) indicate significant differences within AMF species between water regimes ($p<0.05$). Data were analyzed using one-way ANOVA followed by Tukey's honest significant difference test with a significance level of $\alpha = 0.05$; *, $p<0.05$; **, $0.001 \leq p<0.01$; ***, $p<0.001$; $F_{ANOVA}$ is also given. Ns and np mean not significant and not present, respectively.

## 4.4 Results

*Response of the AMF assemblages to different water regimes*

The abundance of the different AMF species within roots, expressed as transcript numbers of rLSU genes per ng RNA, varied strongly when the native AMF assemblage and the *R. irregularis*-invaded assemblage were exposed to the different water regimes (Table 4.1). No significant difference in transcript numbers was measured for *S. africanum*. Conversely, the abundance of *D. aurantia* ($p<0.001$ for both AMF assemblages), of *D. omaniana* and of *Paraglomus* sp. ($p<0.01$ and $p<0.001$ for the native AMF and *R. irregularis*-invaded assemblages, respectively) was significantly decreased under DS conditions, DC or both water regimes. Interestingly, the abundance of *R. irregularis* significantly increased from WW to DS conditions compared to the AMF species from the native AMF assemblage ($p<0.001$). Changing the water regime modified the structure of the native AMF assemblage colonizing the roots (Fig. 1a). Under WW and DC conditions, it was significantly dominated by *D. omaniana* with a relative transcript abundance of 56% and 47%, respectively, whilst under DS conditions, *S. africanum* and *Paraglomus* sp. dominated with a relative abundance of 45% and 34%, respectively.

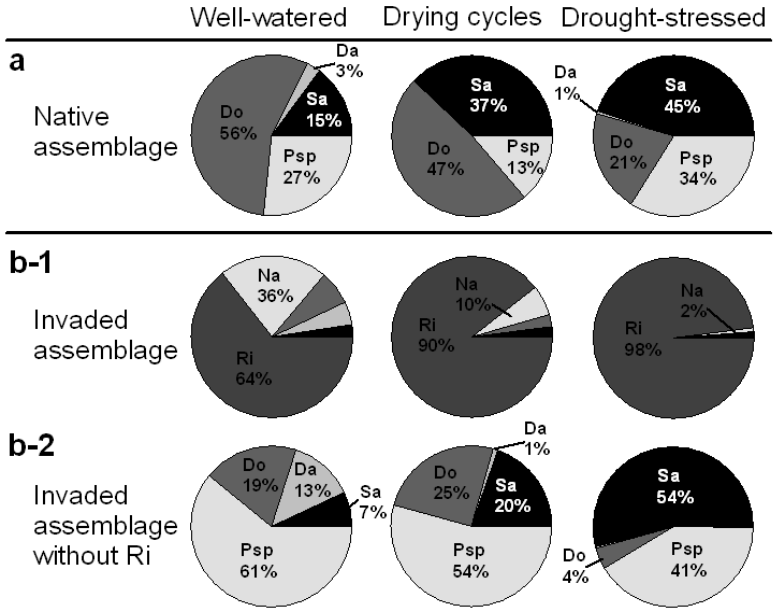


Fig. 4.1 Relative transcripts abundance of large ribosomal subunit (rLSU) genes of the different arbuscular mycorrhizal fungal (AMF) species within (a) the "native AMF assemblage" (NA) containing *Septoglomus africanum* (Sa), *Diversispora omaniana* (Do), *Diversispora aurantia* (Da) and *Paraglomus* sp. (Psp), (b-1) the "*Rhizophagus irregularis*-invaded assemblage", and (b-2) the "*Rhizophagus irregularis*-invaded assemblage minus Ri" (where *Rhizophagus irregularis* (Ri) was excluded for calculations), exposed to the three different water regimes: well-watered, drying cycles and drought-stressed. Each slice represents a mean of six replicates.

*Introduction of* Rhizophagus irregularis *into the AMF-native-assemblage*

*R. irregularis* was clearly the most abundant AMF species colonizing sorghum roots under the three different water regimes, as reflected by a relative transcript abundance of 64% in WW conditions, and of 90% and 98% in DC and DS conditions, respectively (Fig. 4.1b-1). Under DS and DC conditions, the introduction of *R. irregularis* strongly decreased the rLSU transcript numbers of the other species in the native AMF assemblage while no reduction was observed under WW conditions (Fig. 4.2a). Interestingly, the introduction of *R. irregularis* differentially affected the abundance of the native AMF species colonizing roots (Fig. 4.2b-e). The abundance of *S. africanum* (Fig. 4.2b) and of *D. omaniana* (Fig. 4.2d) was significantly decreased under the three different water regimes, while the abundance of *Paraglomus* sp. (Fig. 4.2c) was significantly decreased only under the DS condition. In contrast, the abundance of *D. aurantia* (Fig. 4.2e) was significantly increased under WW conditions. Additionally, the introduction of *R. irregularis* modified the community structure of species from the native AMF assemblage colonizing the roots (Fig. 4.1b-2). Under WW and DC conditions, *Paraglomus* sp. significantly dominated the assemblage with a relative transcript abundance of 61% and 54%, respectively. Under DS conditions, *S. africanum* and *Paraglomus* sp. together dominated the assemblage with a relative transcript abundance of 54% and 41%, respectively.

*Hyphal development and plant biomass production*

Under the DS condition, the fungi from the native AMF assemblage produced significantly more extraradical hyphae than fungi from the *R. irregularis*-invaded assemblage (Fig. 4.3a). No significant differences in HLD were measured under DC and WW conditions. The HLD of the native AMF assemblage was similar under all three water regimes: 23.1 cm g-1, 26.7 cm g-1 and 28.3 cm g-1 soil dry weight for DC, DS and WW conditions, respectively. In the *R. irregularis*-invaded assemblage, the HLD significantly differed between the water regimes: 17.2 cm g-1, 19.9 cm g-1 and 35.6 cm g-1 soil dry weight for DC, DS and WW conditions, respectively.

Plant biomass correlated with the HLD ($p<0.01$). The plant biomass was significantly affected by water regime ($p<0.001$) and the interaction of water regime*AMF assemblage ($p<0.05$). The three water regimes affected the growth of sorghum differently, depending on the AMF assemblage (Fig. 4.3b). Dry weight of plants inoculated with the native AMF assemblage was significantly reduced only under the DC condition (3.9 g), compared to DS (5.0 g) and WW conditions (5.2 g). In contrast, the dry weight of plants inoculated with the R. irregularis-invaded assemblage was generally reduced under dry conditions (4.4 g for DS conditions and 3.6 g for DC) compared to the WW condition (5.5 g). Overall, drought resistance of the plants was reduced when R. irregularis was introduced into the AMF assemblage, as shown by the significant water regime*AMF assemblage interaction.

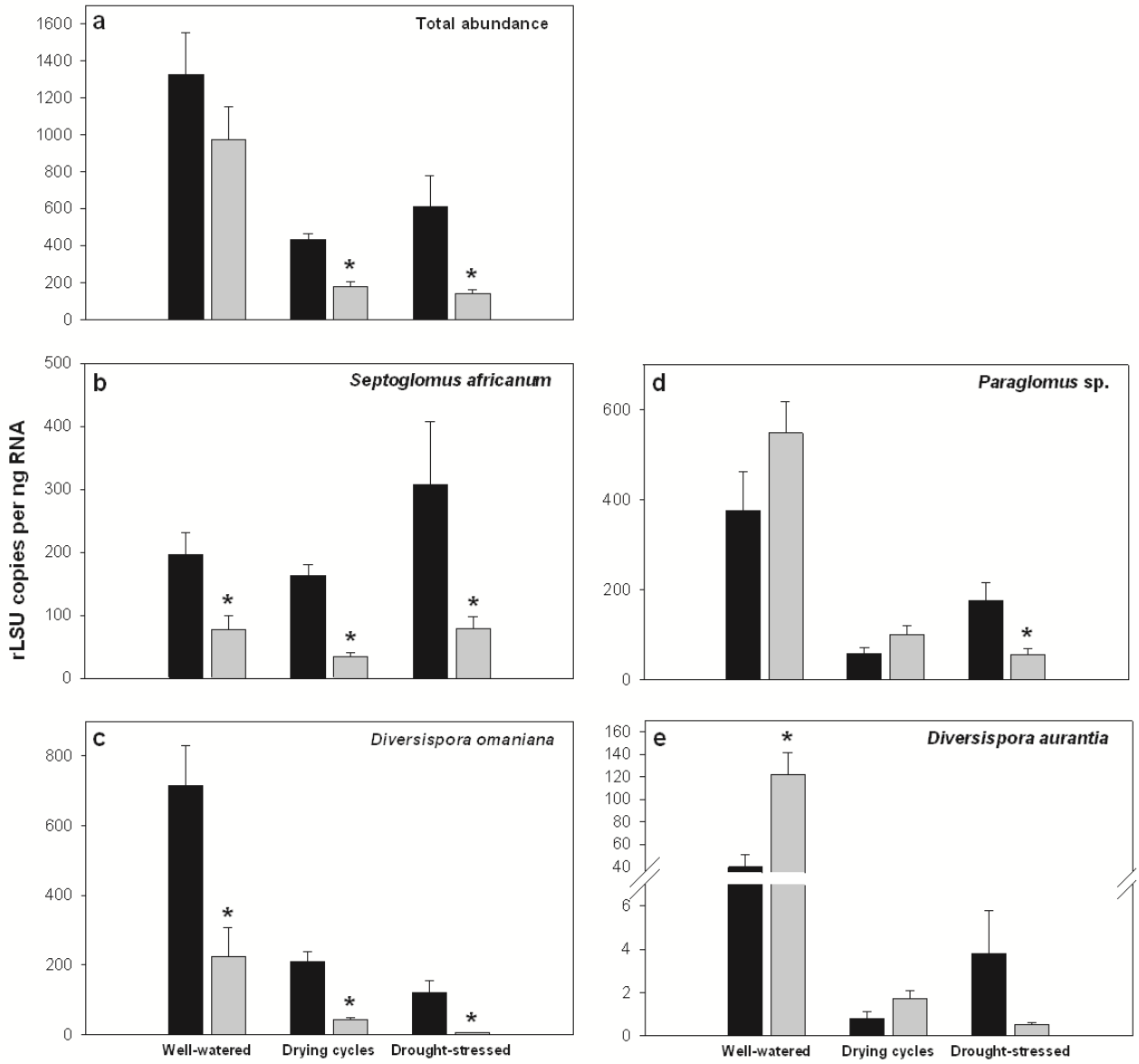


Fig. 4.2 Comparison of the abundance of the native AMF species, measured by transcript abundance of large ribosomal subunit (rLSU) genes, exposed to the three water regimes: well-watered, drying cycles and drought-stressed. The abundances were measured as (a) total abundance of the four native AMF species and (b-e) separately for each species without (black bars) and with (gray bars) introduction of *Rhizophagus irregularis.* Stars indicate significant differences of rLSU transcript numbers with and without the introduction according to the independent-samples *t*-test. Data represent means + SE (n=6).

## 4.5 Discussion

Results from the present greenhouse experiment demonstrate that changes in the water regime can have a strong impact on the abundance of AMF inside a host root, and that AMF species can react differently upon such changes. Data illustrate that, under drought conditions, the introduction of a potentially invasive AMF species can lead to partial displacement of a native, drought-adapted AMF assemblage and to a significant decrease in HLD, which might reduce the plant benefit conferred by the fungi.

Based on rLSU transcript numbers, the four AMF species in the experimental assemblage with sorghum roots were differentially affected by the different water regimes. The abundance of *D. aurantia*, *D. omaniana* and *Paraglomus* sp. decreased when exposed to dry conditions, while the abundance of S. africanum remained constant. Ruiz-Lozano et al. (1995) previously reported variable changes in root colonization of lettuce plants by AMF under different water regimes: under dry conditions, the level of colonization decreased in roots inoculated by *Glomus mosseae* or *G. occultum* but remained constant when inoculated by *G. deserticola* or *G. etunicatum*. Differences in root colonization ability might be a result of the physiological and functional characteristics of AMF species (Fitter 2005), and may be influenced by specific adaptations to environmental conditions prevalent at the place where the AMF species originated from (Marulanda et al. 2007; Lekberg and Koide 2008). Due to such adaptations, AMF species or even different strains of a same species might be either restricted or dominant in an ecosystem, leading to distinct AMF communities in different continents and climatic zones (Opik et al. 2006; Opik et al. 2013). Egerton-Warburton et al. (2007) and Querejeta et al. (2009) reported a dominance of *Glomus* species in AMF communities in soils of xeric habitats. Likewise, *S. africanum* and *R. irregularis*, both belonging to the family of *Glomaceae*, were either not affected or showed an increase in abundance in sorghum roots under DS conditions.

A water regime-dependent shift in the AMF community colonizing sorghum roots was observed under DS conditions; the dominant *D. omaniana* was replaced by *S. africanum* and *Paraglomus* sp. Shifts in AMF community structure due to seasonal changes in precipitation have also been reported under field conditions, and included changes in AMF structures inside host roots (Martinez-Garcia et al. 2012), in the production of extraradical mycelium and glomalin (Clark et al. 2009) or of DGGE- or T-RFLP-band patterns of DNA root extracts (Hawkes et al. 2011). Furthermore, Querejeta et al. (2009) found a shift from *Glomaceae*- to *Gigasporaceae*-dominance in an oak forest between dry and wet years, respectively. Several other studies have revealed the importance of environmental factors in shaping AMF communities, such as host plant identity (Bever et al. 1996; Helgason et al. 1998; Bainard et al. 2014), soil type (Landis et al. 2004 ; Lekberg et al. 2007) or soil management practices (Jansa et al. 2003; Oehl et al. 2003; Al-Yahya'ei et al. 2011).

*R. irregularis* was by far the most abundant AMF species in sorghum roots inoculated with the *R. irregularis*-invaded assemblage under all the three water regimes when comparing the relative abundance of rLSU transcript numbers. The formation by *R. irregularis* of intraradical spores, not formed by species of the native AMF assemblage, probably overestimated the abundance of the fungus as spores have a high nucleic acid content (Gamper et al. 2008). It is therefore difficult to draw conclusions about the physical dominance of *R. irregularis* in terms of intraradical hyphal length or number of active exchange sites by comparing transcript abundance with that of the other species in the native AMF assemblage. Nevertheless, the significant reduction in the total abundance of the rLSU transcripts of the native AMF assemblage under DS and DC conditions does suggest their replacement within the root and consequently a dominant physical presence of *R. irregularis*, even though this might be only by the production of intraradical spores. The dominance of *R. irregularis* over other AMF has already been reported. It was shown to be the most abundant AMF when grown in competition with *G. aggregatum* (Engelmoer et al. 2013; Werner and Kiers 2014), and to dominate over *G. mosseae* when grown under saline stress, phosphorus stress or in association with certain host plants (Alkan et al. 2006). In contrast, Jansa et al. (2008) found that *G. mosseae* was a better competitor compared to *R. irregularis* or *G. claroideum* under certain agricultural practices. These different observations might be explained, in part, by the fact that different AMF strains were used.

Inoculation with *R. irregularis* differentially affected the AMF species in the native AMF assemblage colonizing sorghum roots. Under WW conditions, the introduction of *R. irregularis* modified the abundance of the other AMF species, repressing *S. africanum* and *D. omaniana* and stimulating *D. aurantia*. It has similarly been observed that *G. mosseae* can exert a decreasing and increasing effect on the abundance of *R. irregularis* and *G. claroideum*, respectively (Jansa et al. 2008). In addition, Hart et al. (2012) found that the abundance of *G. custos* colonizing Plantago roots increased when associated with other AMF species, and that the identity of the co-occurring AMF species determined the root colonization level of *G. custos*. The diverse interactions found between co-occurring AMF species might be attributed to different species-specific colonization patterns (Bever et al. 2009; Verbruggen et al. 2012). For example, Hart et al. (2002) observed that AMF species from the *Glomeraceae* invest more in the production of hyphae inside the root whereas species from the *Gigasporaceae* invest more into the extraradical mycelium. Consequently, they suggested that functional traits may be phylogenetically conserved, as also proposed by Maherali and Klironomos (2007).

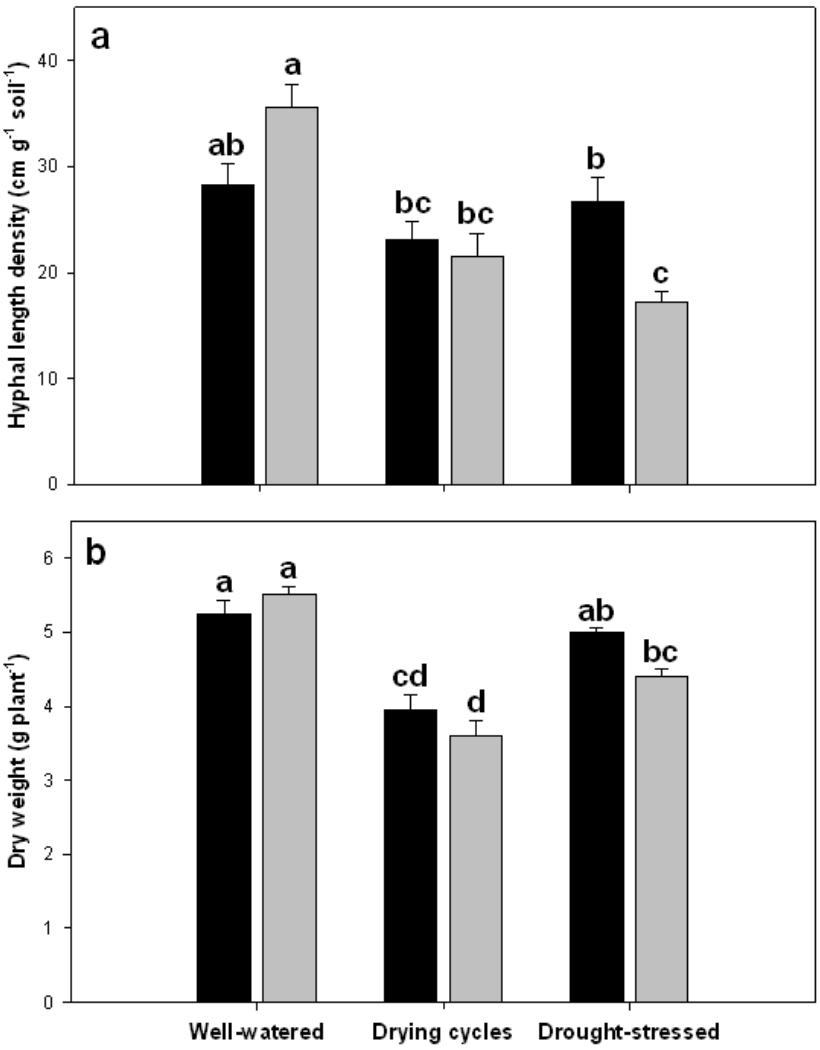


Fig. 4.3 Impact of the introduction of *Rhizophagus irregularis* on (a) the hyphal length density (HLD) in the soil and (b) the combined shoot and root biomass of sorghum plants inoculated with the native AMF assemblage (black bars) and the *R. irregularis*-invaded assemblage (gray bars), exposed to the three different water regimes: well-watered, drying cycles and drought-stressed. Different letters above bars indicate significant differences according to Tukey's honest significant difference test with a significance level of $\alpha = 0.05$. Data represent means + SE (n=6).

Differences in the functional traits of AMF species might also explain the absence of correlation between rLSU transcript abundance inside the sorghum roots and the production of extraradical hyphae. Under the DS condition, extraradical hyphal production and, partially, dry weight accumulation of sorghum plants were reduced when *R. irregularis* was added to the native AMF assemblage, although the rLSU transcript abundance of *R. irregularis* reached its maximum value. This reduction might be explained by the species-specific colonization pattern of *R. irregularis*, and the consequent replacement of AMF species from the native AMF assemblage inside the roots. The fungal species in the native AMF assemblage came from a hyper-arid environment and might, therefore, be better adapted to dry conditions and better

proliferate in dry soils. Up to date, only a few studies have investigated the impact of different water regimes on the performance of AMF species in terms of extraradical mycelium production. For example, exotic and native isolates of *G. mosseae* and *R. irregularis* were found to exhibit diverse adaptation to water availability when grown under drought conditions, with the production of extraradical mycelium as measured by glomalin accumulation being highest in soils colonized by the native AMF (Marulanda et al. 2007). Furthermore, other studies investigating the adaptation of AMF species to distinct environmental conditions by assessing differences in plant growth have revealed that mycorrhizal growth promotion was best when the experimental conditions, e.g. water availability or temperature, were closest to the environmental conditions from where the AMF were isolated (Marulanda et al. 2007; Lekberg and Koide 2008; Antunes et al. 2011). Another aspect that might influence the effect of mycorrhizal colonization on the plant is functional complementarity of AMF in the symbiosis. Several studies have shown that AMF species exhibit different functions for the host plant (Smith et al., 2004; Facelli et al., 2010). The present study similarly suggests that a community of native AMF species can buffer against different water regimes, as reflected by the constant production of extraradical mycelium under all water regimes although changes in the fungal community structure were observed. The results from these different investigations implicitly explain the importance of AMF diversity for host plant performance, especially by buffering the system against diverse stresses.

