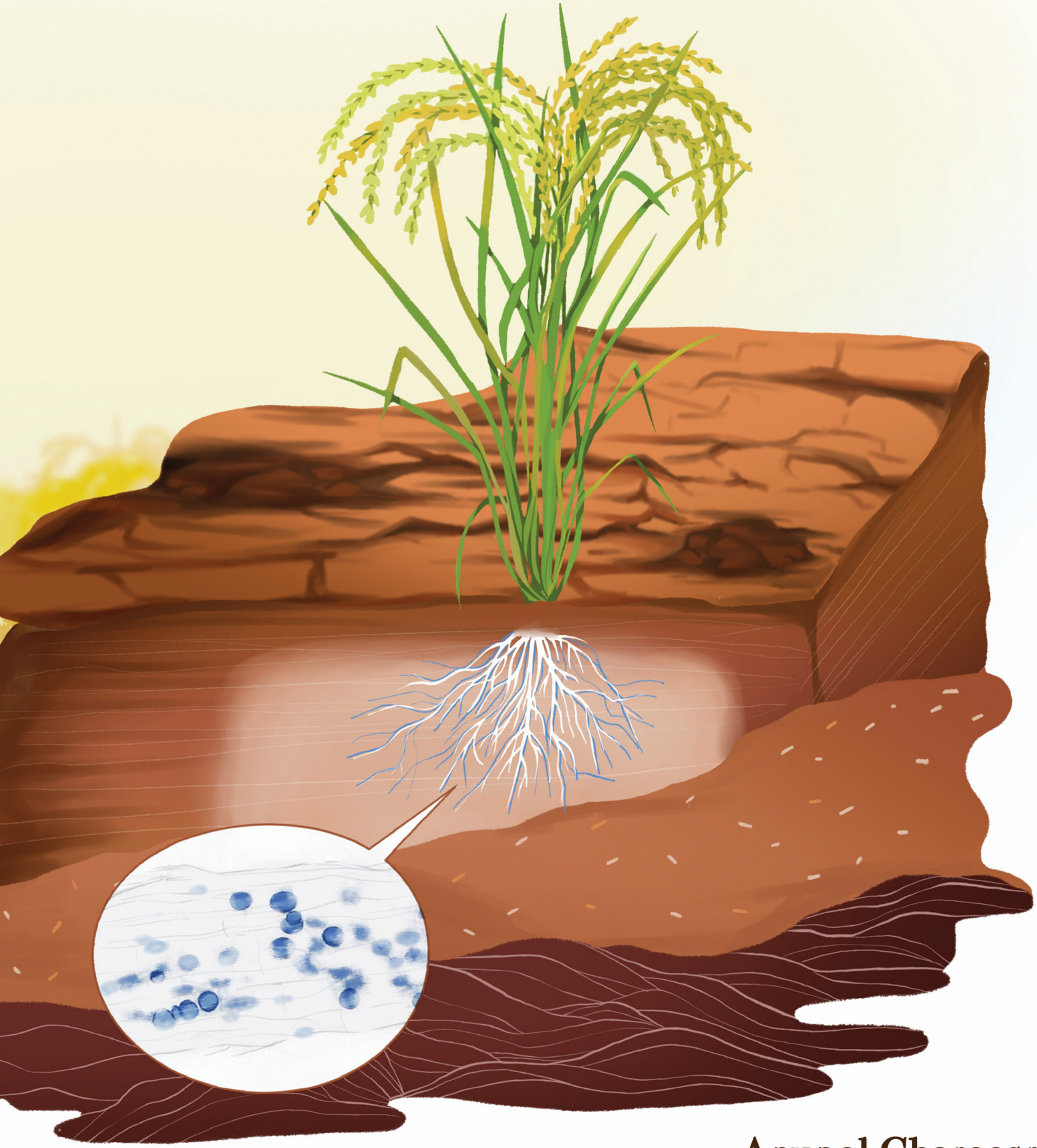
The role of Arbuscular Mycorrhizal Fungi in alleviating drought effects on rice (*Oryza sativa*) productivity



Anupol Chareesri

Propositions

1. Comparing benefits of mycorrhizal plants to non-mycorrhizal plants may not be realistic to assess mycorrhizal performance under agronomically relevant conditions (This thesis)