The observed decrease in extraradical hyphal production under dry conditions when the exotic species *R. irregularis* was introduced into the native AMF assemblage has implications regarding the possible consequences of a commercial application of AMF species. Long-term studies and AMF community surveys of field sites, where AMF inoculants have been applied, would shed light on the question about the impact of their application on the native AMF communities. Molecular tracing of introduced AMF has been successfully applied to investigate their long-term persistence under field conditions (Sýkorová et al. 2012). Additionally, some studies further assessed the impact of AMF introduction on native AMF communities showing contrasting results. Whereas Koch et al. (2011) and Mummey et al. (2009) demonstrated a decrease in the diversity, Antunes et al. (2009) and Alguacil et al. (2011) observed no and even promoting effects on native AMF communities, respectively. Without such knowledge about possible alterations of these communities due to current application practices, there remains a risk of future unwanted consequences (Schwartz et al. 2006). The results reported here should also be considered with respect to revegetation programs, as AMF inocula may help to establish plants, especially in arid and semi-arid ecosystems (Requena et al. 1996; Azcón-Aguilar et al. 2003). These and other studies underline the importance of selecting appropriate fungal ecotypes, preferentially originating from the natural surroundings of the targeted application site, for producing adapted inocula for AMF-assisted revegetation programs (Barea et al. 2011).

In conclusion, changes in soil water availability can have a strong effect on the AMF community structure inside the roots of a host plant. In addition, the introduction of an AMF species widely used in commercial inoculum, such as R. irregularis, can lead to a partial displacement of native AMF species with an associated decrease in the extraradical mycelium. Considering the current worldwide spread of droughts, in addition to widespread biotic exchanges in a globalised world, these two factors may interact to hamper plant benefits provided by drought-adapted native AM fungi and, therefore, may affect ecosystem functioning.

## 4.6 Acknowledgments

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## 4.7 Supplementary data

*RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)*

Roots were ground in liquid nitrogen and total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, Darmstadt, Germany). The DNA-*free* set (Ambion, Austin, USA) was used to digest DNA after RNA purification.

Quantification of transcript numbers of large ribosoma subunit (rLSU) genes was performed using a two-step qRT-PCR procedure. Total RNA was measured with a spectrophotometer (Nanodrop ND-1000, Witec, Switzerland) and then reverse-transcribed (100 ng per reaction) using the iScript cDNA Synthesis kit (Bio-Rad, Paolo Alto, CA, USA). cDNAs were used as templates in qRT-PCR reactions with gene-specific primers designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and amplify 3.1 (http://engels.genetics.wisc.edu/amplify) (table S4.1).

*Design of qRT-PCR markers*

Primers were designed for specific detection and quantification of *Diversispora aurantia*, *Diversispora omaniana*, *Septoglomus africanum*, an undescribed *Paraglomus* sp. and *Rhizophagus irregularis* BEG-75 based on sequencing data published previously (Symanczik *et al.*, *in press a/b*). Primers were targeting species-specific motifs in the rLSU genes and were tested on cDNA synthesized from AMF spores as well as mycorrhized *S. bicolor* roots cultivated separately with the different AMF species.

cDNA was subjected to PCR amplification of the rLSU genes as previously described (Symanczik *et al.*, 2014 a/b) using designed primers. The thermal cycling conditions were modified only: initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30s - annealing at 55°C for 30s- elongation at 72°C for 60 s, and for final elongation at 72°C for 5 min. Amplified DNA fragments were cloned into pGEM-T Vector system (Promega, Madison Wisconsin) and further transformed into competent JM109 *Escherichia coli* cells (Promega, Madison Wisconsin) following the manufacturers' instructions. Plasmids were isolated from overnight cultures of the transformed *E. coli* JM109 (Promega), grown on LB medium supplemented with ampicillin (100 μg $mL^{-1}$) using the ZR Plasmid Miniprep™ -Classic kit (ZYMO Research, Irvine, California) and were sequenced according to Symanczik *et al.* (2014a). The sequences were individually edited and the identity of the sequences was confirmed by alignment with the full rLSU sequence of each AM FUNGAL strain. The concentration of the plasmids was estimated with a spectrophotometer (Nanodrop ND-1000, Witec, Switzerland). The plasmids were linearized using NcoI-HF™ digestion (New England BioLabs®, Ipswich, MA, USA), incubating the samples at 37°C for 2 h. The concentration of plasmid copies per unit of sample volume was calculated according to Jansa et al.(2008), by knowing the concentration of DNA in each sample, length of insert (127bp for *S. africanum*,

126bp for *D. aurantia*, 79bp for *D. omaniana*, 71bp for *Paraglomus sp*. and 76bp for *R. irregularis*), and length of vector (3000 bp), and by assuming molecular weight per nucleotide double-stranded DNA to be 660 Da. Plasmid preparations were serially diluted (10-fold) to achieve a brought range of plasmid concentrations. These were used as templates for generating calibration curves for the conversion of the qRT-PCR detection cycle to rLSU transcript numbers of the AMF species in root samples in an absolute quantification assay.

The primers were confirmed to amplify their target AMF species and to avoid cross-targeting of AMF species as well as sorghum DNA (tested on spores and colonized roots of *S. bicolor*). Cross-amplification and dilution tests were performed using the diluted plasmid preparations as described above to confirm either the species-specificity of the primer-pairs (selection was based either on no cross-amplification with other AMF species or on a Cp value difference of more than ten cycles between target and non-target species) or the effectively of the primer-pairs (correct amplification even if target template is present at low concentrations).

*qRT-PCR measurements*

All qRT-PCR measurements were carried out in 10 µL reaction formats, a LightCycler® 480 System and with Roche SYBR green MasterMix (Roche Diagnostics AG, Rotkreuz, Switzerland). After optimization of the cycling conditions using different concentrations of the primers, the final concentrations in the master mix were 0.4 µM and 0.6 µM for *Paraglomus sp*. and the other four AMF species, respectively. Cycling conditions were the following: initial denaturation at 95°C for 5 min followed by 45 cycles with denaturation at 95°C for 10s, annealing at 60°C for 10s and elongation at 72°C for 10s. Subsequently melting curves were analyzed to confirm the length of amplified DNA fragments and the absence of primer-dimers and non-specific amplification.

Table S4.1 List of PCR primers designed for qRT-PCR analysis

| AMF species | Primer designation | Primer sequence | Amplicon size |
|---|---|---|---|
| *Septoglomus africanum* | G17fw4 | TCTGATGGGTCCTACTTATC | 127 |
| | G17rv4 | CGTATCTTGATGTTAACCATG | |
| *Diversispora aurantia* | G5fw2 | GCCAGTGAAAATTCAGTTTGG | 126 |
| | G5rv2 | TCAGTATCGGTTTCGGGAG | |
| *Paraglomus* sp. | C49fw | GCGTGCGGCAGGGTAA | 71 |
| | C49rv | CCTGCCATGCGGAATAGTG | |
| *Diversispora omaniana* | D73fw | TGGGTCGAGTCAGGGTCAA | 79 |
| | D73rv | CGCTGACCTTCCAACAAAGAA | |
| *Rhizophagus irregularis* | Rifw | TCTGTGGAGTGTGAGGAGCTTAAC | 76 |
| | Rirv | CAACCACACGGGCAAGTACA | |

# 5 Functional characteristics of *Rhizophagus arabicus* in comparison with *Rhizophagus irregularis* - a battle as best symbiont under changing water regimes

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Under preparation

## 5.1 Summary


- It has been suggested that arbuscular mycorrhizal (AM) fungi from contrasting climatic conditions are functionally diverse and that they exhibit optimal performance under conditions resembling their original climate.
- We compared functional traits of two AM fungi from contrasting ecosystems to investigate their ability to improve drought tolerance of *Sorghum bicolor*.
- Sorghum was inoculated or not with *Rhizophagus irregularis* or *Rhizophagus arabicus*, and grown under well-watered or drought conditions in compartmented microcosms.
- *R. arabicus* improved transpiration and nutrition of sorghum plants and was more efficient in extracting $^{15}N$ from soil under both water regimes.

## 5.2 Introduction

Water deficit is considered as one of the most important abiotic stresses limiting plant growth. Plants have evolved different morphological and cellular mechanisms to avoid the stress or increase stress tolerance (Bray 1997). Additionally to their individual stress protection systems, plants growth in association with several soil microorganisms which can alleviate stress symptoms. One of the widespread constituents of soil communities are the symbiotic arbuscular mycorrhizal (AM) fungi. Several studies have demonstrated an improved drought tolerance of AM plants compared to non-mycorrhizal (NM) plants (Ruiz-Lozano, 2003). It was shown that the AM symbiosis often results in altered rates of water movement into, through and out of the host plants, modifying tissue hydration and plant physiology (Augé, 2001). The degree, to which the drought tolerance of a host plants is improved, highly depends on the associated AM fungal species. For example, *Glomus deserticola* stimulated biomass production, transpiration and photosynthesis of lettuce plants more than other AM fungal species when grown under drought-stress conditions (Ruiz-Lozano *et al.*, 1995). However, not only the species of the AM fungus is a decisive factor, also the origin of the fungal isolate affects the plant performance under drought conditions. Different isolates of *G. mosseae* and *Glomus intraradices* varied in their extent to improve the drought tolerance of lavender plants (Marulanda *et al*., 2007). Inoculation with drought-adapted *G. mosseae* and *G. intraradices* isolates increased tissue water content and reduced antioxidant compounds in lavender tissues when plants were exposed to drought conditions. Hence, drought-adapted AM fungi better improve the plants' drought tolerance.
An important aspect in terms of improved drought tolerance is the direct uptake and transfer of mineral nutrients by AM hyphae to the host plant. Inoculation with *Glomus mosseae* increased phosphorus uptake from dry soil in sorghum plants compared to non-mycorrhizal (NM) control plants. However, the ability of AM hyphae to assimilate ammonia under different water conditions was functionally never compared between different AM fungal species.

Here we wanted to assess the impact of two AM fungal species on the performance of *Sorghum bicolor* grown under two water regimes compared to NM plants. Two closely related AM fungal species originating from contrasting climates were tested: *R. arabicus*, isolated from a hyper-arid sand plain in Oman and the well-established and frequently used lab AM fungus, *R. irregularis*, isolated from a humid agricultural field-site in Switzerland. Sorghum is the world's fifth biggest crop (after maize, rice, wheat and barley) and it can grow under relative arid conditions where it serves as an important source of food, feed and fibers. A model system was established where the roots of sorghum were confined in a root compartment whereas the AM hyphae could grow in a connected hyphal compartment (Koegel *et al.*,

2013b). The aim of the study was to compare functional traits of the two AM fungi under two water regimes to give evidence for their ability to improve plants tolerance to drought. These functional traits included the ability of the AM fungi to (i) enhance plants' transpiration (measured by stomatal conductance), (ii) improve plants' nutrition (nitrogen (N) and phosphorus (P) content of plant tissue), (iii) colonize the rhizospere and extent their hyphal network (measured by hyphal length density (HLD) in the root and hyphal compartment), and (iv) their efficiency to colonize new soil patches for nutrient extraction (using $^{15}N$ as tracer).

## 5.3 Materials and Methods

*Plant growth conditions and experimental set-up*

Experiments were performed with sorghum (*Sorghum bicolor* (L.) Moench), cv Pant-5. This cultivar is closely related to BTx623, the sorghum cultivar used for genome sequencing (Paterson et al., 2009). Seeds of cv Pant-5, kindly provided by sorghum breeders of I.G.F.R.I. (CCS Agriculture University of Hissar, Haryana, India) and G. B. Pant University of Agriculture and Technology (Pantanagar, Uttaranchal, India), were surface sterilized (10 min in 2.5 % KClO), rinsed with sterile water several times and soaked in sterile water overnight. Seeds were germinated on sterile moist sand in darkness at 25 °C for 3 days. *Rhizophagus arabicus*, isolated from a hyper-arid sand plain in Oman (Symanczik *et al*., 2014b) and *R. irregularis* BEG-75 (Botanical Institute, Basel, Switzerland) were propagated by trap cultures as previously described (Oehl *et al.*, 2004). To establish AM symbiosis, three pre-germinated seeds were inoculated in the root compartment of compartmented microcosm: one root and one hyphal compartment were connected, but separated by a double layer of 21 μm nylon mesh and an air gap in between to prevent mass flow (Fig S1 modified from Koegel *et al.*, 2013b). Microcosms contained 1620 g of a sterile (120 °C, 20 min) mixture of sand (quartz sand from Alsace, 0.125-0.25mm; Kaltenhouse) and Zeolithe (Clinoptilolite, Chemko Ltd.; Slovakia) (1:1, w/w). Three AM treatments were applied: *R. irregularis*, *R. arabicus* and autoclaved inoculum (NM). Each plant was inoculated with about 200 spores. Each pot received 5 mL of a microbial wash to correct for possible differences in microbial communities (Koide & Elliott, 1989). This wash was prepared by wet sieving 10 g of each inoculum through a 32- mm sieve and a paper filter (FS 14 1/2; Schleicher & Schuell), yielding a final volume of 200 mL.

During four weeks, plants were watered twice a week with distilled water. Then, two water regimes (soil with different field capacity; FC) were applied: "WW" (well-watered condition, 80-100% FC) and "D" (drought condition, 35-55% FC). Soil water content was monitored and regulated by weighing the pots

periodically twice per week. In addition, the pots received 10 mL of Hoagland solution (Gamborg & Wetter, 1975) weekly. In the centre of the hyphal compartment, a 21 µm nylon mesh bag of 15 ml was inserted and kept empty until introduction of the $^{15}N$ labelled substrate three weeks before harvest. Then, the nylon mesh bag was filled with 13 g of sand including 10mg of $^{15}N$-ammonium sulfate (Cambridge Isotope Laboratory). Plants were grown under controlled conditions: 16h of light [220 µE $m^{-2}$ $s^{-1}$] at 25°C and 8 h of dark at 20°C, constant relative aerial humidity of 65%. The experiment was set up in a fully randomized design where each treatment was replicated five times.

Stomatal conductance of sorghum plants was measured on the first fully developed leaf two days post irrigation and three times during the last two weeks before harvest using a steady-state diffusion porometer (Decagon Devices, Pullman, WA, USA). Plants were harvested after 16 weeks of growth. Roots were washed carefully and fresh weight was determined before dividing the root system into different parts. Three subsamples of about 100 mg of fresh roots were snap-frozen and stored at -80°C for further RNA quantification. One subsample of fresh roots was used to determine the degree of AM colonization and the rest was dried at 55°C for 3 days to estimate root dry weight.

*Staining of AM fungi in plant roots*

Trypan Blue staining was used to identify mycorrhizal structures inside the root (Phillips & Hayman, 1970). The percentage of root length colonized by hyphae, arbuscules and vesicles was estimated for each sub-sample by a modified line intersection method (McGonigle *et al.*, 1990). One hundred line-intersections per root sample were scored for AM fungal structures.

*Hyphal length density (HLD)*

HLD was measured separately for the root- and hyphal compartment and was determined by the modified grid-line intersection method (Jakobsen *et al.*, 1992) using 10 g of the growth substrate. After sieving successively through a 400 and a 32 µm mesh, the material was collected and transferred into 50 mL of distilled water and homogenized for 10 s at full speed in a blender. The suspension was transferred into a beaker, diluted to 500 mL and stirred for 1 min before five subsamples were taken every 10 s and loaded onto the Filtration apparatus (MF-Membranfilter 1.2µm; Millipore).

*Nutrient analysis*

Remaining shoots and roots were dried at 55°C for 3 days and weighed. Samples were ground in 1.5 ml Eppendorf ® tubes using 1.1-mm-diameter tungsten carbide balls (Biospec Products Inc., Bartlesville, OK, USA) in a Retch MM301 vortexer (Retch GmbH & Co., Haan, Germany). The total amount of N and $^{15}N/^{14}N$ ratio of bulk samples were determined using an elemental analyzer (EA) and a ThermoFinnigan DeltaV Advantage Continuous-Flow EA-IRMS, respectively. Phosphorus concentration of the plant shoots and roots was measured using the molybdate blue method (Murphy & Riley, 1962) on a Shimadzu UV-160 spectrophotometer (Shimadzu Biotech) after incineration and acid extraction of the plant powder.

*Statistical analyzes*

Data were analyzed using either multifactorial ANOVA followed by LSD's multiple range test with a significance level of 0.05% (three-way ANOVA with the factors AM treatment, water regime and compartment for HLD and two-way ANOVA with the factors AM treatment and water regime for all other data). Data for percentage root length colonized by AM fungi and arbuscular colonization were arcsin-square root transformed and data for $^{15}N$ content in plant tissue, $^{15}N$ concentration in AM hyphae and relative $^{15}N$ hyphal transport were 1/sqrt(x) transformed to fit the assumption of normal distribution. Correlations were calculated by using Pearsons correlation. Analyzes were performed using SPSS software version 20 (IBM, Zürich, Switzerland).

## 5.4 Results

*Effect of water regime and AM treatment on physiological plant parameters*

Stomatal conductance was significantly affected by water regime ($p<0.01$, $F=9.173$) and by AM treatment ($p<0.001$, $F=34.323$). Under D conditions, stomatal conductance was significantly decreased by 38% and 15%, in NM and *R. irregularis* inoculated plants, respectively. Stomatal conductance of sorghum plants inoculated with *R. arabicus* was similar under WW and D conditions (Fig.5.1a).

Plant dry weight of sorghum plants was significantly lower in D than in WW whatever the AM treatment ($p<0.001$, $F=188.133$): from 5.5 g to 3.9 g for *R. irregularis* inoculated plants, from 5.4 g to 3.6 g for *R. arabicus* inoculated plants and from 5.5 g to 3.9 g for NM plants (Fig. 5.1b). Nitrogen (N) and phosphorus (P) content of plant tissue were significantly lower under D conditions ($p<0.001$, $F=123.637$ and $p<0.001$, $F=63.784$, respectively) but significantly higher in AM plant ($p<0.001$, $F=310.277$ and $p<0.001$, $F=68.134$) under both water regimes. Comparing the two AM treatments, N and P content was significantly higher in *R. arabicus* inoculated plants compared to *R. irregularis* inoculated plants under both water regimes (Fig. 5.1c,d).

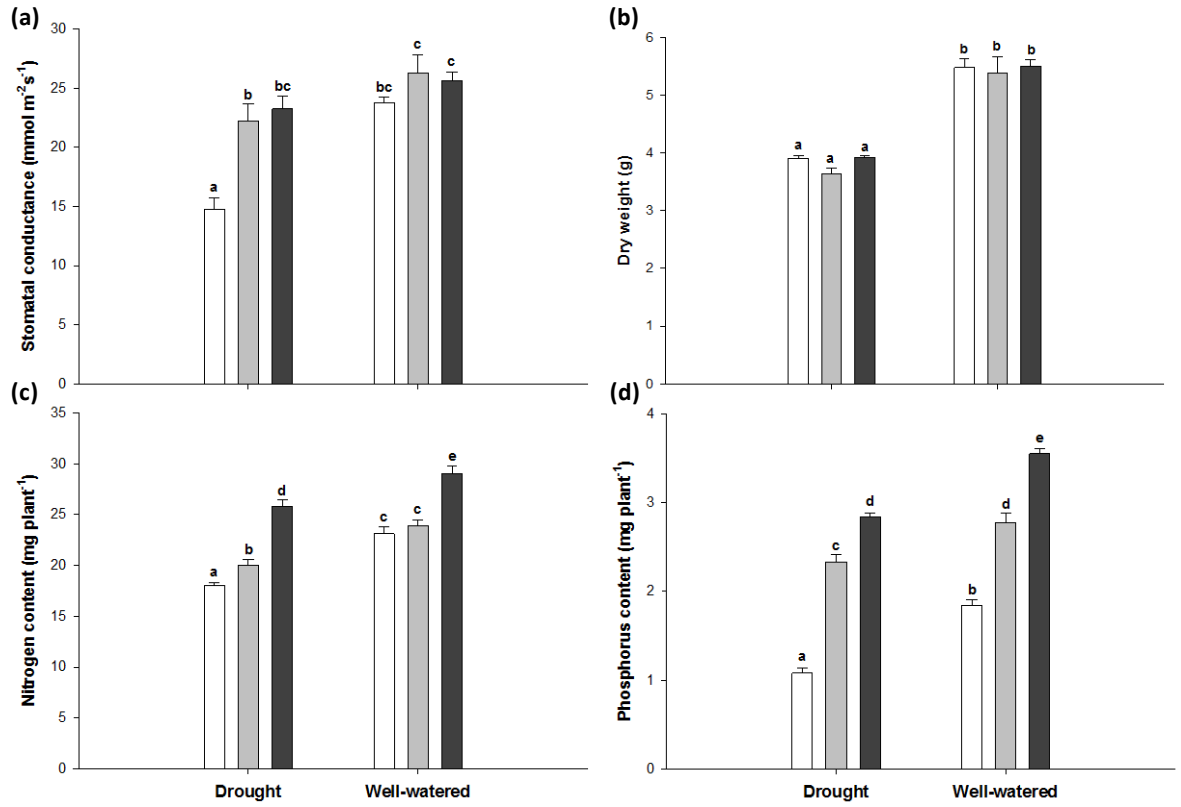



Fig. 5.1 Impact of AM treatment and water regime on (a) stomatal conductance, (b) the total plant dry weight (c) the nitrogen content and (d) the phosphorus content of sorghum plants inoculated with *Rhizophagus irregularis* (light grey), *R. arabicus* (dark grey) or non-inoculated (white) exposed to well-watered and drought conditions. Different letters above bars indicate significant differences according to LSD's multiple range test with a significance level of 0.05%. Data represent means + SE (n=5).

*Effect of water regime on AM fungal performance*

Non-mycorrhizal sorghum roots were free of mycorrhizal structures. Root colonization of AM plants ranged from 60% to 75% and was not significantly different whatever the AM treatment and the water regime. Drought conditions significantly decreased the percentage of arbuscules ($p<0.05$, $F=5.619$): from 43% to 35% in *R. irregularis* inoculated plants and from 43% to 31% in *R. arabicus* inoculated plants.

The soil of NM controls was free of fungal hyphae, indicating that in the AM treatments, all hyphae came from AM fungi (data not shown). Hyphal length density (HLD) in the root and hyphal compartment was examined as a first parameter of AM fungal performance. It was significantly affected by water regime ($p<0.001$, $F=18,855$), AM treatment ($p<0.001$, $F=60,853$) and the interaction AM treatment x compartment ($p<0.01$, $F=10,394$). Drought significantly decreased HLD in the root and hyphal

compartment of *R. irrgularis*, while HLD of *R. arabicus* was only decreased in the hyphal compartment (Fig. 5.2a). HLD of the root compartment significantly correlated with stomatal conductance ($p<0.012$), while HLD of the hyphal compartment significantly correlated with N content ($p<0.001$), P content ($p<0.001$) and $^{15}N$ content ($p<0.01$) in sorghum plants.

We also measured $^{15}N$ concentration in AM hyphae isolated from the hyphal compartment. This parameter was significantly affected by water regime ($p<0.05$, $F=7.062$) and AM treatment ($p<0.001$, $F=83.445$). *R. arabicus* hyphae were significantly more enriched in $^{15}N$ compared to *R. irregularis* hyphae (Fig. 5.2b).

To study the effect of AM fungal performance on the host, we measured the $^{15}N$ content in the sorghum plants colonized by the fungi (Fig. 5.2c). This parameter was significantly different depending on the water regime ($p<0.01$, $F=13.951$), AM treatment ($p<0.001$, $F=152,934$) and their interaction ($p<0.001$, $F=11.68$). $^{15}N$ content was significantly higher in *R. arabicus*-inoculated plants compared to *R. irregularis*-inoculated plants and to NM plants whatever the water regime (Fig. 5.2c).

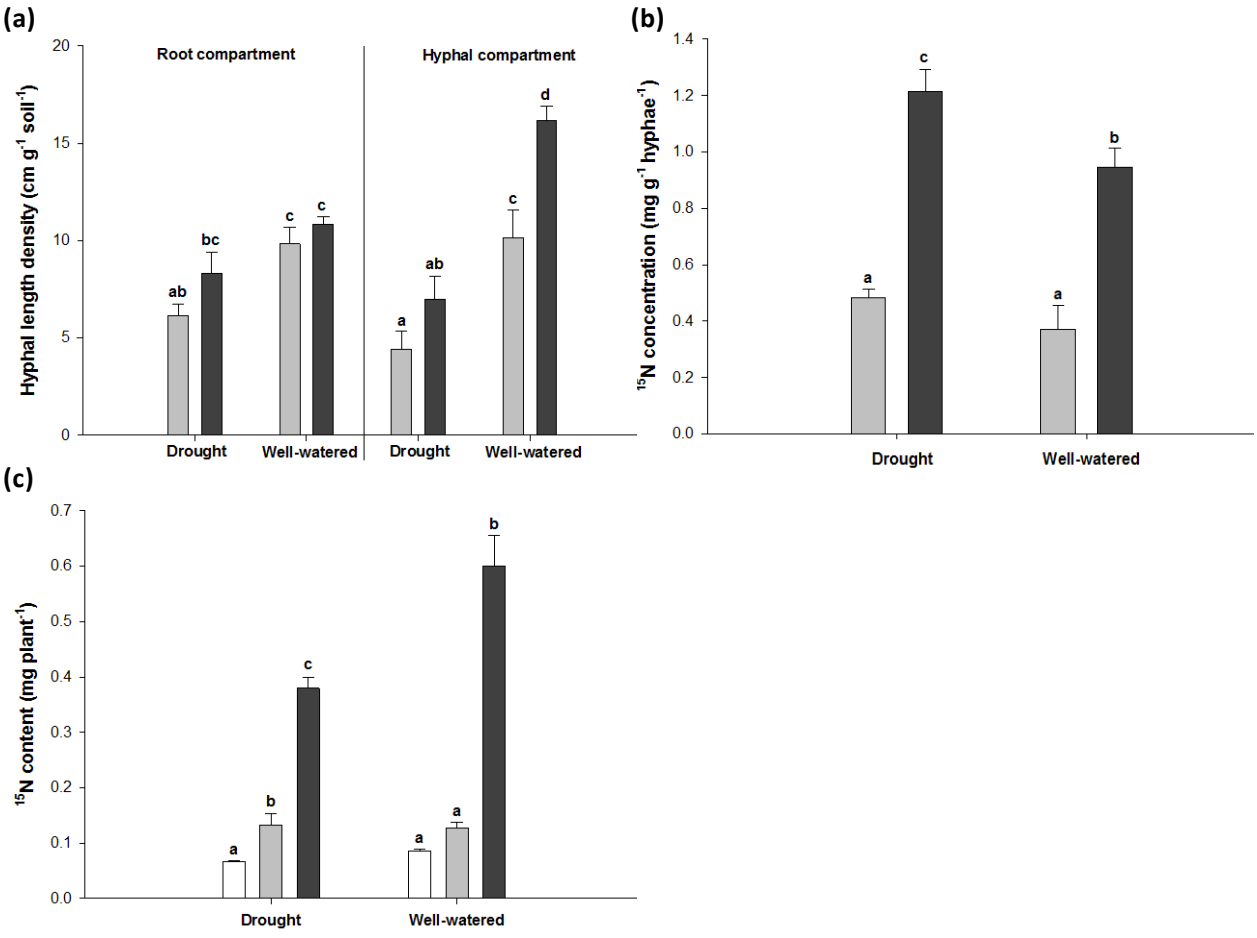


Fig. 5.2 Impact of water regime on (a) the hyphal length density (HLD) in the root and hyphal compartment and (b) the $^{15}N$ concentration of fungal hyphae of *R. irregularis* (light gray) and *R. arabicus* (dark gray). (c) Impact of AM treatment and water regime on the $^{15}N$ content of sorghum inoculated with *Rhizophagus irregularis*, *R. arabicus* or non-inoculated (white) exposed to well-watered and drought conditions. Bars of NM treatments are missing in figure a and b as soil of NM treatments was free of AM hyphae. Different letters above bars indicate significant differences according to LSD's multiple range test with a significance level of 0.05%. Data represent means + SE (n=5).

## 5.5 Discussion

*Effect of water regime on the AM fungi and their function in nitrogen acquisition*

Under drought conditions, *R. arabicus*-inoculated plants maintained transpiration while NM and *R. irregularis*-inoculated plants decreased transpiration. AM plants were shown to maintain higher rates of gas exchange during soil drying compared to NM plants (Ruiz-Lozano *et al.*, 1995; Neumann & George, 2004). Similarly, *R. irregularis*-inoculated sorghum displayed higher rates of stomatal conductance under drought stress condition compared to NM plants (Cho *et al.*, 2006). It was suggested that the AM-mediated increase in transpiration under drought conditions results from an enhanced ability of the AM-root system to gather water from dry soil (Duan *et al.*, 1996). This might be achieved as (i) AM hyphae increase the root surface area and have access to a bigger soil volume and (ii) they can exploit solution-filled soil pores which are not accessible for plant roots. Thus, AM hyphae can extract water from the soil which is not accessible for plant roots (Khalvati *et al.*, 2005). The significant correlation between HLD in the root compartment and stomatal conductance give further evidence for an increased accessibility of water by AM root. The maintenance of gas exchange under drought conditions is only one effect among others which were attributed to the increased drought tolerance mediated by the AM symbiosis (Augé, 2001; Ruiz-Lozano, 2003).

In our study, nutrient content (P and N) in plant tissue significantly decreased under drought conditions. Although, drought decreased the mobility of P and other mineral elements in the soil solution (Marschner, 1995), AM fungi increased nutrient content in sorghum tissue especially when inoculated with *R. arabicus*. Also Neumann and George (2004) observed higher P content in *G. mosseae*-inoculated sorghum compared to NM plants grown under drought conditions. The improved nutrition of AM plants might be also accounted for the enhanced assimilation ability of AM roots due to their increased surface area as explained previously. Thus, AM hyphae can reach nutrients not available for plants (Smith & Read, 2008). Observed correlations between HLD in the hyphal compartment and nutrient content in sorghum support the significance of hyphal nutrient uptake for plant nutrition.

*Effect of water regime on AM fungal performance*

In our study, root colonization was not affected by water regime as previously described (Neumann & George, 2004) as the AM fungus inside the root is not directly exposed to the stress condition. In contrast, HLD significantly decreased for *R. irregularis* in the root and hyphal compartments whereas HLD was only decreased in the hyphal compartment of *R. arabicus*. In previous studies, estimations of HLD under drought conditions were controversial and either decreased (Staddon *et al.*, 2004; Neumann *et al.*, 2009) or increased (Khalvati *et al.*, 2005). Here, we also showed that *R. arabicus* took up and transferred significantly more $^{15}N$ under both water regimes compared to *R. irregularis*, indicating a higher ability of *R. arabicus* to extract nutrients from the soil. The increased uptake might have resulted from a more efficient colonization of the label compartment by hyphae of *R. arabicus*. These functional differences might have to do with the area of origin of the AM fungal isolates. Maruland et al. (2007) observed that drought-adapted AM fungal strains improved growth and nutrient content of lavendar plants and enhanced glomalin in the soil under drought conditions compared to non-adapted AM fungal strains. However, measurements were only taken under drought conditions without their comparison to well-watered conditions.

Generally, it can be stated, that a fast and effective colonization of soil patches by AM hyphae is of great importance, as in nature, soil nutrients are patchily distributed. Furthermore, competition for soil nutrients is strong. Consequently, the ability to effiiently extract nutrients from the soil displays an exellent functional trait.

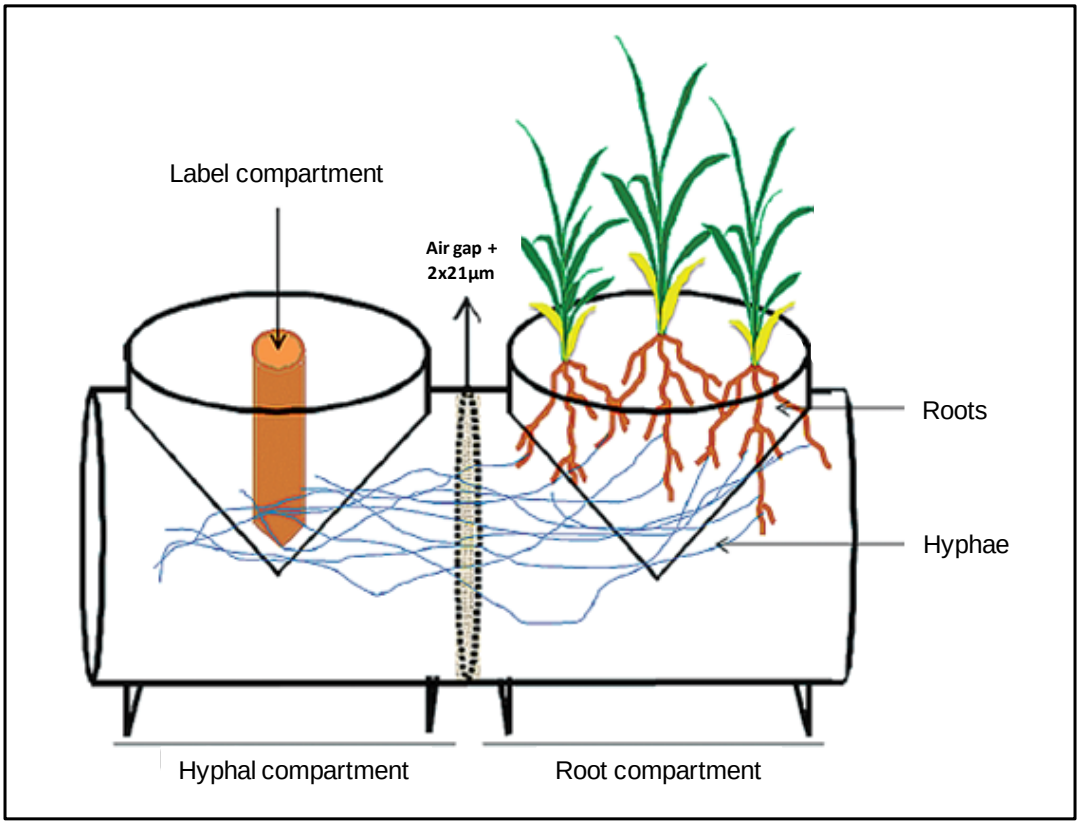
In summary, *R. arabicus* was found to excerts better symbiotic abilities compared to *R. irregularis* under both water regimes. *R. arabicus* originated from a desert ecosystem, which is characteriszed by long dry periods and rarly occuring rainy events. Thus, a desert AM fungus needs to perform good under drought conditions but at the same time, it needs to adapt quickly to changing water conditions in the case of a short rain event.

## 5.6 Acknowledgments

This project was supported by the Swiss National Science Foundation (grant nos. 127563 to Thomas Boller, PZ00P3_136651 to Pierre-Emmanuel Courty, 130794 to Andres Wiemken and the R'Equip grant no. 121258 to Moritz F. Lehmann and Thomas Boller). We thank Mark Rollog for laboratory assistance and scientific support in the stable isotope lab.

## 5.7 Supplementary data

Fig. S5.1 Compartmented microcosms. One root and one hyphal compartment are separated by two 21µm nylon meshes and an air gap to prevent mass flow. A 21µm nylon mesh bag of 15 ml was inserted in the center of the hyphal compartment (label compartment) and filled with 13 g of sand labeled with 10 mg of $^{15}N$ ammonium sulfate three weeks before harvest Modified from Koegel *et al*. (2013).



# 6 The potential of using native symbiotic mycorrhizal fungi as a biotechnological tool in the Arabian Peninsula

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In preparation

## 6.1 Abstract

The vegetation in the Arabian Peninsula has to cope with drought, heat, soil salinity, and low fertility, particularly due to low phosphorus (P) availability. The beneficial mycorrhizal symbiosis between plants and arbuscular mycorrhizal (AM) fungi is a key factor supporting plant growth under such environmental conditions. As a multifunctional symbiosis, the mycorrhiza is well known to play a crucial role in the P acquisition by plants. It also enhances soil aggregate stability, a feature of particular relevance for the sandy soils prone to erosion. Under arid conditions, mycorrhizal plants were found to maintain higher drought tolerance and to have better access to P and other soil nutrients than non mycorrhizal ones and consequently, and consequently resulting in a better yield. Most importantly, native AM fungal communities from Arabian Peninsula soils must be "stress-adapted" to withstand these conditions, and they might have specialized physiological properties. Therefore, it is of particular interest to apply stress-adapted AM fungal strains isolated originally from these lands as a biotechnological tool for revegetation practices in this region of the world.

Here we present a pioneering program aiming to isolate, identify and apply the AM fungi from Omani agricultural and natural habitats. We have recovered taxonomically unique AM fungal species and obtained them in pure cultures forming a core of an expanding germplasm bank for arid zone-adapted ecotypes of AM fungi. First experiments have been conducted to evaluate the abilities of some of the strains in promoting the seedling growth of a natural keystone plant in Arabia, namely, *Prosopis cineraria* (Ghaf), under nursery conditions. The results show that inoculation with certain AM fungal species is beneficial to increase the growth of *P. cineraria.*

## 6.2 Introduction

The natural vegetation growing in the desert ecosystem of the Arabian Peninsula has to cope with drought, heat, soil salinity, and low fertility, particularly due to low phosphorus (P) availability (Fisher & Membery, 1998; Glennie & Singhvi, 2002; Al-Yahya'ei *et al.*, 2011). Additionally, sandy soils possess a loose structure with a low water holding capacity. Under such environmental conditions, the beneficial mycorrhizal symbiosis between plants and arbuscular mycorrhizal (AM) fungi plays an important role for the survival and growth of naturally occurring plants. AM fungi are well known to convey multiple benefits for plant growth and health, above all in stressful environments (Newsham *et al.*, 1995; Smith & Read, 2008). Under arid conditions, for example, mycorrhizal plants were found to maintain higher drought tolerance (Augé, 2001), have better access to phosphorus (Neumann & George, 2004a) and consequently, possess increased yield compared to non-mycorrhizal ones (Ceballos *et al.*, 2013). Furthermore, the AM symbiosis may also enhance soil aggregate stability and soil structure (Rillig & Mummey, 2006), a feature of particular relevance for the sandy soils prone to erosion and hence, positively affecting soil moisture characteristics (Fusconi and Berta, 2012).

The application of AM fungal inocula in horticulture, agriculture and revegetation programs became more prominent within the last decades as the number of studies demonstrating improved plant growth after inoculation has steadily increased (Requena *et al.*, 2001). The application of AM fungal inocula which involves growing plants in the presence of AM fungi prior to the planting to the field appears to be especially beneficial. This is because the plants possess an already established, tailored AM symbiosis and colonization by fungal hyphae that can start immediately after transplantation into the field. In addition, AM hyphae were shown to be important infection structures in arid and semi-arid ecosystems (Requena *et al.*, 1996; Azcón-Aguilar *et al.*, 2003). Therefore, the nursery-application of AM fungi can increase the infectivity of soils in such habitats (Requena *et al.*, 2001).