2. Arbuscular mycorrhizal fungi enhance plant drought tolerance through maintaining photosynthetic activity rather than through direct water uptake.
   (This thesis)

3. Control of natural enemies in an experimental design is crucial

4. Climate change constitutes both threats and opportunities for food security

5. It is costly to be environmentally friendly

6. Cooking for one is both a fine art and a mental-health therapy

Propositions belonging to the thesis, entitled

The role of Arbuscular Mycorrhizal Fungi in alleviating drought effects on rice (*Oryza sativa*) productivity

Anupol Chareesri

Wageningen, 16 April, 2024

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Anupol Chareesri

Thesis committee

Promotors

Prof. Dr T.W. Kuyper
Personal chair at the Soil Biology Group
Wageningen University & Research

Prof. Dr G.B. De Deyn
Personal chair at the Soil Biology Group
Wageningen University & Research

Other members

Prof. Dr L. Mommer, Wageningen University & Research
Dr A.E. Bennett, Ohio State University, Columbus, United States
Dr B. Andreo Jimenez, Wageningen University & Research
Prof. Dr P.C. Struik, Wageningen University & Research

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Anupol Chareesri

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Anupol Chareesri

The role of Arbuscular Mycorrhizal Fungi in alleviating drought effects on rice (*Oryza sativa*) productivity

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Chapter 1

Introduction

Rice (*Oryza sativa* L.) is one of the main foods feeding half of the world's population. It has been domesticated for over 5000 years and is currently cultivated worldwide. More than 90% of rice production comes from lowland rice varieties that are grown under flooded condition, while upland rice contributes only 4% to total world rice production (GRiSP 2013). Lowland rice is mostly grown under flooded conditions, in fields where the water level is sufficient by natural rain or under conditions of irrigation. Farmers flood rice fields to increase phosphorus (P) availability, control weeds, and create a suitable condition for the growth of nitrogen (N_2)-fixing plants such as *Azolla*. In irrigation areas, water is sufficient for rice cultivation, but the scarcity of water becomes a major problem for rice that is grown in rainfed conditions. Presently, climate change induces increased variation in rainfall, resulting in either serious drought or flooding in many regions, and both drought and flooding subsequently induce a stress condition for the growth of plants. Rice is not an aquatic plant and while it has some physiological mechanisms for drought tolerance, its tolerance is far less than that of other cereals. Under drought stress, rice growth declines, and the yield can decrease up to 64% (Venuprasad et al. 2007). Drought is therefore one of the most threatening factors for rice production.

The effects of drought on rice

Drought can occur naturally, and humanly. Lack of precipitation and increasing frequency of heatwaves with higher temperatures can cause (physiological) drought. Shortage of water can directly decrease transpiration and photosynthesis of rice (Lauteri et al. 2014). Yoshida and Hasegawa (1982) observed a decrease in transpiration rate of upland rice when soil water potential was reduced to below -20kPa. The decrease of transpiration can lead to a reduction of nutrient uptake that then limits the growth of plants (Serraj et al. 2011). Moreover, drought can affect nutrient acquisition by plants by reducing the availability of soil nutrients. With inadequate soil moisture, nutrients will become less available for plant uptake, especially phosphorus (P) (Suriyagoda et al. 2014) and potassium (K) (Sardans and Peñuelas 2007). Besides, plants can be more susceptible to weeds and pests under drought. Certain pests (e.g., thrips, brown leafhopper) tend to develop and spread rapidly under dry conditions (BRRD 2007a).

The effects of drought depend on timing and severity. Timing refers to the onset, duration and specific growth stage of plants that are exposed to drought stress, while severity is related to the level of soil moisture. In rainfed-rice cultivation, drought may occur when soil moisture is reduced due to lack of rainfall for a long-time. For instance, in Thailand drought occurs in late June till August, which is the period that rice plants are already in the field at vegetative stage. Another drought period is around mid-October, which is the reproductive stage for rice. Drought stress during vegetative stage reduces the expansion of leaves, delays the maturity of rice, and affects the grain yield (Lilley and Fukai 1994). Yield loss is most severe when drought occurs during panicle formation stage (Farooq et al. 2009).