Native AM fungal communities inhabiting soils of desert ecosystems, such as found in the Arabian Peninsula, must be "stress-adapted" to withstand these harsh environmental conditions, and may possess particularly interesting physiological properties. Adaptations of AM fungal species to distinct environmental conditions were already shown (Marulanda *et al.*, 2007; López-Gutiérrez *et al.*, 2008; Lekberg & Koide, 2008; Antunes *et al.*, 2011). Plant drought tolerance was best improved when the experimental conditions were similar to the environmental conditions from where the AM fungal isolate originated from. Exotic and native isolates of *Glomus mosseae* and *Glomus intraradices* were shown to exhibit diverse adaptations to water availability when grown under drought conditions (Marulanda *et al.*, 2007). Inoculation with native, drought-adapted isolates of *Glomus mosseae* and *Glomus intraradices* improved drought tolerance of lavender plants as reflected by increased water content and declined

antioxidant compounds in plant tissues. Similar observations were made with regard to temperature. AM fungi exhibit optimal performance when the experimental conditions were closest to the environmental conditions from where the AM fungus was isolated (López-Gutiérrez *et al.*, 2008; Antunes *et al.*, 2011).
Nowadays, the use of commercial AM inoculants is growing and certain AM fungal species (mainly *Rhizophagus irregularis*) are traded globally and used in agriculture and revegetation programs (Gianinazzi-Pearson, 2002) even if they are not native in the applied soil. How the introduced exotic AM fungal species would alter the existing native AM fungal community is still poorly understood as only few greenhouse studies have addressed this question. Koch *et al*. (2010) studied the impact of inoculation with two stains of *R. irregularis* on the structure of a native Canadian AM fungal community inhabiting *Sorghum bicolor* roots. They detected a dramatic decrease in the diversity of the native AM fungal community after inoculation. Similarly, Mummey *et al*. (2009) found that pre-inoculation with two *Glomus* species reduced the AMF richness richness of another native AMF community in roots of *Leucanthemum vulgare* compared to uninoculated controls after exposure to the field soil. However, pre-inoculation with *Gigasporaceae* species had no impact on the AM fungal community richness. The application of exotic AM fungi and possible alterations of the native AM fungal communities should be considered, as there remains a risk of future unwanted consequences (Schwartz *et al.*, 2006).
Therefore, the application of native AM fungi should be favored due to (i) their better adaptation to the prevalent environmental conditions and (ii) the lower risk of potential negative impacts on the native AM fungal communities.

In the current project a culture collection of AM fungal strains derived from single-spores isolated originally from Oman was established including different Omani ecosystems and a variety of native plant species from which the fungal cultures where recovered (Symanczik et al., 2014a, b). This germplasm bank is crucially needed as a basis for various basic and applied research related to the environmental conditions of the region. Isolation, characterization and propagation of AM fungi from Omani habitats opens the door to apply them as a “biotechnological tool” (Gianinazzi-Pearson, 2002) to promote sustainable agriculture and environmental conservation in the country and the region as a whole.
In this study we evaluated the efficiency of some of the isolated AM fungi in promoting growth of Ghaf (*Prosopis cineraria*) seedlings. Ghaf is a keystone tree in the whole Arabian Peninsula, Iran, Pakistan and India. It plays an important role as an agro-forestry species as it is able to form a tripartite symbiosis including AM fungi and rhizobial nitrogen-fixing bacteria. It has been chosen also because of its relatively fast development under nursery conditions. This makes it a good model for short-term experiments to evaluate the efficiency of AM fungal inocula in growth promotion.

## 6.3 Materials and methods

*Study ecosystems*

The isolated species of AM fungi listed here were recovered from the Al-Sharqya region of Oman. The geography of the area, sampling sites, environmental conditions and soil properties are described in Al-Yahya'ei *et al*. (2011) and Symanczik *et al*. (2014a,b). The ecosystem with its dominant vegetation is described in figure 6.1.

*Establishment of the AM fungal germplasm bank*

The methods used to establish trap cultures and the green house conditions have been described previously (Al-Yahya'ei *et al.*, 2011). More than 1000 attempts to establish single-spore derived cultures were initiated essentially as described (Symanczik *et al.*, 2014a,b). After single-spore essays were checked for sporulation, positive cultures were used as inocula for further propagation by culturing with a consortium of *A. porrum*, *Hieracium pilosella* L. and *Plantago lanceolata* L. as AM fungal host plants. The resulting mycorrhizal inocula were then used for application under nursery conditions. The complete establishment process with indicated time scale is illustrated in figure 6.2.

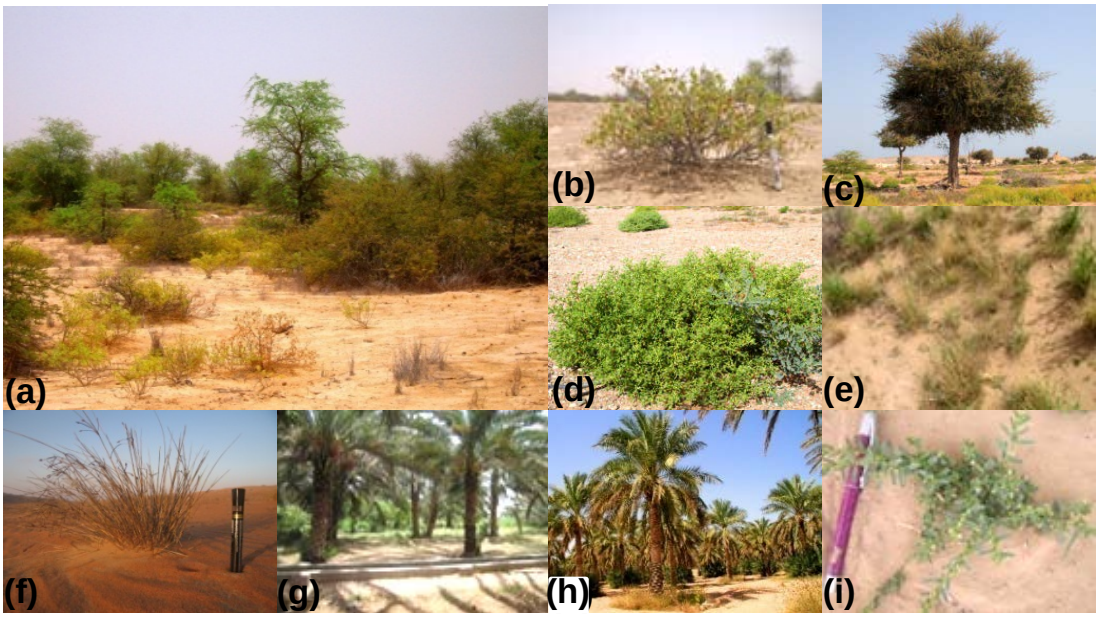


Fig. 6.1 The ecosystem and explored habitats. (a) General picture of the natural undisturbed habitat in the Experimental station in Al Kamel inhabited by the natural vegetation from which soil samples were taken (b) *Salvadora persica* (c) *Prosopis cineraria* (d) *Tetraena qatarense* (e) and the natural inter-plant-area. (f) Typical sand dune habitat with *Heliotropium kotschyi* in the region of Al-Sharqiya Sands. (g) Traditional date palm plantation close to the Experimental station in Al Kamel. (h) Modern date palm plantation in the Experimental station in Al Kamel (**i**) with *Polygala erioptera* growing between date palms.

*Identification of AM fungal strains*

Morphological and molecular identification of AM fungal strains were performed as described previously (Symanczik *et al.*, 2014a,b).

*Inocula preparation*

Four single-strain (*Paraglomus* sp., strain C49; *Funnelliformis nakheelum*, strain G90; *Diversispora spurca*, strain K46; *Rhizophagus arabicus*, strain F80) two mixed-strain (mix F69+F41, *Diversispora omaniana* (strain F69) and *Claroideoglomus drummondii*, (strain F41) and mix G8+G14 (*Diversispora aurantia*, strain G8 and *Septoglomus africanum*, strain G14), and four non-mycorrhizal (NM) controls (NM control, "NM-control", nothing added; NM plus microbial wash, '"NM-wash"; NM plus autoclaved inocula, "NM-carrier"; NM plus microbial wash and autoclaved inoculum, "NM-wash+carrier". Eight seeds were used as replicates for mycorrhizal treatments and 20 seeds for NM-control treatments (the higher number of seeds in NM control treatments resulted from low germination-rates observed in preliminary experiments, data not shown). A total of 250 spores were used for the single-strain treatments and 200 spores for the mixed-strain treatments. Each pot except the NM-control and NM-carrier treatment received 5 mL of filtered washings of AM fungal inoculum to correct for possible differences in microbial communities (Koide & Elliott, 1989). This filtrate was prepared by wet sieving 100 g of each inoculum through a 32-mm sieve and a paper filter (FS 14 1/2; Schleicher & Schuell), yielding a final volume of 1 L.

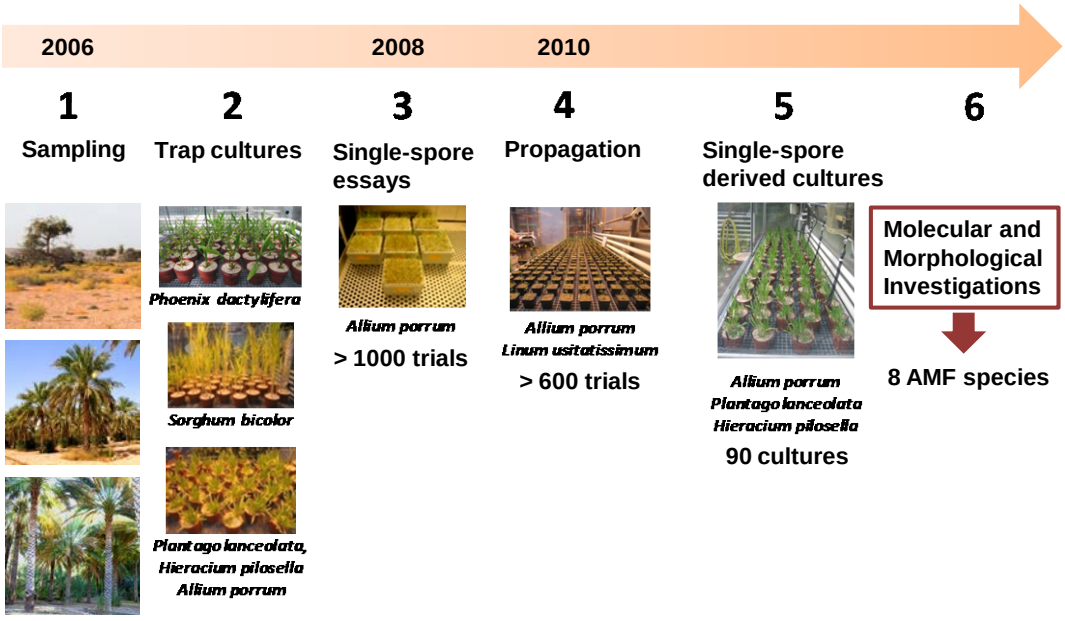


Fig. 6.2 The six-steps process of the germplasm bank establishment started from soil sample collections in the field in 2006 until maintaining the pure AM fungal single-spore derived cultures at the end of 2010 followed by the identification process.

*Growth of P. cineraria*

The *P. cineraria* experiment was conducted in the Oman Botanic Garden. *P. cinereria* seeds were soaked in water overnight before they were sawn in compartmented germination trays. Each seed was placed in a 50 ml pot filled with peat moss (Plantafior, Germany) and vermiculite (1:1;v/v) and supplemented either with mycorrhizal inocula or sterilized inocula (later referred to as "carrier"). After one month, seedlings were transplanted into 300 mL pots filled with original soil from the surrounding area of the garden and fine peat moss (1:1;v/v), and grown for two months. Plants were further transplanted into 3 L pots and grown for additional nine months in the green house (temperature: 24-32°C; relative humidity: 65-70%) before they were transferred to the field.

Germination rates were monitored three months after inoculation of seeds and calculated as the percentage of seedling numbers present after three month divided by the initial amount of inoculated seeds. Survival rates were assessed 12 month after sowing and calculated as the percentage of seedling numbers present after 12 month divided by the number of germinated seedlings. Plant growth performance was estimated by measuring the total branch length (accumulated length of all branches to estimate total biomass). Data were collected three months after sowing (in June 2012) and one year after sowing (in February 2013).

*Statistical analyses*

Data were analyzed using one-way ANOVA followed by LSD's multiple range test with a significance level of 0.05%. Analyses were performed using SPSS software version 20 (IBM, Zürich, Switzerland).

## 6.4 Results

*Establishment of the arbuscular mycorrhizal germplasm bank*

Out of more than 1000 attemps, 90 single-spore derived cultures were established successfully. Eight different AM fungal species were identified; four described AM fungal species including *Claroideoglomus drummondii*, *Diversispora aurantia*, *Diversispora spurca* and *Septoglomus africanum* (Symanczik *et al.*, 2014b) and three previously unknown AM fungal species: *Diversispora omaniana*, *Rhizophagus arabicus* and *Septoglomus nakheelum* (Symanczik *et al.*, 2014a). In addition, a still undescribed AM fungal species *Paraglomus* sp. was used in the experiment. Numbers of established AM fungal single-spore derived cultures in relation to their original host plant species from which the AM fungal culture was recovered from and the total number of cultures for each AM fungal species is given in table 1. A phylogenetic tree showing the position of the eight isolated species of AM fungi is given in figure 6.3.

Table 6.1Frequency of AM fungal single-spore derived cultures in relation to the original host plant from which the AM fungal culture was recovered from and the total number of cultures for each AM fungal species.

| AM fungal species | Original associated plant species | Number of AM fungal cultures | |
|---|---|---|---|
| | | Plant species$^{-1}$ | AM fungal species$^{-1}$ |
| *Claroideoglomus drummondii* | *Tetraena qatarense* | 1 | |
| | *Salvadora persica* | 6 | |
| | *Prosopis cineraria* | 8 | |
| | Inter-plant area (IPA natural) | 8 | 23 |
| *Diversispora aurantia* | *Prosopis cineraria* | 3 | |
| | *Phoenix dactylifera* (Trad) | 3 | 6 |
| *Diversispora omaniana* | *Tetraena qatarense* | 1 | |
| | *Salvadora persica* | 1 | |
| | Inter-plant area (IPA natural) | 2 | 4 |
| *Diversispora spurca* | *Tetraena qatarense* | 9 | |
| | *Salvadora persica* | 16 | |
| | *Prosopis cineraria* | 4 | |
| | Inter-plant area (IPA natural) | 3 | |
| | *Heliotropium kotschyi* | 3 | |
| | *Polygala erioptera* (IPA Mod) | 4 | |
| | *Phoenix dactylifera* (Mod) | 1 | |
| | *Phoenix dactylifera* (Trad) | 3 | 43 |
| *Funelliformis nakheelum* | *Phoenix dactylifera* (Trad) | 3 | 3 |
| *Paraglomus* sp. | *Tetraena qatarense* | 3 | |
| | *Salvadora persica* | 2 | 5 |
| *Rhizophagus arabicus* | Inter-plant area (IPA natural) | 2 | 2 |
| *Septoglomus africanum* | *Phoenix dactylifera* (Trad) | 4 | 4 |

*Growth of P. cineraria*

Germination and survival rates strongly varied for NM-control treatments, while those of mycorrhizal treatments were constant except for germination rate of the *C. drummondii+D. omaniana* treatment (Table 6.2).

After three months of growth, the total branch length was significantly higher for all mycorrhizal treatments except for the mix F69+F41 compared to all control treatments (Fig. 6.3a, c). In the control treatments, total branch length ranged between 27.3cm and 29.9cm, while in mycorrhizal plants, it ranged between 37.8cm and 42.4cm. After one year, the total branch length was significantly higher only in the mix G8+G14 compared to control treatments. The total branch length of all other mycorrhizal plants was similar as control plants (Fig. 6.4b).

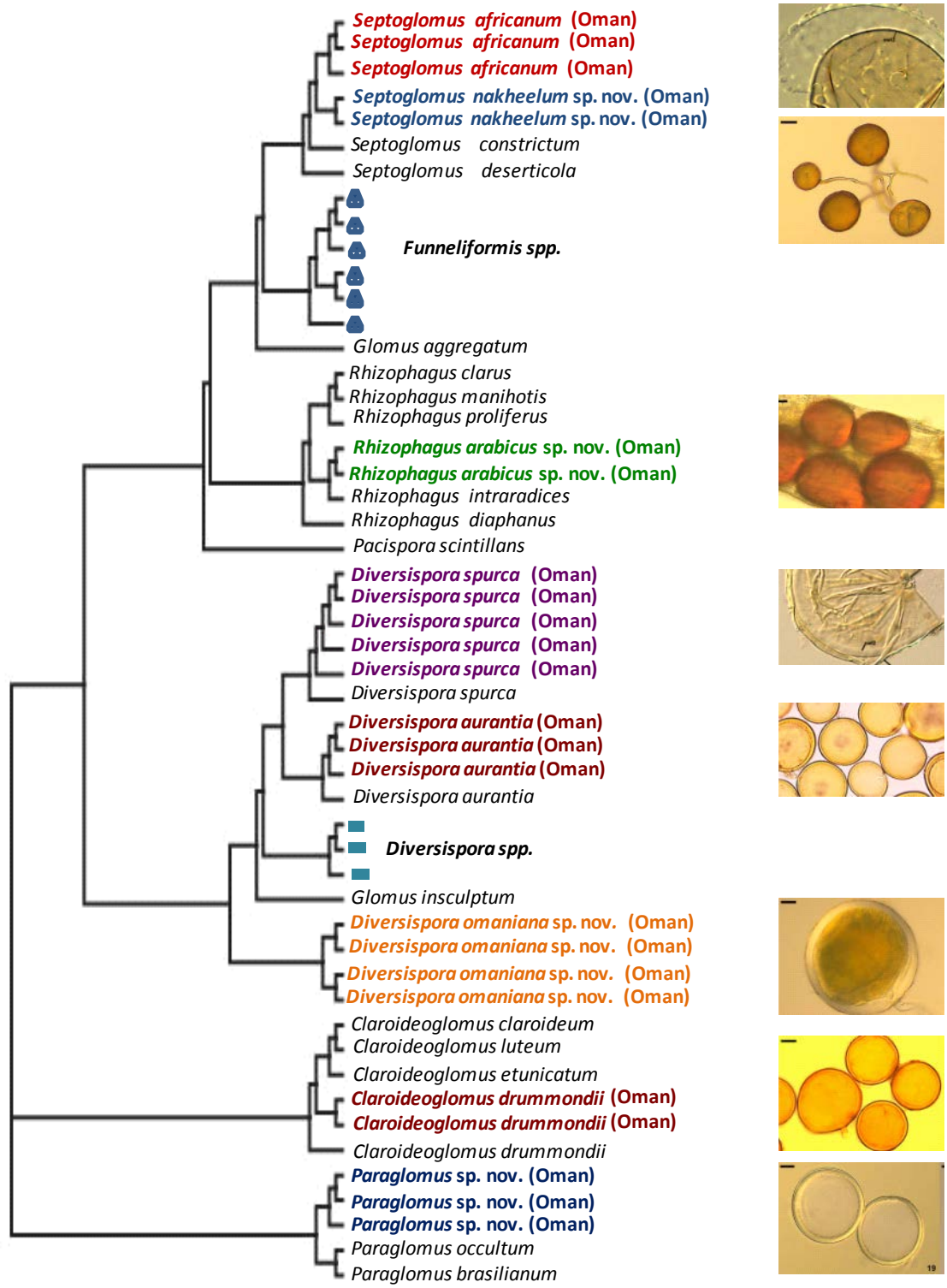


Fig. 6.3 A phylogenetic tree which was inferred from the partial LSU rDNA region (628 dataset characters), showing the positions of the eight isolated species of AM fungi (shown in coloured boldface). An image of each of the AM fungal species was associated with its phylogenetic position. Triangles (for Funneliformis spp.) and squares (for Diversispora spp.) substitute species names.

Table 6.2 Germination- and survival rates for *Prosopis cineraria* seedlings.

| AM fungal treatment | Initial seeds treatment$^{-1}$ | Germination rate | | Survival rate | |
|---|---|---|---|---|---|
| | | Number | % | Number | % |
| NM-control | 40 | 10 | 25 | 6 | 60 |
| NM-wash | 40 | 7 | 18 | 7 | 100 |
| NM-carrier | 40 | 12 | 30 | 8 | 67 |
| NM-wash+carrier | 40 | 13 | 33 | 7 | 54 |
| *D. aurantia + F. africanum* | 8 | 5 | 63 | 5 | 100 |
| *C. drummondii + D. omaniana* | 8 | 3 | 38 | 3 | 100 |
| *R. arabicus* | 8 | 5 | 63 | 5 | 100 |
| *D. spurca* | 8 | 5 | 63 | 5 | 100 |
| *Paraglomus* sp. | 8 | 5 | 63 | 5 | 100 |
| *F. nakheelum* | 8 | 5 | 63 | 5 | 100 |

## 6.5 Discussion

The establishment of a germplasm bank of arid zone-adapted ecotypes of AM fungi is the first step and a prerequisite for a conservation effort for *Glomeromycota* in Oman. The importance and significance to establish a germplasm bank specific to a target ecosystem was already demonstrated for degraded semi-arid Mediterranean ecosystems (Barea et al. 2011). Similar efforts were made at the Centre for Mycorrhizal Culture Collection (CMCC) which houses and maintains cultures from different agro-ecological zones of India (TERI) and for AM fungi in China (Gai *et al.*, 2006). Germplasm banks of AM fungi from specific ecosystems are fundamental in the process of applying these beneficial fungi in revegetation programmes in the same ecosystem.

Utilization of AM fungal inocula improved germination- and survival rates of *P. cineraria* during seedling establishment. Similar observations were made in regard to micropropagated plants. Application of AM fungi during the propagation process of *Sesbania sesban* (Subhan *et al.*, 1998), *Leucaena leucocephala* (Puthur *et al.*, 1998) and banana (Orivaldo *et al.*, 1999) reduced the transplantation shock and improved survival of micropropagated plantlets.

After the first three months, the growth of mycorrhizal *P. cineraria* seedlings was increased compared to NM-control seedlings. However, the differences between mycorrhizal and non-mycorrhizal seedlings mostly disappeared after one year. A possible explanation for this observation might be the restricted pot size. *P. ceneraria* seedlings were transplanted only once (after three month) and subsequently grown in 3 L pots for the remaining nine month. The positive correlation of plant growth and pot size was demonstrated for a wide range of plant species (Poorter *et al.*, 2012). The limited pot size restrained the

prosopis seedlings in their optimal growth what might have led to the loss of the previously observed growth differences.

The application of AM fungal inoculants in the nursery proved to be an effective strategy to provide the seedlings with a tailored mycorrhizal status before their transplantation into the field. The potential of this strategy in improving the transplant performance was successfully demonstrated in the nursery production of *Pinus halepensis* seedlings mycorrhizal with *Pisolithus tinctorius* or *Lactarius deliciosus* (Díaz *et al.*, 2009, 2010).

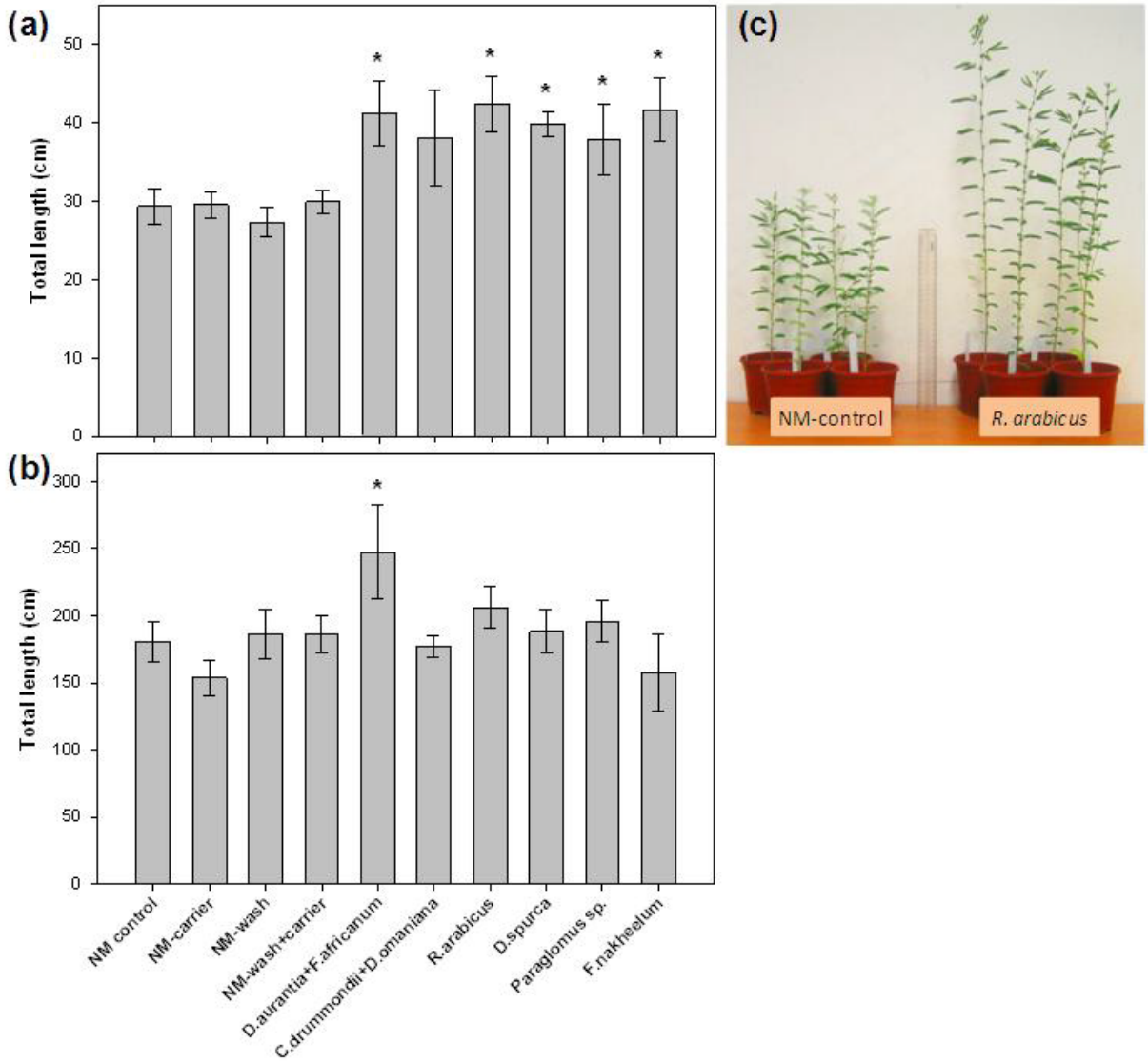


Fig. 6.4 Impact of inoculation with different AMF species on the total length of *Ghaf* seedlings (a) three months and (b) one year after inoculation. (c) Growth of non-mycorrhizal control (NM-control) seedlings and seedlings inoculated with *Rhizophagus arabicus* after three months. Stars above bars indicate significant differences compared to non-mycorrhizal control plants according to LSD's multiple range test with a significance level of 0.05%. Data represent means + SE (n=3-13).

The use of native AM fungi as biotechnological tool was successfully demonstrated in several field experiments highlighting the important role of these fungi in the early stages of native shrub species establishment and development, after the restoration of degraded Mediterranean ecosystems (Azcón-Aguilar & Barea, 1997; Requena *et al.*, 2001; Caravaca *et al.*, 2003). Further positive effects after AM fungal application were the increased resistance of mycorrhizal plants to environmental stresses, enhanced plant nutrient acquisition and improved soil quality (Jeffries *et al.*, 2003; Alguacil *et al.*, 2005). A long-term study comparing the effect of native AM fungi and an exotic *Glomus intraradices* strain reported that the exotic *G. intraradices* was effective only during the first year after transplanting with similar growth promotion as native AM fungal species. However, five years after transplantation, plants associated with *G. intraradices* displayed similar growth as non-inoculated controls and spores of *G. intraradices* were barley present in the rhizosphere of the shrub while those of native AM fungi established well (Requena *et al.*, 2001). Possible explanations may be the adaptation of native AM fungi to prevalent environmental conditions, as mentioned earlier. Also Klironomos (2003) illustrated the importance of fungal origin on plant growth performance for a wide range of AM fungal and plant species.

These findings emphasize the importance of selecting native AM fungal strains for inoculation practices. It is reasonable to propose that the isolated species of this study belong to populations exhibiting unique traits and physiological properties adapted to withstand desert conditions such as desiccation in drought seasons, hot summer temperatures and low soil nutrients. Therefore, these species represent good candidates to be used as future biofertilizers to enhance crop productivity and the fitness and survival of native plants and in soil revegetation and reclamation measures under the environmental conditions prevailing in their original ecosystem. Our endeavor of isolation, characterization and first practical experiments with some AM fungi from arid habitats of Oman represents a first step towards their application in sustainable land management practices.

The first experiment included only a small proportion of the total available AM fungal single-spore derived cultures and one native plant species. Therefore, to arrive to solid conclusions about the potential of a widespread use of AM fungi in sustainable land use practices, there is a need for more complex and larger experiments. In future, the germplasm bank will serve various basic and applied research with efficiency assessment of different AM fungal isolates to promote plant growth, conservation and agriculture in Oman.

## 6.4 Acknowledgments

This project was supported by the Swiss National Science Foundation (grant nos. 127563 to Thomas Boller and 130794 to Andres Wiemken) and by Oman's Ministry of Agriculture and Fisheries (internal grant and study leave (2966/2008) to Mohamed Al-Yahya'ei). Special thanks go to the staff of the Oman botanic garden for their help in conducting the *P. cineraria* experiments

# 7 Inoculation of date palm (*Phoenix dactylifera*) using native arbuscular mycorrhizal fungi

## 7.1 Introduction

Date palms (*Phoenix dactylifera*) are the main crop in Oman and in many other desert areas in the world (FAO, 2009). They are believed to be the most ancient crop in the world with a high cultural and economical value (Chao & Krueger, 2007). Furthermore, they represent a survival crop due to the high nutritional value of its fruit (Al-Shahib & Marshall, 2003). Within the last decade, the date production had significantly decreased in some regions partially due to the spread of bayoud disease (caused by *Fusarium oxysporum* f. sp. albedinis) (Zaid *et al.*, 2002). The date palm not only has to struggle with diseases, but it also has to cope with poor soil structure of newly planted areas, low organic matter and nutrients, soil salinity and drought (Zaid *et al.*, 2002).

Fortunately, date palms are able to form symbiosis with arbuscular mycorrhizal (AM) fungi (Dreyer *et al.*, 2006; Al-Yahya'ei et al., 2011). The symbiotic AM fungi are well known to convey multiple benefits for plant growth and health, above all in stressful environments (Newsham *et al.*, 1995; Smith & Read, 2008). Under arid conditions, mycorrhizal plants were found to maintain higher drought tolerance (Augé, 2001) and have better access to phosphorus and other nutrients (Neumann & George, 2004a) leading to increased yield compared to non-mycorrhizal plants (Ceballos *et al.*, 2013). Additionally, AM fungal hyphae and hyphal exudates improve physical and chemical soil conditions (Bearden & Petersen, 2000), enhance soil aggregate stability and soil structure (Rillig & Mummey, 2006), and consequently improve soil moisture characteristics (Fusconi and Berta, 2012).

Positive impacts of the AM symbiosis on the growth and health of date palms were already observed. Under greenhouse conditions, AM fungi promoted the growth of date palm seedlings, especially on nutrient-poor soils (Shabbir et al., 2011). Furthermore, they have been shown to protect date palm seedlings against bayoud disease (Jaiti *et al.*, 2007).

From these observations it follows that the AM symbiosis is an important factor which needs to be considered in the propagation and cultivation processes of date palms. Often, date palms are produced by tissue culture (micropropagation) techniques. The first period involves the growth of date palm seedlings *in vitro*. After seedling establishment, the plantlets are transferred to artificial soil lacking AM fungi and grown for several months under greenhouse conditions for acclimation.

In this study, tissue cultured date palm seedlings were inoculated with desert-habituated AM fungi strains under greenhouse conditions. Inoculation with AM fungi proved to be as beneficial as foliar fertilization after two years of date palm growth. The experiment emphasizes the importance of integrating efficient and native AM fungal strains in the process of date palm seedling production.

## 7.2 Materials and Methods

The experiment was established in an agricultural research station belonging to the Ministry of Agriculture and Fisheries, Oman. The seedlings were tissue-cultured in the tissue culture lab of Jemah (varaiety of Khalas Al Daherah). Date palms were established for 28 month in callus stage under tissue culture conditions, transplanted into 100 mL pots filled with peat moss (Plantafior, Germany) and vermiculite (1:1;v/v) and grown for five month in a shade house (temperature: 24-32°C; relative humidity: 65-70%). Six single-strain (*Septoglomus africanum*, strain G14; *Diversispora aurantia*, strain G8; *Claroideoglomus drummondii*, strain F41; *Diversispora omaniana*, strain F69; *Paraglomus* sp., strain C49; *Paraglomus* sp., strain C57) one mixed-strain (including all six AMF strains), a non-mycorrhizal control (NM-control) and a non-mycorrhizal fertilizer control (NM-fert) treatment were applied. The morphological and molecular identity and origin of the AM fungal species used are previously described (Symanczik *et al.*, 2014a,b). A total of 250 spores were used for the single-strain treatments, 200 spores for the mixed-strain treatment and NM-fert plants received foliar-fertilizer (Micromix X200 SP, Pioneers Chemicals Factory CO, Saudi Arabia) weekly. The NM-fert treatment represents the conventionally applied handling practice of date palm seedlings in the tissue culture lab. Date palm seedlings were selected on the basis of homogeneity in total height and leaf number, inoculated and transplanted into 3 L plastic bags in March 2011 until March 2013. Plant growth performance was estimated by measuring the amount of leaves, the total height (accumulated length of all leaves to estimate total biomass) and the stem diameter at the base of the date palm.

## 7.3 Results

The amount of leaves was significantly higher in all single-strain inoculated plants and the NM-fert plants compared to NM-control plants (Fig. 1a). The total height was significantly higher for date palms inoculated with *S. africanum*, *D. aurantia* and *C. drummondii* and for NM-fert plants compared to NM-control plants (Fig 1b, c). Stem diameter was similar for all treatments and ranged between 44.1cm and 47.7 cm.

## 7.4 Discussion and conclusion

With the integration of AM fungi inocula into the propagation process, similar growth results of mycorrhizal date palm seedlings were achieved without applying mineral fertilizers compared to the fertilizer control treatment. Date palms possess a shallow and coarse root system and therefore, they are highly dependent on the AM symbiosis. By introducing AM fungi early in the propagation, we enabled the plants to acquire sufficient amounts of nutrients from the soil itself, without the need of additional fertilizers. However, not all AM fungal species improved growth to a similar extent. Only inoculation with *S. africanum*, *D. aurantia* and *C. drummondii* resulted in increased biomass production compared to non-mycorrhizal control plants. *S. africanum* and *D. aurantia* were originally recovered from date palm plantations (Symanczik *et al.*, 2014a,b) and hence, might be specially adapted to interact with date palms. However, only few studies on the interaction of AM fungi and date palms have been conducted so far. Inoculation with a commercial AM fungal inoculum enhanced growth of tissue cultured date palm seedlings. Mycorrhizal seedlings without fertilizer input grew better than non-mycorrhizal full-fertilized control seedlings and furthermore, the mycorrhizal seedlings performed better when subjected to saline conditions (Shabbir et al., 2011). Also Al-Whaibi and Khaliel (1994) observed an improved potassium and phosphorous nutrition of date palm seedlings inoculated with *Glomus deserticola* compared to non-mycorrhizal control seedlings grown under nursery conditions.

Normally, during the hardening process of tissue-cultured date palm seedlings are grown in artificial growth substrate (peat moss, vermiculite) lacking AM fungi. Additionally, mineral fertilizers are commonly used to increase seedling growth. After transplantation into the field, it is difficult for the date palms to establish AM symbiosis due to the low infection potential of desert soils (Requena *et al.*, 2001) and the high amounts of fertilizer added to the nutrient deficient soils (Smith and Read, 2008). By providing the date palm seedlings with an already established AM symbiosis prior the transplantation into

the field, the high input rates of mineral fertilizers might be reduced to achieve the same growth and yield, as it was shown for oil palms (Schultz, 2001).

Another important aspect which favors the application of mycorrhizal inocula is the potential of improving the out-planting success. Date palms propagated by tissue culture often have difficulties to establish after transplantation into the field and only about 50-90% survive the first period after transplantation (Zaid & De Wet, 2002) By providing the date palm seedlings with an already established AM symbiosis prior the transplantation into the field, such low success rates might be improved. Studies performed with oil palms increased the survival rate after transplantation from 55% for non-mycorrhizal seedlings up to 83% and 100% for mycorrhizal ones (Schultz, 2001). Additionally, with an already established AM symbiosis, date palm seedlings were shown to be better protected against diseases as mentioned earlier.

The application of AM fungi in the propagation process of tissue cultured date palms harbors a huge potential. In terms of economical aspects, overall production costs could be reduced by lowering the input of mineral fertilizers and improving the survival rates of date palm seedlings. And, even more importantly, the production process will turn to be more sustainable by alleviating the problems associated with high chemical inputs. However, more studies are needed to test and confirm the above mentioned assumptions.

## 7.5 Outlook

After transplantation into the field in 2013, we will continue to monitor the growth performance of inoculated date palms. We will assess if yields and nutrient contents of dates will be improved in mycorrhizal date palms and if the identity of the associated AM fungus will also result in differences of those parameters. Furthermore, we will assess the persistence of introduced AM fungi in the rhizosphere of date palm roots as propagules in the soil but also within the date palm roots.

Further studies investigating the timing of inoculation should be performed to assess the most beneficial point of time for AM fungal application. Inoculation during the *in vitro* growth phase of seedlings might be one of the best moments as the transfer from *in vitro* to the soil displays the most critical step with low survival rates. Plants grown *in vitro* with almost 100% relative humidity often show structural and physiological differences as reduced cuticular wax deposits and excessive water losses. AM fungi were shown to improve water relations of mycorrhizal plants hence, the application of AM fungal inocula during the *in vitro* phase might enhance the transplantation success of date palm seedlings to the greenhouse.

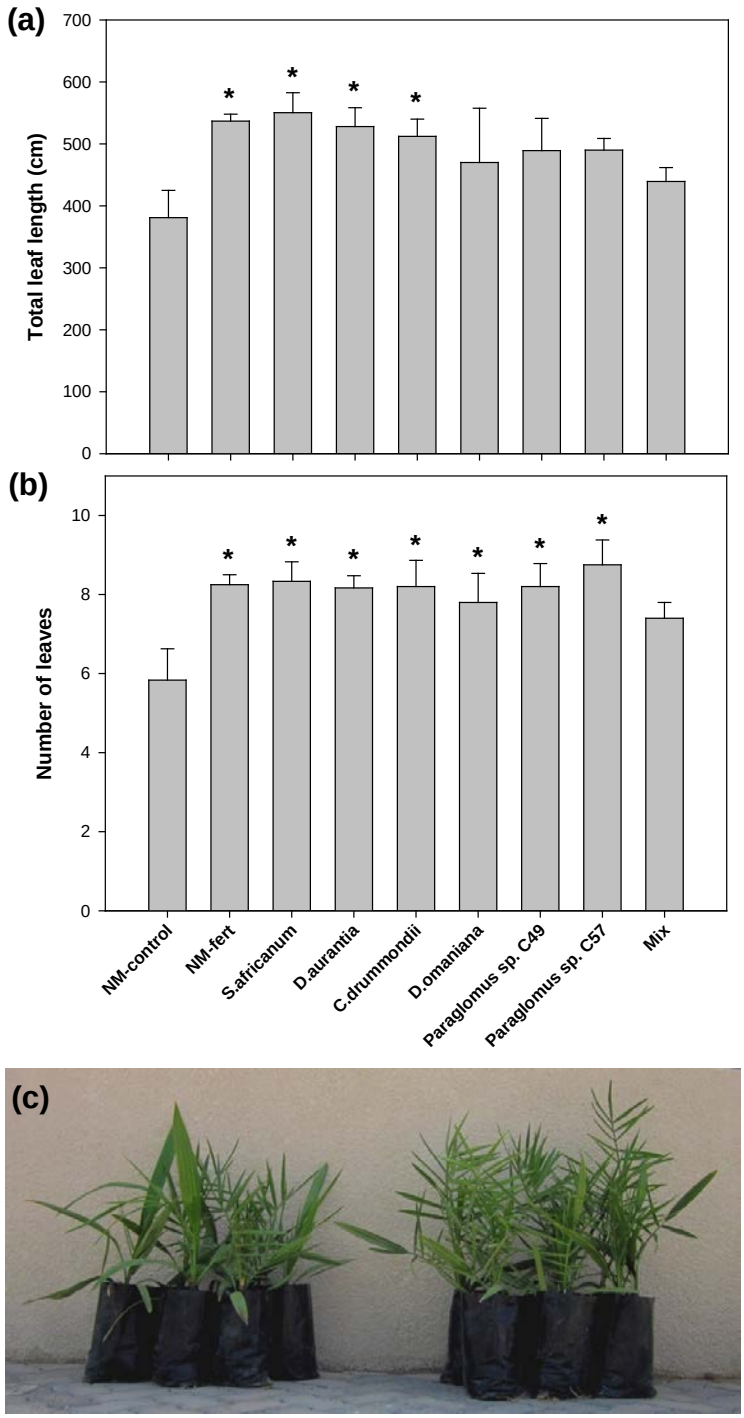


Fig.1 Impact of inoculation with different AM fungal species on (a) the total leaf length and (b) the number of leaves of *Phoenix dactylifera* seedlings. (c) Growth differences between non-mycorrhizal control seedlings (left) and seedlings inoculated with *Septoglomus africanum* (right). Stars above bars indicate significant differences compared to non-mycorrhizal control plants according to LSD's multiple range test with a significance level of 0.05%. Data represent means + SE (n=4-6).