Responses of rice to drought stress

The effects of drought can be minimized by proper irrigation management and farming practices. Nevertheless, plants do also have their own adaptation mechanisms under drought stress. Fukai and Cooper (1995) have described the adaptation mechanisms in plants as: drought escape, dehydration avoidance, dehydration tolerance, and drought recovery. *Drought escape* is a mechanism whereby plants cope with drought by developing rapid phenological changes. Some rice cultivars escape from drought by early maturing or flowering to complete their life cycle (Fukai and Cooper 1995). *Dehydration avoidance* is the mechanism by which plants maintain leaf water potential. This may be facilitated by altered plant allocation to shoots and roots. The root:shoot ratio is usually increased under drought stress, and drought-tolerant plants will develop more roots to extract more water from the soil. Drought-tolerant rice varieties develop thicker and deeper roots in conditions where soil moisture is low (Farooq et al. 2009). However, Kassam (2018) observed no difference in root diameter between drought-sensitive and drought-tolerant rice varieties under well-watered conditions, but increased root length under drought, especially in the drought-sensitive cultivar. In addition, plants can maintain leaf water potential by reducing water loss from transpiration. Under drought stress, plants will produce abscisic acid (ABA) to stimulate stomatal closure and reduce leaf expansion to prevent water loss (Zhang et al. 2006), and a shoot-stimulating hormone like indole-3-acetic acid (IAA) will decrease.

High ABA levels reduce the growth of plants because closure of stomata reduce transpiration and CO_2 exchange ultimately resulting in reduced photosynthesis. *Dehydration tolerance* is a mechanism to maintain plant metabolism, like that of conditions of adequate water availability. The osmotic adjustment (OA) is likely playing an important role for this mechanism by maintaining cell turgor (Levitt 1980). The last mechanism is *Drought recovery*, which is related to the ability of plants to retain green leaves and thereby continue growing during and after a drought period.

Growing Rice like Wheat

Rice is much more sensitive than other cereals such as wheat (*Triticum aestivum* L.). Wheat requires far less water and despite its lower water use than rice, can achieve high yields. Considering the same phylogenetic (both are members of the Poaceae) and ecophysiological (both are C3 plants) properties, it has been considered a worthy effort to grow rice like wheat, that is maintaining (or even enhancing) rice yields while substantially lowering water use. A major program to grow rice like wheat was undertaken by Wageningen University. This program yielded five PhD theses, dealing with several aspects of drought tolerance of and water use by rice. More specifically the following topics have been addressed. Kadam (2018) studied the morphological, anatomical, physiological and genetical basis for responses of rice to water deficit. For that, different rice genotypes were compared at the same low water availability. Responses of rice to drought included a higher specific root length, due to lower root tissue density and to a lesser extent by thinner roots due to a lower cortical area. Wheat, on the other hand, responded to drought by a lower specific root length. Ouyang (2021) also studied the morphological, anatomical, and physiological mechanisms that determine the limited adaptation potential of rice to drought. Drought reduced root growth and root proliferation and also resulted in increased suberization of roots, resulting in reduced water transport. The study concluded that root properties that are conducive for rice productivity under inundated conditions are not conducive for root growth under drought. Melandri (2019) focused on drought-induced changes in plant metabolism and oxidative stress. The thesis strongly focused on leaf metabolism, with far less attention on roots. Vijayaraghavareddy (2021) investigated the physiological basis for drought adaptation of different rice varieties. The highest sensitivity to drought, as evidenced by yield reduction under these conditions, was noted during the flowering stage. Drought increased root length, an effect that was largest at the vegetative phase. Unfortunately, data on root length were not connected to simultaneous changes in root diameter and specific root length, contrary to the study by Kadam (2018). While these four studies took a predominant above-ground view on the morphological, anatomical and physiological responses of rice to drought, Andreo Jimenez (2017) focused on below-ground processes. She explored the relationship between two plant hormones, abscisic acid and strigolactones, under the influence of drought. She also investigated root-endophytic Ascomycota in rice roots and the effect of drought on community changes of those fungal endophytes, but mechanisms that allowed those endophytes to confer drought tolerance were not studied. A positive correlation with a certain group of fungi, belonging to the Pezizomycotina, and rice yield under drought was observed. A genome-wide association study found a number of single-nucleotide polymorphisms (SNPs) that were associated with specific endophytic fungi, but most of these associations were found under well-watered conditions, not under conditions of water deficit.