# 8 General discussion

## 8.1 AM fungal species diversity and related challenges

Öpik *et al*. (2006, 2013) convincingly demonstrated that the global diversity of AM fungal species is far underestimated. They screened data sets of several publications which investigated the community composition of AM fungal species colonizing field roots deriving from a variety of different ecosystems. They found many sequence types which could not be related to any known species, thus displaying so far potentially undescribed AM fungal species. Similarly, at our study site located in Southern Arabia, Al-Yahya'ei *et al*. (2011) found a unique AM fungal community composition by investigating on i) AM fungal species colonizing date palm roots of an agricultural field site and ii) AM fungal spore abundance and richness in two date palm sites and, under natural vegetation, in undisturbed habitats around these sites. Our efforts in isolating, propagating and characterizing AM fungi from Southern Arabia led to the discovery of undescribed species at both locations: one species (*F. nakheelum*) originated from one of the agricultural date palm plantations and two (*R. arabicus* and *D. omaniana*) from the undisturbed habitats. Considering the novelties detected at only two locations, it is very likely that much more undescribed species are present. Therefore, exploring more habitat types and plant species, especially endemic and rare plant species, there is a potential for detecting a plenitude of novel AM fungal species in the so far unexplored region of Southern Arabia.

What are the reasons for the discrepancy between the low numbers of so far 252 described AM fungal species and the plenitude of new undescribed phylotypes detected by molecular techniques in roots from environmental samples? Species description in the Glomeromycota involves several steps (the process is described in chapter 2 and illustrated in Fig. S6.1) and comprises several challenges. Firstly, trapping the new AM fungal species by so-called trap-cultures. Different factors might restrict the successful multiplication of the target AM fungal species in the trap-cultures: (i) AM fungal spores are not detected in the soil at the time of sampling either because of seasonality of sporulation (Klironomos *et al.*, 2001) or because of the low numbers of spores in the soil characteristically for arid and semiarid ecosystems (Requena *et al.*, 1996; Azcón-Aguilar *et al.*, 2003). (ii) The plant species used in the trap culture might not be suitable as host for certain AM fungal species, due to host plant preferences (Bever *et al.*, 1996; Yang *et al.*, 2012). (iii) Growth conditions in terms of temperature or water conditions during the propagation process might be not favorable for the AM fungus to colonize the plant (Klironomos *et al.*, 2001). After successful establishment of trap-cultures, AM fungal single-spore derived cultures have to be established. The deposition of such cultures in a gene bank is of crucial importance to preserve them as

references on one hand and for further investigations on the other hand. The single-spore derived cultures serve further as basis for morphological and molecular investigations. The second challenge involves the morphological characterization (described in chapter 1.3.2). Investigated characters, comprising mainly spore morphology, are difficult to recognize, and it needs years of experience to acquire the ability. Especially in ancestral lines of AM fungi, as e.g. in the genus *Paraglomus,* AM fungal species possess a simple morphological arrangement. It is challenging to find morphological differences between AM fungal species in these clades. For example, one of the AM fungi, we have recovered from the natural undisturbed habitat, and propagated in single-spore derived cultures, we were unable to scientifically name it so far, because of high morphological similarity with related members of the same family (under preparation, personal communication Janusz Blaskowski). Further problems arise when short DNA sequences of low resolving power are used to identify a new species and submit it to the public databases (Redecker *et al.,* 2013). *Claroideoglomus drummondii* isolated from the natural habitat is clustering in a sister clade with sequences of *C. drummondii* and *Claroideoglomus walkeri* obtained from the National Center for Biotechnology Information (NCBI) data base (chapter 3). It should be expected that our sequence of *C. drummondii* is clustering directly with the sequence of the data base. Since the sequence deposited in the data base is spanning the full ITS region, but only a small part of the LSU region, comparisons are difficult and similarity scores are weak leading to unexpected and false clustering.
Generally, it can be stated that AM fungal species description is a difficult task as summarized above. Furthermore, AM fungi, as obligate symbionts, can be propagated only in association with a host plant and, as they are hidden under ground without producing noticeable and easily visible structures, it is difficult to spot and collect them in the field.

## 8.2 From AM fungal species to AM fungal communities

Members of the Glomeromycota were shown to be either “generalists”, with worldwide distribution or “specialists” which are restricted to only one type of ecosystem, field site or even a single plant species. It is generally accepted that AM fungal species are adapted to environmental conditions prevalent at their original ecosystem. These two factors can be accounted for the formation of distinct AM fungal communities inhabiting all kinds of different ecosystems around the world. Several studies have demonstrated that plant diversity and community structure are influenced by AM fungi and by the composition of AM fungal communities (van der Heijden *et al.,* 1998; Klironomos *et al.,* 2000; Bever *et al.*, 2001). Consequently, a change in the AM fungal community composition might lead to a change in the plant community composition. In chapter 4, we have demonstrated that the introduction of a potentially invasive AM fungal species, in this case *R. irregularis* can lead to a displacement of native

adapted AM fungal species especially under drought conditions. Simultaneously, crucial symbiotic functions, as plant growth promotion and extraradical mycelium production were decreased under drought conditions. This may lead to an overall loss of beneficial interactions of adapted native AM fungi with the local plant community. Such a displacement of AM fungi becomes even more important when considering "specialist" AM fungal species with a restricted distribution. Öpik *et al*. (2006) found that half of the investigated AM fungal taxa were detected from only one site, and furthermore that about one-third of them were recorded even from only one host plant species. This observation is in accordance with our own study in which we discovered undescribed AM fungal species which have never been detected elsewhere on earth (chapter 2). *Otospora bareai* is another example of "specialist" AM fungus which was recovered from a dolomitic shrub land in Sierra de Baza National Park in Spain (Ferrol *et al.*, 2008). The authors hypothesized that this rare or even endemic AM fungal species might be of special importance for the presence of rare endemic plant species in this type of ecosystem. Therefore, it is of special interest to uncover AM fungal "specialists" in the roots of co-occurring endangered plant species and further propagate them in single-spore derived cultures to be used in inoculation approaches. AM fungal inocula consisting of native AM fungal species were often found to be superior in growth promotion of endangered plants compared to the general AM fungal species often used in the lab (Vosátka & Dodd, 2002). Even if in some cases, improved plant growth was obtained after inoculation with "generalist" AM fungi under controlled conditions, their performance in the field should be considered in terms of selecting the appropriate AM fungal species (Zubek *et al.*, 2009). Therefore, it is important to study each endangered species individually, exposed to the particular environmental conditions prevalent at its original habitat, in order to determine the most effective AM fungal inocula for practical applications.

In our study, the abundance of each AM fungal species was measured by detecting transcript abundance of ribosomal large subunit (rLSU) genes within the host root to reveal the active and, thus, functional part of the AM fungal species colonizing the roots (chapter 3). However, we did not investigate the sporulation ability of the AM fungal species before and after inoculation with the invasive *R. irregularis*. By analyzing the formation of spores, the end of the glomeromycotan life cycle, we might have obtained clues on the propagation success of the different AM fungal species under the pressure of competition. Such additional information would have shed new light on the impact of *R. irregularis* on a native AM fungal assemblage especially under drought conditions.

## 8.3 Functionality of drought-adapted AM fungal species

Several studies have demonstrated differences in plant performance in terms of plant water relations, growth rates or nutrient acquisitions after inoculation with different AM fungal species. In our study, we

particularly addressed the efficiency to extract $^{15}$N-ammonium sulfate ($^{15}$N) under different water regimes to elucidate if AM fungal species exhibit differences in their efficiency to take up $^{15}$N from drying soil. Studies on the diversity and functionality of mycorrhizal nutrient transporters from different AM fungal species are missing. Therefore, we cannot make comparisons with previous data. We can propose several mechanisms which might have led to the observed differences in $^{15}$N uptake of extraradical hyphae between *R. arabicus* and *R. irregulare*: (i) hyphal N-transporters display different affinities for N uptake; (ii) AM fungi differentially regulate and express their N-transporters in the extraradical mycelium; (iii) AM fungi possess different sets of hyphal N-transporter homologues. To resolve the question on the mechanisms behind, it would be important to identify and functionally assess AM hyphal ammonium and nitrate transporters.

When considering the impact of drought on plant performance it is of interest to investigate the expression of aquaporins (AQPs) as they are important in the regulation of root water uptake (Luu & Maurel, 2005). The improvement of drought tolerance of plants by AM fungi is well accepted but whether AQPs are involved in this process is not well understood. Several studies have demonstrated that plant AQPs are differentially regulated upon inoculation with different AM fungal species and two different hypotheses were proposed including either water facilitation (by maintenance or up-regulation of AQP expression) or water conservation (by down-regulation of AQP expression) (as described in the appendix). However, to make assumptions about the involvement of AQPs in promoting plant drought tolerance it would be important to (i) conduct tissue-specific analyses of gene expression and protein localization for each AQP in the host root and (ii) to resolve the function of each AQP as they were shown to transport a variety of different molecules in addition to water (e.g. urea, boric acid, silicic acid, ammonia, carbon dioxide). It might be very likely that AQP transporters in roots might help to take up ammonia which becomes less mobile when soil is drying. So far, only few studies investigated the function of AQP transporters and mostly they studied only some selected AQP homologues (Ruiz-Lozano & Aroca, 2010). To make overall assumptions about the involvement of AQPs in the AM fungal-mediated promotion of drought tolerance it would be important to study each AQP transporter, its localization in the root and its exact function as they all play in concert with each other. Furthermore, a full comprehensive study including all functional traits of an AM fungal species (root colonization, HLD and expression of fungal transporter in the intra- and extraradical hyphae) would provide deeper insights about possible mechanisms involved. But it would still remain difficult to apply this knowledge to natural ecosystems in which plants are simultaneously associated with several AM fungal species which all might provide different functions to their host (as mentioned in chapter 1.4).

Thus, the often used statement “the contribution of AM symbiosis to plant drought tolerance is the result of accumulative physical, nutritional, physiological and cellular effects” (Ruiz-Lozano, 2003) well

describes the observed impact of AM fungi on plant drought tolerance and gives further evidence that it is not possible to separate those effects from each other for explaining the overall picture.

## 8.4 Application of native AM fungi as biotechnological tool

The application of AM fungi as biotechnological tool in horticulture, agriculture and revegetation programs harbours a huge potential. The number of studies, which demonstrate improved plant growth and nutrition and amendments of soil characteristics after inoculation steadily increases. We were able to show that inoculation with native AM fungi positively affected growth of *Prosopis cineraria* and *Phoenix dactylifera* under nursery conditions.

The use of AM fungi for extensive, agricultural applications requires huge amounts of fungal inocula and its provision need to be covered by mass production. Several companies started to produce and sell a variety of commercial AM fungal inocula which were mainly comprised of *R. irregularis* strains (Gianinazzi-Pearson, 2002). However, a quality check is crucially needed as commercial products were partially shown not to fulfill their beneficial functions. A study was performed to investigate the infectivity of ten commercially available mycorrhizal inocula (Corkidi *et al.*, 2004). Maize plants were inoculated with recommended rates of each inocula type and grown on three different substrates. The observed percentages of root colonization ranged from highly insufficient (0%) to satisfactory (50%) and might be explained by the presence or absence of viable propagules, the content and type of infective propagules, the amount of recommended application rates as well as unbeneficial soil-microbial interactions. This study highlights the restrictions associated with the use of commercial mycorrhizal inocula. For this reason, an own, small-scale production of mycorrhizal inocula should be favoured. Additional advantages are the own choice of trap plants for propagation, suitable for the target ecosystem, and the choice of an appropriate growth substrate, which will be used for subsequent plant growth. This way of inocula production is especially suitable for nursery applications, where smaller amounts of fungal inocula are needed. Another striking approach is the integration of so-called “nurse plants” into agricultural field. Studies have shown that certain plant species can act as “nurse plants” by providing multifunctional benefits. *Lavandula stoechas* improved the establishment of *Cupressus atlantica* by positively affecting soil microbial characteristics and AM fungal development (Duponnois *et al.*, 2011). The presence of *L. stoechas* increased the total microbial activity, phosphate-solubilizing fluorescent pseudomonads, AM fungal networks and dehydrogenase activity in the soil as well as the nutrient uptake and growth of the accompanied *C. atlantica*. The use of “nurse plants” proved to be highly efficient especially in revegetation approaches (Ouahmane *et al.*, 2006; Bashan *et al.*, 2009) and is actually an

imitation of the naturally occurring “resource islands” (patchily distributed vegetation with improved rhizospere properties) characteristically for arid and semiarid ecosystems (Schlesinger *et al.*, 1996). However, all those beneficial impacts could be of great value for agricultural purposes as it is done in agroforestry systems. *Prosopis cineraria* would be a perfect candidate to be a “nurse plant” as it is deep rooting (hydraulic lift) and forms a tripartite symbiosis with AM fungi and rhizobial nitrogen-fixing bacteria. The integration of *P. cineraria* into agricultural fields would provide a multitude of direct and indirect benefits as e.g. improved microbial activity, nutrient availability and soil structure, enhanced water infiltration into the soil and the provision of shade. Furthermore, *P. cineraria* was shown to best improve organic carbon and available N, P and K in the soil compared to other tree species, due to its valuable litter input (Yadav *et al.*, 2008). This kind of vegetation assemblage can improve its own environment by self-maintaining several functions (Fig. 7.1) In summary, the combination of AM fungal inoculation approaches integrated into agoforestry-systems might be a highly valuable practice especially suited for sustainable, low-input systems with additional economic cost improvement.

## 8.5 Perspectives

The unique AM fungal communities uncovered at two locations in the desert ecosystem of Oman (Al-Yahya’ei *et al.*, 2011) and the observed differences in these community’s structures indicate the adaptation and specialization of those AM fungal communities. The detection of four new AM fungal species isolated at a single harvest time suggests that there might be more undescribed AM fungal species present. Reasons leading to this assumption are the following: i) Spore isolation from trap cultures took place only once to start single-species cultures. It was shown that AM fungal species have different sporulation times dependent on the season and that they undergo a kind of succession (Stutz & Morton, 1996; Hart *et al.*, 2001). By continuously isolating spores from the trap cultures during the year over the whole period we could have increased the isolated AM fungal species richness. ii) Variety of trapping plants was rather low. It was shown that plant identity influences which AM fungal species colonize the host plant and further sporulate in the trap culture (Bever *et al.*, 1996). iii) Growth conditions

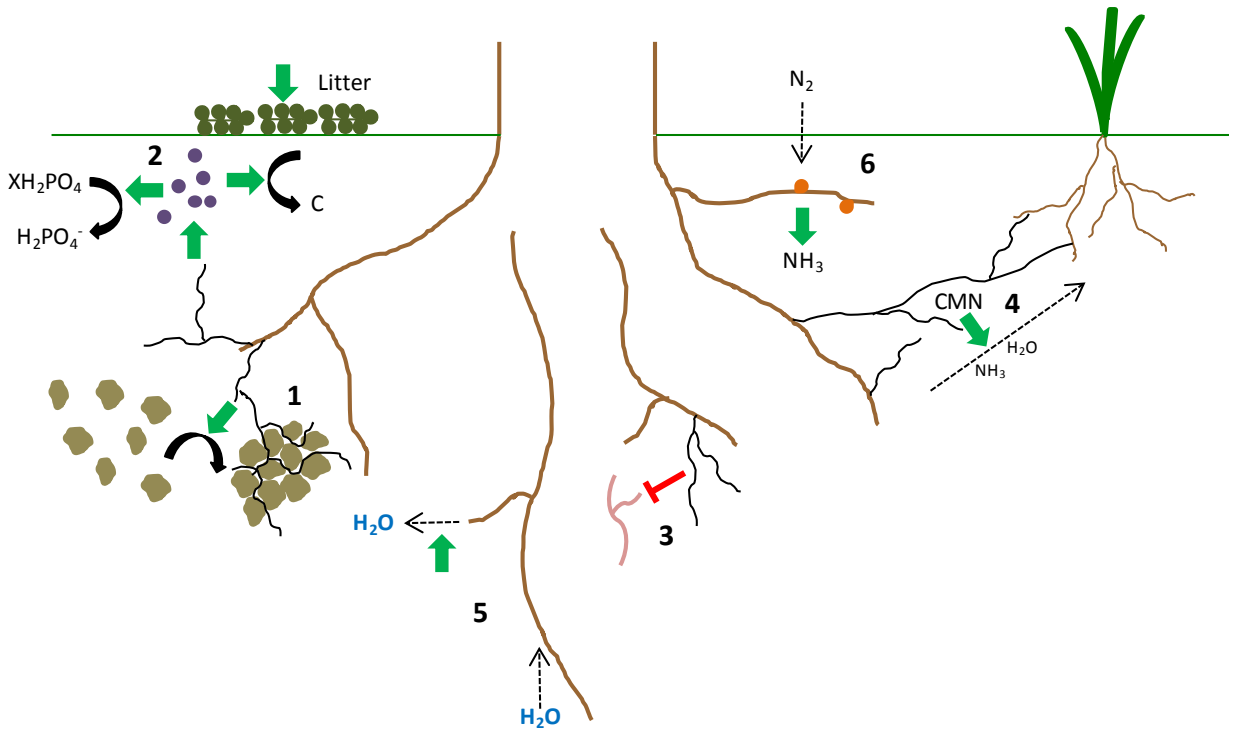


Fig. 7.1 Schematic view of possible interactions among different components within the soil of an agroforestry-system with *P. cineraria*. AM fungi can (1) improve soil structure by physical and biological actions of AM hyphae; (2) enhance microbial abundance and activity (mentioned later), and thus indirectly affecting nutrient provision (mobilization of P) and recycling (e.g. from litter). (3) Further, AM fungi can protect the plant against pathogens. (4) AM hyphae can connect the root systems of neighbouring plants, to build a common mycorrhizal network (CMN) by which ressources can be shared. (5) The deep-rooting *P. cineraria* can lift water from deeper to upper soil layers (hydraulic lift) and improve water infiltration in the soil. (6) Rhizobia of *P. cineraria* can fix nitrogen which can be further allocated to neighbouring plants.

of trap cultures were uniform. By introducing a range of growth conditions as e.g different water regimes in the trap culture pots, different temperature or nutrient application, we could have triggered the sporulation of different AM fungal species.

Furthermore, it would be interesting to explore more different habitat types in Southern Arabia. Habitats located close to the sea might include AM fungal species adapted to saline conditions, as it was demonstrated for AM fungal species isolated from coastal vegetation on Okinawa Island, Japan (Yamato *et al.*, 2008). Also AM fungal communities from mountain regions of Oman might harbor so far undescribed AM fungal species, similarly as for *Acaulospora alpina*, isolated in the Swiss Alps (Oehl *et al.*, 2006). Besides the exploration of new habitat types, endemic plant species should be further included

in the observation as they were shown to be associated with unique AM fungal species (Ferrol *et al.*, 2008).

AM fungal community assembly is simultaneously driven by many factors as summarized in chapter 1.5.2. In general, it can be stated that environmental conditions and interspecific competition among AM fungal species are responsible for the formation of distinct AM fungal communities adapted to the inhabited ecosystem.