The program growing Rice like Wheat considerably increased insight in the differences between rice and wheat under drought and suggested pathways for genetic improvement of rice to harness its tolerance to drought. At the same time these studies, with a focus on the rice plant itself, left a niche for further studies how other soil biota could contribute to conferring drought tolerance. One such group are the Arbuscular Mycorrhizal Fungi (AMF), which I selected as the topic for my study. Their role in conferring tolerance to unfavorable conditions, including drought, is well-known. Both direct (enhanced water acquisition) and indirect (through hormonal changes) mechanisms are likely relevant for enhanced drought tolerance.

Arbuscular Mycorrhizal Fungi (AMF)

Apart from adaptations to drought that plants possess, soil organisms can also help plants to cope with drought stress (Rodriguez and Redman 2008). While the potential role of drought tolerance of rice due to endophytic Ascomycota was studied by Andreo

Jimenez (2017), another group of mutualistic fungi has received more attention in conferring drought tolerance. Arbuscular Mycorrhizal Fungi (AMF) are amongst the soil microorganisms that increase drought tolerance of plants (Augé 2001; Ruiz-Lozano and Aroca 2010; Ruiz-Sanchez et al. 2010). The symbiosis between AMF and plants is well known for its benefits in increasing nutrient and water uptake of the plant. The hyphae of AMF can reach water and nutrient sources in small soil pores that plant roots cannot access (Smith and Read 2010) and translocate those resources to the host plant. Besides, AMF can maintain transpiration and photosynthesis of the plants by maintaining (or less reducing) leaf water potential and stomatal conductance under drought (Augé 2001; Querejeta et al. 2007). Submaranian (1995) found that AM-inoculated maize under drought stress had higher leaf water potential and lower stomatal closure (higher stomatal conductance) than non-AM maize plants. The contribution of AMF to changes in stomatal conductance is related to plant growth hormones, for instance ABA and IAA. These hormones are important for regulating shoot and root development of plants, and they can stimulate AM symbiosis (Lüdwig-Müller and Güther 2007). On the other hand, it has also been reported that AMF may downregulate the production of ABA to maintain plant photosynthesis activity. Duan et al. (1996) indicated that the flux of ABA in the shoot of AM plants is lower than in non-AM plants, resulting in higher transpiration and leaf water potential.

Rice and arbuscular mycorrhizal fungal symbiosis

Like the majority of plants, including almost all members of the Poaceae, rice is an arbuscular mycorrhizal plant. The community composition and abundance of AMF in rice roots and rice fields depend on soil properties, but also on cultivars, farming practices and different growing stages of rice plants. In the systems of rice intensification (SRI), Watanarojanaporn et al. (2013) found two main AM fungal groups that colonized rice roots, belonging to the genera *Glomus* and *Acaulospora*, while only *Glomus* was found in rice roots from conventional cultivation system. Although AMF prefer aerobic or upland conditions, a species such as *Glomus etunicatum* (currently *Claroideoglomus etunicatum*) can still survive under submerged conditions (Purakayastha and Chhonkar 2001). The abundance of AMF in rice can decrease under anaerobic conditions (Vallino et al. 2014) and under conditions of intensive farming (soil tillage, use of fungicides, high doses of fertilizer especially P) (Oehl et al. 2003). Less-intensive farming practices, that is less disturbance and lower doses of mineral fertilizer and additions of organic amendments, may increase the colonization of AMF in rice (Lumini et al. 2011). The extent to which increased colonization by AMF translates into enhanced drought tolerance of rice is currently unknown. This knowledge gap is also due to the fact that most experiments compare plants with and without AMF, whereas under most field conditions plants are mycorrhizal, albeit to a different degree.