In the experiment of chapter four, we have chosen four desert AM fungal species and investigated the impact of water regime on their assembly. We observed that water regime had a strong impact on the assembly of those AM fungal species inside the roots of sorghum. Competition was included as additional factor by introducing *R. irregularis* to the native AM fungal assemblage. We could observe that the abundance of the native AM fungal species was either decreased (*Septoglomus africanum, Diversispora omaniana*), not affected (*Paraglomus* sp. exposed to dry conditions) or even increased (*D. aurantia* under well-watered conditions). To get insights into the mechanisms responsible for AM fungal species assembly, it would be interesting to include different combinations of AM fungal species. The individual colonization of each AM fungal species alone would serve as reference, measured by quantitative real-time PCR. Further, plants could be inoculated with different combinations of AM fungal species, starting with dual-inoculation, triple-inoculation etc. to resolve the mechanisms influencing competition among AM fungi and AM fungal community assembly. It would also give further evidence if community assembly is mainly based on phylogenetic relatedness as proposed by Maherali *et al.* (2007). These authors have shown that phylogenetic relatedness, which may comprise functional relatedness, determines AM fungal community assembly. By constructing experimental communities consisting of either closely related (within one family) or distinct (from three different families) AM fungal species, they found that realized species richness was highest in communities including AM fungal species from all three families. Therefore, phylogenetic relatedness displays another driver for AM fungal community composition. By quantifying internal root colonization and external hyphal production by quantitative real-time PCR in all different AM fungal species combinations and comparing their functional abilities in terms of intra- and extraradical colonization, competitive interactions among AM fungal species might be better understood.

Furthermore, the sporulation ability in response to competition should be observed as the formation of spores represents the end of the AM fungal lifecycle. AM fungi possess different sporulation strategies with some AM fungal species producing small spores in huge amounts whereas others forming big spores in low amounts. To investigate the sporulation strategy of each AM fungal species would be important to better interpret the data of external colonization. By measuring the spore abundance in single-inoculation pots, the general trait of each AM fungal species could be estimated and would serve as standard to make

further comparisons in terms of competition. Furthermore the growth period should be extended and different sampling points could be included for the same reasons as mentioned above. Additionally, these evaluations could be extended by including different abiotic parameters as water availability, as in our study, or temperature or plant species.

It is reasonable to believe that the members of the AM fungal community found in the desert ecosystem of Oman, which harbors newly discovered species, possess unique physiological properties adapted to withstand desert extreme conditions such as dehydration in long drought seasons, high summer temperatures and low soil nutrients. Therefore, functional characteristics of these AM fungal species should be further investigated. By demonstrating that inoculation with *R. arabicus* increased transpiration of sorghum under drought and that the fungus exhibited greater capacity to extract $^{15}N$ from dry soil, we can propose that *R. arabicus* is better adapted to drought conditions. *R. arabicus* has never been detected somewhere else and might represent a specialist for dry habitats as it was only found in association with the natural vegetation. We can assume that also the newly described *D. omaniana* and *Paraglomus* sp., which were recovered from the natural vegetation, might exhibit adaptations to dry conditions. Functional experiments, including those AM fungal species, would be necessary to reveal their functional characteristics.

Our efforts in applying native mycorrhizal fungi in the propagation process of *Prosopis cineraria* under nursery conditions and the subsequent transplantation of *P. cineraria* to the field opens the door for further experiments: to test the suitability of mycorrhizal *P. cineraria* as agroforestry tree and to reveal if the identity of associated AM fungal species differentially affects (i) growth of *P. cineraria* under field conditions and (ii) physico-chemical and biological soil properties such as nutrient availability, microbial activity or soil structure.

Studies performed in arid and semiarid Mediterranean ecosystems revealed that inoculation with AM fungi may enhance total microbial abundance and activity in the soil (Duponnois *et al.,* 2011) and that it might differ according to AM fungal and plant species (Alguacil *et al.*, 2005). Dehydrogenase is a suitable indicator to estimate the overall soil microbial activity, especially in arid ecosystems. Hence, it would be a good tool to measure the microbial activity in the rhizospere of *P. cineraria* in relation to the inoculated AM fungal species. Additionally, inoculation with native AM fungi improved soil aggregate stability (Jeffries *et al.,* 2003), an important feature for the loose and sandy soils particular for desert ecosystems, which can be easily assessed by measuring the percentage of stable aggregates.

To evaluate the suitability of *P. cineraria* with its tailored mycorrhizal symbiosis for agroecosystems, we could establish intercropping systems with separated root zones to focus on the involvement of the

mycorrhizal network. Several aspects could be studied: (i) the transfer of fixed N from *P. cineraria* to the neighbouring plant via the CMN; (ii) the transfer of hydraulically lifted water; (iii) the importance of AM fungal species and (iv) the compatibility of neighbouring plant species with *P. cineraria* and the associated AM fungus.

## 8.6 From basic to applied research – concluding remarks

In this thesis we initiated a pioneering international collaborative program aiming to isolate, identify and functionally assess the AM fungi from Omani agricultural and natural habitats. We have recovered taxonomically unique communities of AM fungi and obtained them in pure single-spore derived cultures forming a core of an expanding germplasm bank. The ecosystems of Oman have to cope with drought, heat, soil salinity, and low fertility, particularly due to low phosphorus (P) availability. In such environmental conditions, the beneficial multifunctional mycorrhizal symbiosis is a key factor to promote sustainable agriculture. Most importantly, native AM fungal communities inhabiting the soil of such ecosystems must be "stress-adapted" to withstand these conditions, and they may have particularly interesting physiological properties. Therefore, it is a worthy scientific effort to investigate the functionality of these “stress-adapted” AM fungal strains and to apply them as a biotechnological tool to improve agriculture in this region and to use them in conservation ventures. Furthermore, we could give evidence that the application of native adapted AM fungal species should be considered instead of making use of commercially traded exotic AM fungi. In our experiment, *R. irregularis*, a commercially traded species, significantly displaced the native AM fungal species colonizing sorghum roots with consequent reduction in their beneficial functions under drought conditions in the greenhouse. This underlines the importance of selecting adequate fungal ecotypes originating from the targeted application site for producing appropriate inocula for mycorrhizal-based inoculation programs.

# Appendix: *Sorghum bicolor* aquaporins are differentially regulated in response to changing water regimes and symbiosis with arbuscular mycorrhizal fungi

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## Introduction

In roots, water transport occurs via two major pathways, the apoplastic and cell-to cell (symplastic and transcellular) pathway (Steudle, 2000). After water is taken up from the soil, it passes the root cortex mainly via the apoplastic pathway, driven by the water potential gradient. However, at the endodermis, the water is forced into the cell-to-cell pathway due to the Casparian strip, which blocks the apoplastic pathway. As "gate keeper" cell layer, the endodermis functions as an important regulatory organ where the plant is able to control the uptake of water and mineral nutrients. Water movement across the endodermis occurs along the water potential gradient as well, but it is mediated, and potentially regulated, by aquaporins (AQP) in the plasma membrane at the outer and inner surface of endodermis cells (Luu & Maurel, 2005), thereby affecting the hydraulic properties of the root system.

AQPs are a family of small pore-forming integral membrane proteins. They have a well-conserved structure where the pore is formed by the interaction of six membrane-spanning helices and two loops containing characteristic asparagine-proline-alanine (NPA) motifs. They have been shown to facilitate the transport of water, small neutral solutes (i.e. urea, boric acid, salicylic acid) or gases (i.e. ammonia, carbon dioxide). In plants, they form a large family of 35 members in Arabidopsis (Johanson *et al.*, 2001), of 33 members in maize (Chaumont *et al.*, 2001) and rice (Sakurai *et al.*, 2005), and of 55 in poplar (Cohen *et al.*, 2013). Based on amino-acid sequence comparison, AQPs of most plant species can be divided into five subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and, only in dicots, uncharacterized intrinsic proteins (XIPs) (Maurel *et al.*, 2008; Lopez *et al.*, 2012). Among these groups, especially PIPs and TIPs are thought to be involved in the regulation of radial transcellular water transport in root tissues (Maurel *et al.*, 2008). For instance, the expression of PIPs in maize roots correlated with the presence of an apoplastic barrier (the exodermis) suggesting an important role in the transcellular water flow when its movement through the apoplastic path is hindered (Hachez *et al.*, 2012).

Since it has been shown that the AM symbiosis regulates root hydraulic properties, including root hydraulic conductivity (Ruiz-Lozano & Aroca, 2010), researchers started to investigate the involvement of the AM symbiosis on the regulation of plant AQPs (Ruiz-Lozano and Aroca 2010). Roussel *et al.* (1997) and Krajinski *et al*. (2000) first demonstrated mycorrhiza-induced expression of TIPs in parsley and *Medicado truncatula*, respectively. However, AQP encoding genes may be differentially regulated by AM symbiosis and drought conditions. Three out of four PIP encoding genes were differentially regulated in NM roots and in roots of *Rhizophagus irregularis*-inoculated *Phaseolus vulgaris* under drought

conditions. The expression of *PvPIP1.3* and *PvPIP2.1* increased whereas the expression of *PvPIP1.2* decreased and that of *PvPIP1.1* was not affected in NM roots. In contrast, the expression of *PvPIP1.1* slightly increased whereas the expression of *PvPIP1.2* and *PvPIP1.3* decreased and that of *PvPIP2.1* was not affected in AM roots (Aroca *et al.*, 2007).

Here, we focus on the AQPs encoding genes of sorghum (*Sorghum bicolor*), an important crop plant the genome of which has been fully sequenced (Paterson et al., 2009). Sorghum is the world's fifth biggest crop (after maize, rice, wheat and barley) and it can grow under relative arid conditions where it serves as an important source of food, feed and fibers. Bioinformatic analyses of the sorghum genome revealed a set of 35 aquaporin (AQP) encoding genes in the fully sequenced genome of sorghum. The transcript abundance of 14 selected AQPs was measured in roots of sorghum in the presence or absence of AM fungi under two water regimes (well-watered and drought). Two closely related AM fungal species originated from contrasting climates were tested: *R. arabicus*, isolated from a hyper-arid sand plain in Oman and *R. irregularis*, isolated from an agricultural field in Switzerland. Gene expression analysis revealed that some AQP genes were differentially regulated, either by water regime, AM treatment or by both factors.

## Materials and Methods

*Plant growth conditions and experimental set-up*

The experimental design was the same as described in chapter 5. For this study only sorghum roots were used for analyzes of plant aquaporins (AQPs).

*Identification and characterization of sorghum aquaporin (AQP) genes*

Identification and characterization of sorghum genes encoding AQPs was performed as described by Koegel et al. (2013a). A selection out of all identified AQPs for further investigation was made by screening the literature for homolog AQP genes of other plant species which were shown to be either mycorrhizal- or drought-regulated: *PcTIP*, (Roussel *et al.*, 1997); *MtTIP1.1*, (Krajinski *et al.*, 2000); *MtNIP1.1* and *MtPIP2.1*, (Uehlein *et al.*, 2007); *HaTIP7* and *HaTIP18*, (Sarda *et al.*, 1999); *GmPIP1*, *GmPIP2*, *LsPIP1* and *LsPIP2*, (Porcel *et al.*, 2006); *PvPIP1.1*, *PvPIP1.2*, *PvPIP1.3* and *PvPIP2.1*, (Aroca *et al.*, 2007); *PttPIP2.2* and *PttPIP2.4*, (Marjanović *et al.*, 2005); *PttPIP2.8*, *PttPIP2.10*, *PttNIP2.1*, *PttTIP1.2*, *PttTIP2.2-2.4*, (Cohen *et al.*, 2013); *LjNIP1*, (Giovannetti *et al.*, 2012); *OePIP1.1* and *OePIP2.1*, (Secchi *et al.*, 2007); *OsPIP1.1-1.3* and *OsPIP2.1-2.6*, (Lian *et al.*, 2006).

Sequencing, assembly, and annotation of the *S. bicolor* genome was described by Paterson et al. (2009). All *S. bicolor* sequences are available at the Phytozome website (http://www.phytozome.net/search.php?show=blast) and have been deposited at GenBank/European Molecular Biology Laboratory/ DNA Data Bank of Japan. Using BLAST search at the Phytozome website, we identified gene models coding for putative AQP genes in the draft genome. Gene prediction at the JGI was performed using gene predictors (FGENESH, and GENEWISE), and gene models were selected by the JGI annotation pipeline (Paterson *et al.*, 2009). Selection of the AQP genes was based on expressed sequence tag (EST) support, completeness, and homology to an accurate set of proteins. The detected putative homologs were characterized based on conserved domains, identities, and E-values in comparison with the use of a range of AQP gene sequences available from plants at the NCBI GenBank (http:// www.ncbi.nlm.nih.gov/) and Phytozome (http://www.phytozome.net/search.php?show=blast). The 35 identified AQPs are given in Table SA1.

For phylogenetic analysis, the AQP amino acid sequences were aligned with MEGA5 (Tamura *et al.*, 2011) using the following multiple alignment parameters: gap opening penalty 15, gap extension penalty 0.3, and delay divergent sequences set to 25%; and the Gonnet series was selected as the protein weight matrix. Neighbor joining trees were constructed using Poisson correction model for distance computation in MEGA5 (Tamura *et al.*, 2011). Gene accession numbers of amino acids sequences from sorghum are given in the table SA1, accessions of homologs from the other plant species are given in methods SA1.

*Quantification of AQP genes by quantitative reverse-transcription PCR*

To compare the relative abundance of AQP genes in sorghum roots after inoculation in the three AM treatments and the two water regimes, RNA extraction and cDNA synthesis were performed as described by Courty et al. (2009), using the conditions specified in Methods SA1. The relative abundance of selected AQP genes was analyzed by quantitative reverse-transcription PCR (qRT-PCR). cDNAs were used as templates in qRT-PCR reactions with gene-specific primers (Microsynth AG, Balgach, Switzerland) designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and amplify 3.1 (http://engels.genetics.wisc.edu/amplify) (Table SA2). The following criteria were used: product size between 100 and 150 bp, melting temperature 60°C, and a GC percentage > 50%. Target gene expression was normalized to the gene encoding the *S. bicolor* ubiquitin (Protein ID 5060159). Reactions of qPCR were run using the LightCycler® 480 System (Roche). The following cycling parameters were applied: 95°C for 5 min and then 45 cycles of 95°C for 15 s and 60°C for 1 min. A control with no cDNA was run for each primer pair. For data analysis, the geometric mean of the biological replicates ($n$ = 5) was calculated. The primer efficiency ranged between 90% and 110%. Fold-change expression was calculated relative to NM-well-watered conditions (control conditions).

*Statistical analyses*

Data were analyzed using either multifactorial ANOVA followed by LSD's multiple range test with a significance level of 0.05% (with the factors AM treatment and water regime) or an independent-samples *t*-test. Analyzes were performed using SPSS software version 20 (IBM, Zürich, Switzerland).

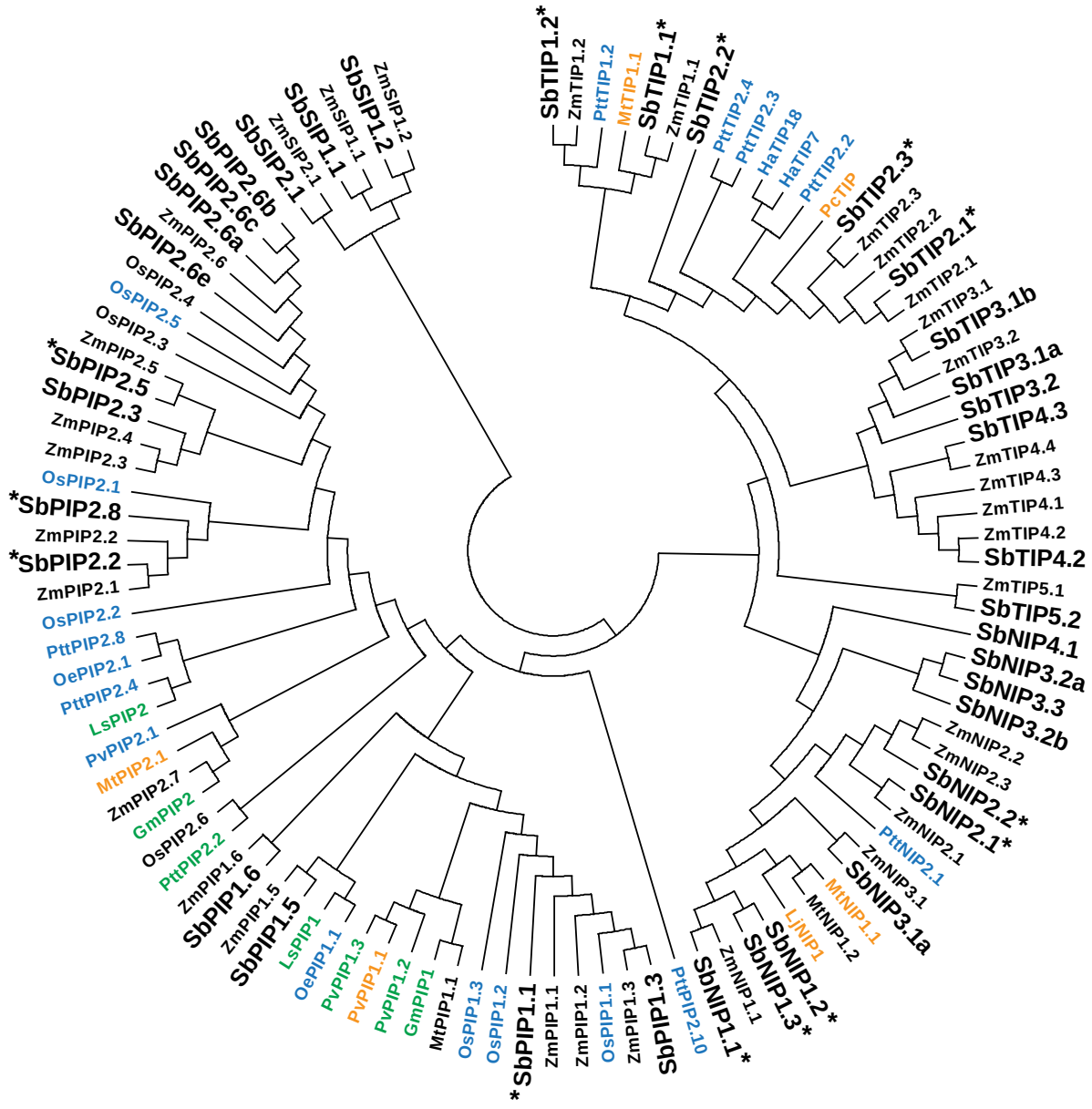


Fig. A.1 Neighbor-joining tree for aquaporins (AQPs) based on the full open reading frames. Sequence names consist of species code (first letter of genus and first letter of species name) and gene name. Species codes: Lj, *Lotus japonicus*; Ls, *Lactuca sativa*; Mt, *Medicago truncatula* Oe, *Olea europeae*; Os, *Oryza sativa;* Ptt, *Populus trichocarpa*; Pc, *Petroselinum crispum*; Pv, *Phaseolus vulgaris*; Sb, *Sorghum bicolor;* Zm, *Zea mays*. *S. bicolor* AQPs are enlarged in font size. Selected sorghum AQPs are marked with an asterix. Homologs regulated by drought (blue), mycorrhizal symbiosis (orange) or both factors (green) are highlighted.

## Results

*Identification of AQP genes in* S. bicolor

Thirty-five genes coding for putative AQP genes were identified in the predicted gene catalog resulting from the automated annotation of the whole *S. bicolor* genome assembly (v1.0, http://genome.jgi-psf.org/sorghum/sorghum.home.htm). A phylogenetic analysis was performed including the newly identified sequences and published sequences from other plant species (Fig. A.1). Twelve, 10, 10 and three of the AQP genes identified in the *S. bicolor* genome are members of the PIP, NIP, TIP and SIP subfamily, respectively. NIP1.4a and NIP1.4b were identified as truncated putative AQP (Table SA.1).

According to their clustering in the phylogenetic tree and similarities with homologues from other plant species, subfamilies were further divided into subgroups: Sorghum PIPs, NIPs, TIPs and SIPs have two (SbPIP1 and SbPIP2), four (SbNIP1, SbNIP2, SbNIP3 and SbNIP4), four (SbTIP1, SbTIP2, SbTIP3 and SbTIP4) and two (SbSIP1 and SbSIP2) subgroups, respectively. In the NIP subfamily, SbNIP3.1 has substitutions in both NPA motifs (NPS/NPV), while SbNIP4.1has a substitution from Ala (A) to Ile (I) only in the second NPA motive. In the SIP subfamily all first NPA motives possess substitutions, where the A residue is substituted by Thr (T) or Leu (L) (Table SA.1).