Mixed cropping of rice and arbuscular mycorrhizal fungi

Application of AMF as so-called biofertilizer in rice fields, in order to increase mycorrhizal colonization, is cost and labor intensive. It is not always successful, as these AMF have to be competitive against indigenous inoculum. An alternative to adding external inoculum could be a management practice that enhances mycorrhizal colonization. Intercropping, the simultaneous cultivation of different crop species on the same field, and cropping with varietal mixtures, the simultaneous cultivation of different varieties of the same crop species, have been suggested as potential practices to achieve that aim. From a mechanistic point of view both practices are comparable as the metrics used to calculate benefits, through the use of relative parameters (Relative Yield Increase, RYT; or Land Equivalence ratio, LER) or absolute parameters (biomass increment), are similar; additive partitioning into selection and complementarity effects can be applied in the same way; and complementarity effects can be disentangled in three different mechanisms that apply in both kinds of mixed systems. Selection effects imply a larger benefit by the plant species or variety that is already more productive. Complementarity effects can be due to niche differentiation by both plant species or varieties and include a.o. differences in rooting depth, temporal niche differentiation, but also abiotic facilitation and differential sensitivity to pathogens or differential association with or benefit from mutualistic fungi such as AMF (biotic feedbacks). Li et al. (2009) found that intercropping mung bean with rice increased the colonization of AMF in rice roots and nodulation in mung bean, which complementarily enhanced P uptake in rice, and N and P acquisition in mung bean. Wang (2020) found that mixing two maize varieties increased mycorrhizal colonization and mycorrhizal benefit. Similar studies on mycorrhizal roles with rice varietal mixing have not been executed. It is also still questionable through what mechanisms intercropping and varietal mixing enhance AMF colonization and whether and how such practices can improve drought tolerance. *Rhizobium* can improve root morphology of rice plants (Choudhury and Kennedy 2004), therefore it may increase water uptake as well. Impacts of AMF on root morphology and anatomy have yielded partly contradictory results, with some studies reporting that AMF reduced SRL and other studies reporting no effect. In addition, intercropping crops with different rooting depth can create hydraulic lift or hydraulic redistribution which causes movement of water in different layers of soil via uptake of water. The hydraulic lift in rice and legumes has been demonstrated by Zegada-Lizarazu and Iijima (2004), and a mycorrhizal role in translating the impact of hydraulic lift into increased productivity has been demonstrated by Singh et al. (2019, 2020) for a cereal (finger millet) and legume (pigeon pea) intercropping system.

Research questions and hypotheses

Although several studies have found positive effects of AMF on drought tolerance of various plant species, they did not clarify the various mycorrhizal-mediated mechanisms of drought tolerance of rice. Thus, my research aimed to gain more insight in the symbiosis between AMF and rice under drought stress. The main research aims were to investigate (i) the effects of AMF on the growth of rice under drought compared with well-watered conditions, (ii) the relation between the occurrence of AMF and plant-growth regulating hormones under drought and well-watered conditions, (iii) the temporal effects of drought and the potential benefits of AMF on rice photosynthetic activity (iv) the contribution of AMF in rice varietal mixtures in increasing mycorrhizal colonization and thereby enhancing drought tolerance of rice (fig. 1.1).

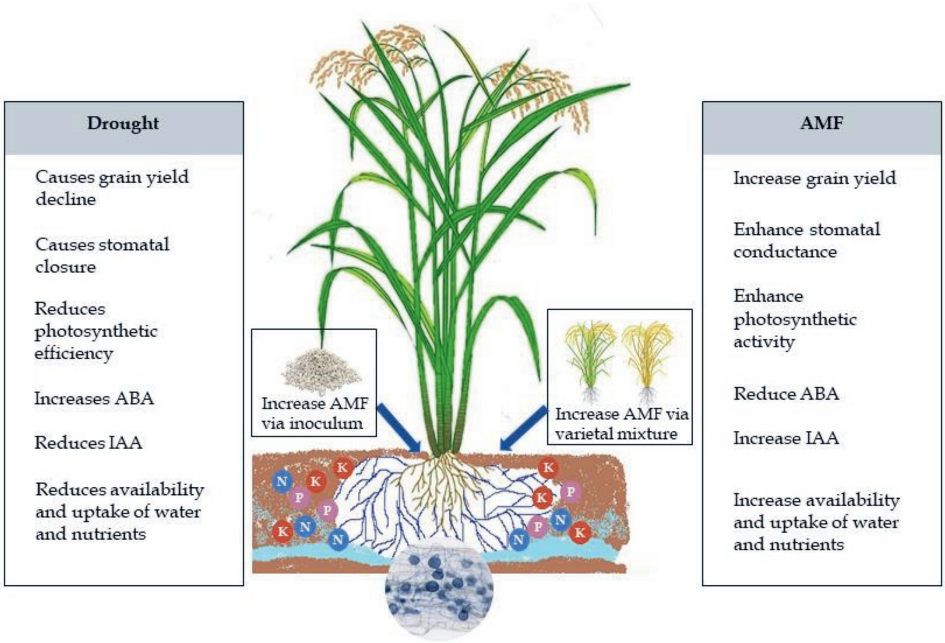

Figure 1.1: The (negative) effects of drought (left) and potential benefits of (increased) AMF colonization (right) by inoculation and varietal mixtures. These opposite impacts could imply a significant mycorrhiza × drought interaction in experiments.

R1: The effects of AMF on the growth of rice under drought compared with well-watered conditions

I hypothesized that increasing AMF colonization by inoculation to non-sterile field soil maintains the growth of rice under drought. Increasing AMF colonization enhances N and P uptake of rice plants and maintains photosynthesis activity. These effects translate to higher shoot biomass, root biomass and grain yield. I expected less grain yield reduction in rice plants with higher AMF colonization compared with plants with lower AMF colonization. These hypotheses were tested in both pot and field experiments (Chapters 2-5).

R2: The relation between the occurrence of AMF and plant-growth regulating hormones under drought and well-watered conditions

I hypothesized that AMF regulate ABA and IAA hormone in rice plants under drought conditions. Drought increases ABA and reduces IAA resulting in less water loss when plants are subjected to drought. With higher AMF colonization, I expected that the ABA production decreases, and IAA production is maintained. This hormone regulation results in higher photosynthesis and biomass and grain yield in plants with higher AMF colonization than in plants with lower AMF colonization. The hypothesis was tested in pot experiments (Chapter 2).

R3: The temporal effects of drought and the potential benefits of AMF on rice photosynthetic activity

Under drought conditions, plant stomatal conductance and photosystem II activity are reduced, which causes reduction in plant growth. So, I hypothesized that high AMF colonization in plants could maintain photosynthesis efficiency by maintaining stomatal conductance and photosystem II activity. I studied this mechanism by testing the effects during temporal monitoring of the changes of those factors and the recovery of the rice plants. I also expected to see higher ability for recovery from drought in plants with higher AMF colonization than plants with lower AMF colonization. Augé (2001) noted that mycorrhizal plants show higher water use efficiency than non-mycorrhizal plants; he listed 10 cases of enhanced WUE versus 4 cases of reduced WUE. He also reported that the hydration status of mycorrhizal plants is likely higher than that of non-mycorrhizal plants. A higher acquisition of water (maybe improved contact between hyphae and soil water in small soil pores compared to roots) and nutrients also contributes to better withstand drought. Next to these nutritional effects, there could also be non-nutritional (hormonal) effects. Finally, through the presence of mycorrhizal fungi the hydraulic status of the soil may be improved. Taken together these data indicate that AM plants would likely suffer less from drought and recover better from drought. These experiments are described in Chapters 2, 3 and 4.

R4: The contribution of AMF in rice varietal mixtures in increasing mycorrhizal colonization and thereby enhancing drought tolerance of rice

I hypothesized that varietal mixing increases AMF colonization and enhances the benefits of AMF for rice plants under drought conditions. The beneficial effects of mixing two different rice varieties will be due to complementarily effects. The grain yield of rice will be increased due to increasing AMF colonization by varietal mixing, both under conditions of natural inoculum potential and after inoculation. The hypothesis was tested in Chapter 5.