Sorghum AQP genes are spread over eight of the ten chromosomes (except chromosomes V and VIII). Six AQP encoding genes were located on chromosome VI, and between two and five AQP encoding genes on the other seven chromosomes. One AQP gene is located on scaffold12 not yet assigned to a chromosome (Table SA.1). The expression of 25 of the putative sorghum AQPs is supported by EST sequences (Table SA.1).

Five, five and four AQP encoding genes from the TIP, NIP and PIP subfamily were selected for further gene expression studies. Selection was based on a literature screen for homologs in other plant species which were shown to be regulated by mycorrhization or drought (Fig. A.1).

*Effect of water regime and AM treatment on AQP expression*

From the 14 selected AQPs, *NIP1.3* and *TIP2.2* were not expressed in roots and *TIP1.2* was expressed only at low levels in roots of *S. bicolor*. The other AQP genes were found differentially regulated, either by AM treatment, by water regime or by both factors. Relative fold-changes above 2.0 and below 0.5 were considered as significant. In the NIP subfamily, *NIP1.2* and *NIP2.2* were significantly down-regulated only in *R. arabicus*-inoculated plants grown under drought (D) conditions. The selected TIP encoding genes were regulated only under D conditions. *TIP1.1* and *TIP2.3* were significantly up-regulated in NM and *R. irregularis*-inoculated plants. *TIP2.1* was significantly down-regulated in *R. arabicus*-inoculated plants. The regulation of the selected PIP encoding genes was highly variable.

*PIP1.1*, *PIP2.2* and *PIP2.5* were significantly up-regulated in *R. irregularis*-inoculated plants under well-watered (WW) conditions. Under D conditions, *PIP2.2* and *PIP2.5* were up-regulated in NM and *R. irregularis*-inoculated plants. *PIP2.8* was down-regulated under D whatever the AM treatment. Fold-changes in AQP gene expression compared to NM-WW conditions are given in table A.1.

## Discussion

Aquaporins (AQPs) are transmembrane proteins which facilitate the diffusion of water across membranes. In roots, they may play an important role in water relations especially in so-called "gate keeper" cell layers, as e.g. the endodermis, where water movement is forced from the apoplastic to the cell-to-cell pathway.

*Thirty-five AQP genes in sorghum*

Bioinformatic analyses of the sorghum genome revealed a set of 35 aquaporin (AQP) encoding genes in the fully sequenced genome of sorghum. The number of sorghum AQPs was the same as in *A. thaliana* and similar to those of *Zea mays* and *Oryza sativa* (33 for both plant species). However, comparisons to the moss *Physcomitrella patens* with 23 AQP genes (Danielson & Johanson, 2008) and *Populus trichocarpa* with 55 AQP genes (Cohen *et al.*, 2013) reveal considerable differences in the numbers of AQP homologues within different plant taxa.

Based on amino-acid sequence comparison, AQPs of most plant species can be divided into five subfamilies which are associated to their specific membrane localization: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs) first identified in the symbiosomes of legumes, but also present in the plasma membrane and endoplasmic reticulum (ER), small basic intrinsic proteins (SIPs) found in the ER and only in dicots, uncharacterized intrinsic proteins (XIPs) localized in the plasma membrane (Maurel *et al.*, 2008a; Bienert & Chaumont, 2011; Lopez *et al.*, 2012). The composition of sorghum AQP subfamilies slightly differed compared to the composition of AQP subfamilies in other plant species (Table SA.4). Similar as in maize, rice and *A. thaliana*, sorghum have four AQP subfamilies (PIP, TIP, NIP and SIP). But, differences were more pronounced for poplar and *P. patens*: additionally, they have a subfamily of uncategorized intrinsic proteins (XIPs) and in *P. patens* only (specific of dicots), two more subfamilies of uncategorized intrinsic proteins (GIP and HIP).

Another common feature found in the NIP and SIP subfamilies of AQP genes are alterations in the asparagine-proline-alanine (NPA) motifs. NPA motives are located in loop B and E and meet at the center

of the pore, determining the substrate selectivity of the AQP. Similar as the poplar PttNIP3.1 and PttNIP3.2, SbNIP3.1 has substitutions in both NPA motifs. Within the SIP subfamily, such substitutions are even more frequent. The Ala (A) residue in the first NPA motif can be substituted by Thr (T) or Leu (L) as observed for sorghum, maize and poplar or by Thr (T), Leu (L) or Cys (C) as observed for *A. thaliana*.

*Effect of water regime and AM treatment on AQP expression in roots*

Several studies have investigated the regulation of AQP expression, as influenced by drought stress and association with AM fungi (Porcel *et al.*, 2006; Lian *et al.*, 2006). However, the biological significance of these regulation patterns remain unclear. Regulation of AQP gene expression might be of particular importance in "gate-keeper" cell layers as the endodermis. Here, it is important that water movement can be quickly adapted and fine-tuned by inducing changes in the activity or density of AQPs to counteract sudden environmental changes. Studies of the regulation of AQP encoding genes should, in the future, assess tissue-specificity of AQP gene expression in view of further functional studies of the AQPs in the movement of water through the root in relationship to the water regime and AM treatment.

Under drought conditons, the soil water potential steadily decreases and might lead to an efflux of water from the root (with higher water potential) to the soil (with steadily decreasing water potential). Thus, under such conditions, AQP genes of endodermal cells might be down-regulated to prevent water lost from the vascular root cylinder to the root cortex and subsequently to the soil. In our study, *PIP2.8* was the only AQP gene which was strongly down-regulated under drought conditions in all AM treatments, suggesting a potential role in preventing this kind of water loss. To address this in more detail, it would be important to measure gene expression separately for each tissue (rather than working with whole root extracts), and to determine functional characteristics of the PIP2.8 protein in terms of its substrate specificity and biochemical regulation.

To better resolve the involvement of AM symbiosis in drought mediated AQP regulation, it would be important to assess AQP gene regulation patterns of cortical cells and endodermal cells under different levels of water stress. Cortical cells are in close contact with the AM fungus, and thus directly exposed to possible AM mediated changes in AQP gene regulation.

## Acknowledgments

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Table A.1 Mean relative fold-changes in AQP gene expression in *Sorghum bicolor* roots inoculated with *Rhizophagus irregularis*, *Rhizophagus arabicus* or non-inoculated (NM) exposed to well-watered and drought conditions.

| Gene | Fold change expression | | | | | | $F_{ANOVA}$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | Well-watered | | | Drought | | | | | |
| | NM | *Rhizophagus irregularis* | *Rhizophagus arabicus* | NM | *Rhizophagus irregularis* | *Rhizophagus arabicus* | W | AM | WxAM |
| TIP1.1 | 1 | 1.1 | 0.9 | 2.6 ** | 3.1 ** | 0.6 | 38.27 *** | 20.50 *** | 14.05 *** |
| TIP1.2 | — | — | — | — | — | — | — | — | — |
| TIP2.1 | 1 | 1.2 | 0.8 | 1.1 | 1.0 | 0.2 *** | ns | 4.27 * | ns |
| TIP2.2 | — | — | — | — | — | — | — | — | — |
| TIP2.3 | 1 | 2.0 | 0.7 | 2.0 * | 2.0 * | 1.1 | ns | 5.54 * | ns |
| NIP1.1 | 1 | 1.3 | 0.9 | 0.8 | 1.0 | 0.8 | ns | ns | ns |
| NIP1.2 | 1 | 0.7 | 1.1 | 1.3 | 0.8 | 0.5 ** | ns | ns | ns |
| NIP1.3 | — | — | — | — | — | — | — | — | — |
| NIP2.1 | 1 | 1.1 | 1.3 | 1.0 | 0.8 | 0.9 | ns | ns | ns |
| NIP2.2 | 1 | 0.8 | 0.5 | 0.9 | 0.7 | 0.3 ** | ns | 5.34 * | ns |
| PIP1.1 | 1 | 2.0 * | 1.3 | 1.2 | 1.3 | 1.3 | ns | 2.68 * | ns |
| PIP2.2 | 1 | 2.5 * | 1.1 | 2.0 ** | 2.2 * | 0.8 | ns | 22.13 *** | 6.25 ** |
| PIP2.5 | 1 | 7.5 * | 0.7 | 5.7 * | 6.9 ** | 2.0 | 4.75 * | 17.71 *** | 3.52 * |
| PIP2.8 | 1 | 1.1 | 1.4 | 0.2 *** | 0.1 *** | 0.1 *** | 27.70 *** | ns | ns |

Fold change expression was calculated relative to non-mycorrhizal (NM)-well-watered conditions (set as 1). Ubiquitin was used as referenz gene. Data were analyzed using independent samples *t* -test for fold change expression with a significance level of 0.05% compared to control conditions. Regulation higher than two fold-changes and lower than 0.5 fold-changes were considered as significant. 2-way-ANOVA including the factors water regime (W) and AM treatments (AM) followed by LSD's multiple range test with a significance level of 0.05% was performed over all expression values within each AQP gene; $F_{ANOVA}$ is given; *, $p<0.05$; **, $0.001\leq p<0.01$; ***, $p<0.001$. Ns and — mean not significant and low or not expressed in roots, respectively.

## Supplementary data

## Supplementary methods

*RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)*

Roots were ground in liquid nitrogen and total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, Darmstadt, Germany). The DNA-*free* set (Ambion, Austin, USA) was used to digest DNA after RNA purification.

Relative gene expression was performed using a two-step qRT-PCR procedure. Total RNA was measured with a spectrophotometer (Nanodrop ND-1000, Witec, Switzerland) and then reverse-transcribed (100 ng per reaction) using the iScript cDNA Synthesis kit (Bio-Rad, Paolo Alto, CA, USA). cDNAs were used as templates in qRT-PCR reactions with gene-specific primers designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and amplify 3.1 (http://engels.genetics.wisc.edu/amplify) (Table S5.2).

*Accession numbers of AQP homologs*

GmPIP1(AJ937960); GmPIP2 (AJ937961); HaTIP7 (X95950); HaTIP18 (X95951); LsPIP1(AJ937962); LsPIP2 (AJ937963); LjNIP1 (HE860041); MtPIP1.1 (AF386739); MtPIP2.1(AY059380); MtNIP1.1(AY059381); MtNIP1.2 (AY539750); MtTIP1.1 (Q9FY14.1); OePIP1.1 (DQ202708); OePIP2.1 (DQ202709); OeTIP1.1 (DQ202710); OsPIP1.1 (AP005108); OsPIP1.2 (AK098849); OsPIP1.3 (AP004026); OsPIP2.1 (AP003802); OsPIP2.2 (AP006168); OsPIP2.3 (AL662958); OsPIP2.4 (AP004668); OsPIP2.5 (AP004668); OsPIP2.6 (AL731636); PcTIP (CAA88267.1); PttPIP2.2 (AJ849325.1); PttPIP2.4 (AJ849327.1); PttPIP2.8 (XM_002313474.2); PttPIP2.10 (XM_002327697.1); PttNIP2.1 (XM_002324021.1); PttTIP1.2 (EF144587.1); PttTIP2.2 (XM_002303264.2); PttTIP2.3 (XM_002303321.2); PttTIP2.4 (XM_002326421.1); PvPIP1.1(U97023); PvPIP1.2 (AY995196); PvPIP1.3 (DQ855475); PvPIP2.1 (AY995195). ZmPIP1.1 (X82633); ZmPIP1.2 (AF131201); ZmPIP1.3 (AF326487); ZmPIP1.4 (AF326488); ZmPIP1.5 (AF326489); ZmPIP1.6 (AF326490); ZmPIP2.1 (AF326491); ZmPIP2.2 (AF326492); ZmPIP2.3 (AF326493); ZmPIP2.4 (AF326494); ZmPIP2.5 (AF130975); ZmPIP2.6 (AF326495); ZmPIP2.7 (AF326496); ZmTIP1.1 (AF037061); ZmTIP1.2 (AF326500); ZmTIP2.1 (AF326501); ZmTIP2.2 (AF326502); ZmTIP2.3 (AF326503); ZmTIP3.1 (AF326504); ZmTIP2.3 (AF342809); ZmTIP4.1 (AF326505); ZmTIP4.2 (AF326506); ZmTIP4.3 (AF326507); ZmTIP4.4 (AF326508); ZmTIP5.1 (AF326509); ZmSIP1.1 (AF326497); ZmSIP1.2 (AF326498); ZmSIP2.1 (AF326499); ZmNIP1.1 (AF326483); ZmNIP2.1 (AF326484); ZmNIP2.2 (AF326485); ZmNIP2.3 (A342810); ZmNIP3.1 (AF326486)

## Supplementary tables

Table S A.1 Characteristics of *S. bicolor* AQP genes (obtained from Phytozome database)

| Name[a] | Accession | Protein Id | Chromosome location[b] | NPA motif[c] | EST presence[d] | Number exons | Number introns |
|---|---|---|---|---|---|---|---|
| SbNIP1.1 | Sb04g008360 | 5054938 | IV | -/- | ok | 4 | 3 |
| SbNIP1.2 | Sb03g002490 | 5035054 | III | -/- | Na | 5 | 4 |
| SbNIP1.3 | Sb09g006390 | 5060249 | IX | -/- | ok | 4 | 3 |
| SbNIP1.4a* | Sb10g021770 | 5031762 | X | - | Na | 2 | 1 |
| SbNIP1.4b* | Sb10g021780 | 5031763 | X | - | Na | 3 | 2 |
| SbNIP2.1 | Sb04g028020 | 5055557 | IV | -/- | ok | 5 | 4 |
| SbNIP2.3 | Sb10g008090 | 5061544 | X | -/- | ok | 5 | 4 |
| SbNIP3.1 | Sb01g017230 | 5048640 | I | NPS/NPV | ok | 4 | 3 |
| SbNIP3.2a | Sb07g003370 | 5043039 | VII | -/- | Na | 4 | 3 |
| SbNIP3.2b | Sb07g003270 | 5043032 | VII | -/- | Na | 3 | 2 |
| SbNIP3.3 | Sb07g003360 | 5043037 | VII | -/- | Na | 4 | 3 |
| SbNIP4.1 | Sb03g008210 | 5052792 | III | -/NPI | ok | 5 | 4 |
| | | | | | | | |
| SbTIP1.1 | Sb01g047140 | 5050203 | I | -/- | ok | 2 | 1 |
| SbTIP1.2 | Sb03g047470 | 5054474 | III | -/- | ok | 2 | 1 |
| SbTIP2.1 | Sb04g032900 | 5055859 | IV | -/- | ok | 2 | 1 |
| SbTIP2.2 | Sb10g019360 | 5061735 | X | -/- | ok | 3 | 2 |
| SbTIP2.3 | Sb06g024590 | 5057642 | VI | -/- | ok | 2 | 1 |
| SbTIP3.1 | Sb01g049870 | 5030626 | I | -/- | Na | 3 | 2 |
| SbTIP3.2a | Sb01g018430 | 5048709 | I | -/- | ok | 2 | 1 |
| SbTIP3.2b | Sb06g023240 | 5042227 | VI | -/- | Na | 3 | 2 |
| SbTIP4.2 | Sb09g007940 | 5060291 | IX | -/- | ok | 3 | 2 |
| SbTIP4.3 | Sb03g000790 | 5034964 | III | -/- | ok | 2 | 1 |
| | | | | | | | |
| SbPIP1.1 | Sb06g025150 | 5057675 | VI | -/- | ok | 4 | 3 |
| SbPIP1.3 | Sb04g032430 | 5055837 | IV | -/- | ok | 4 | 3 |
| SbPIP1.5 | Sb04g037800 | 5056164 | IV | -/- | ok | 2 | 1 |
| SbPIP1.6 | Sb10g007610 | 5061522 | X | -/- | ok | 2 | 1 |
| SbPIP2.2 | Sb02g010760 | 5050878 | II | -/- | ok | 3 | 2 |
| SbPIP2.3 | Sb04g026650 | 5055487 | IV | -/- | ok | 4 | 3 |
| SbPIP2.5 | Sb06g022840 | 5057539 | VI | -/- | ok | 3 | 2 |
| SbPIP2.6a | Sb02g010780 | 5050880 | II | -/- | ok | 3 | 2 |
| SbPIP2.6b | Sb02g010790 | 5033196 | II | -/- | Na | 3 | 2 |
| SbPIP2.6c | Sb02g010800 | 5033197 | II | -/- | Na | 3 | 2 |
| SbPIP2.6d | Sb02g010830 | 5050882 | II | -/- | ok | 3 | 2 |
| SbPIP2.8 | Sb0012s010440 | 5047265 | * | -/- | Na | 1 | 0 |
| | | | | | | | |
| SbSIP1.1 | Sb05g007520 | 5056497 | V | NPT/- | ok | 3 | 2 |
| SbSIP1.2 | Sb09g019780 | 5060469 | IX | NPT/- | ok | 3 | 2 |
| SbSIP2.1 | Sb01g036970 | 5049530 | I | NPL/- | ok | 3 | 2 |

[a] The symbol '*' indicates that this gene is a truncated putative AQP

[b] The symbol '*' indicates that this gene is located on a scaffold not yet assigned to a chromosome

[c] Deviations of NPA motifs on first or second location (-/-) are reported. '-' indicates that NPA motif is conserved

[d] Na means that EST is not available

Table S A.2 List of PCR primers designed for qRT PCR.

| **Primer** | **Sequence 5'→ 3'** |
|---|---|
| SbNIP1.1fw | CCCCTGCCTGCTCTCTCT |
| SbNIP1.1rv | GTTCGTCTGGGCGGAGTT |
| SbNIP1.2fw | GCAGGCGGGGTATCAGAG |
| SbNIP1.2rv | CCGTTGGTGCTCAGGTTC |
| SbNIP1.3fw | GCTGCCACCCAAACAAG |
| SbNIP1.3rv | CCTCTGCTCCTGCCATAA |
| SbNIP2.1fw | TGTGTGTGTCGGTGCTCA |
| SbNIP2.1rv | GCAACAAGTGCGTGCGTA |
| SbNIP2.2fw | GCATCCACACCTCACCAT |
| SbNIP2.2rv | GCGAGCGAGCACACAGTA |
| SbTIP1.1fw | GGACCCGTTTGCTTCTGT |
| SbTIP1.1rv | CGTGGAACTCGGTGAATACT |
| SbTIP1.2fw | CGCACTTCGTGTCTGTCAA |
| SbTIP1.2rv | GGGCGTGTGTTGCTGTTC |
| SbTIP2.1fw | TTCATCGGCGGCAACTAC |
| SbTIP2.1rv | ATCCAAACAGCCCAGACG |
| SbTIP2.2fw | CCCTGCCTCTGCCTCAG |
| SbTIP2.2rv | AATGCTGCGAGTTCATCG |
| SbTIP2.3fw | CGGCAACTGGGTCTACTG |
| SbTIP2.3rv | TAGGAGGCGATGAACACG |
| SbPIP1.1fw | CTCCTCCCCTCTGCTTCC |
| SbPIP1.1rv | GGTGCGGTGCTTGTGTC |
| SbPIP2.2fw | GGTCGCTCTTGTTTCAGCA |
| SbPIP2.2rv | GAATCCCTTGGACAGCAGA |
| SbPIP2.5fw | TCGTCGTTGGTTGCTTCT |
| SbPIP2.5rv | CATTTTTGCCCAGTTGTCG |
| SbPIP2.8fw | GGGTTCCTTGCCTTCTGT |
| SbPIP2.8rv | TGGGGGTGGGTCACTGTA |
| Ubiquitin-fw | CAAGGAGTGCCCCAACAC |
| Ubiquitin-rv | GGTAGGCGGGTAAAGCAAA |

Table S A.3 Composition of AQP subfamilies in different plant species

| AQP subfamily | ***Sorghum bicolor*** | *Zea mais* | *Oryza sativa* | *Arabidopsis thaliana* | *Physcomitrella patens* | *Populus trichocarpa* |
|---|---|---|---|---|---|---|
| Plasma membrane (PIP) | **12** | 12 | 11 | 13 | 8 | 15 |
| Tonoplast (TIP) | **10** | 12 | 10 | 10 | 4 | 18 |
| NOD-26 like (NIP) | **10** | 5 | 10 | 9 | 5 | 11 |
| Small basic (SIP) | **3** | 3 | 2 | 3 | 2 | 6 |
| Uncategorised (XIP) | | | | | 2 | 6 |
| Others (GIP, HIP) | | | | | 2 | |
| Total | **35** | 32 | 33 | 35 | 22 | 55 |

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