Research Approach

Study sites

The experiments were executed in Khon Kaen, Thailand. The pot experiments (Chapter 2, 3, and 5) were done in the greenhouse at Khon Kaen University, while the field experiment (Chapter 4) was done in Ban Fang District, Khon Kaen (fig. 1.2). Khon Kaen has the average highest temperature from November to April is about 32.9 ^{o}C, and the average lowest temperature is about 22.6 ^{o}C (Upper North-Eastern Region Meteorological Center accessed on 12th February 2024). In the greenhouse the average temperature time was 42 ^{o}C during day time, and 27 ^{o}C at night.

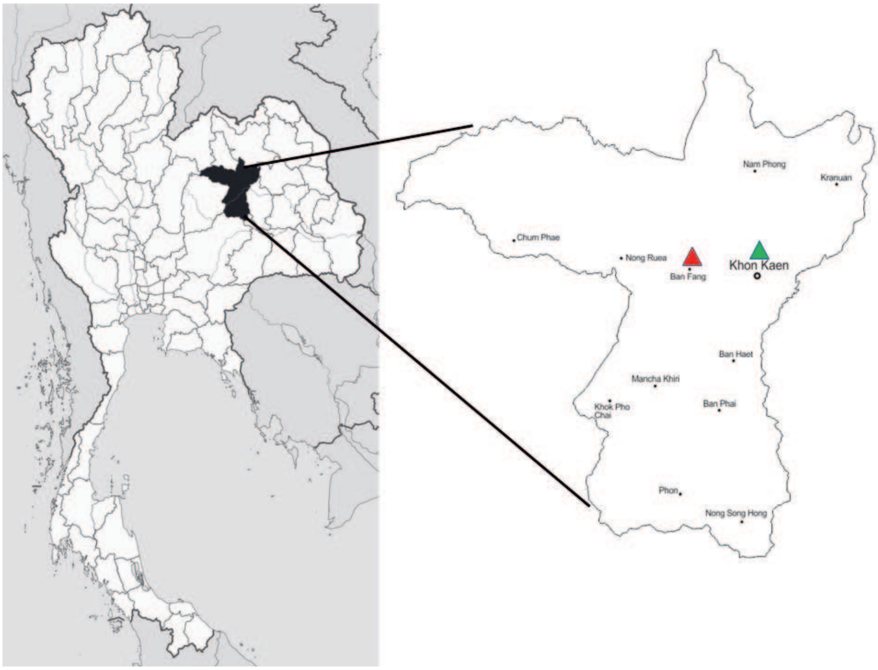

Figure 1.2: A map of Khon Khan, the green triangle showing the area where the pot experiments were conducted, and the red triangle was the field experiment location.

Rice varieties

In pot experiments (Chapter 2), and field experiments (Chapter 4), I used six rice varieties, viz., Surin 1 (SR1), Khao Dowk Mali 105 (KDML105), RD6, Chainart 1 (CNT1), RD22 and RD33. In pot experiments Chapter 3 and 5, I used SR1 and KDML 105. These rice varieties are amongst the most widely grown varieties in Thailand. They are lowland rice varieties that are commonly grown under submerged conditions. Surin 1 is reported to be highly tolerant to drought, while KDML 105 is a moderately drought-tolerant variety, and RD6, CNT1, RD22 and RD33 are lower drought-tolerant varieties. The relationship of AMF and these rice varieties has not been widely studied.

Arbuscular mycorrhizal fungi

For the pot and field experiments, I used commercial AMF inoculum (RootGrow Professional, Kent, UK) which contained *Funneliformis mosseae, F. geosporus, Claroideoglomus claroideum, Glomus microaggregatum,* and *Rhizophagus irregularis* (Robinson Boyer et al. 2016). The soil for the pot experiments was collected from a rice field where we conducted the field experiments. This field has been used for rice cultivation for more than 20 years, with a rotation of maize cultivation in some years. This soil contained around 5 AMF spores per 100g, which is considered low. I used non-sterilized soil in pot and field experiments to compare the effects of higher and lower AMF colonization.

Drought

In this study, I implemented a Type III drought treatment according to the classification by He and Dijkstra (2014) (fig. 1.3), by reducing soil water to a certain level, after which I rewetted the soil. At the beginning, all rice seedlings received sufficient water to stimulate growth and facilitate AMF symbiosis. Drought treatments were applied 6 weeks after the rice had been planted.

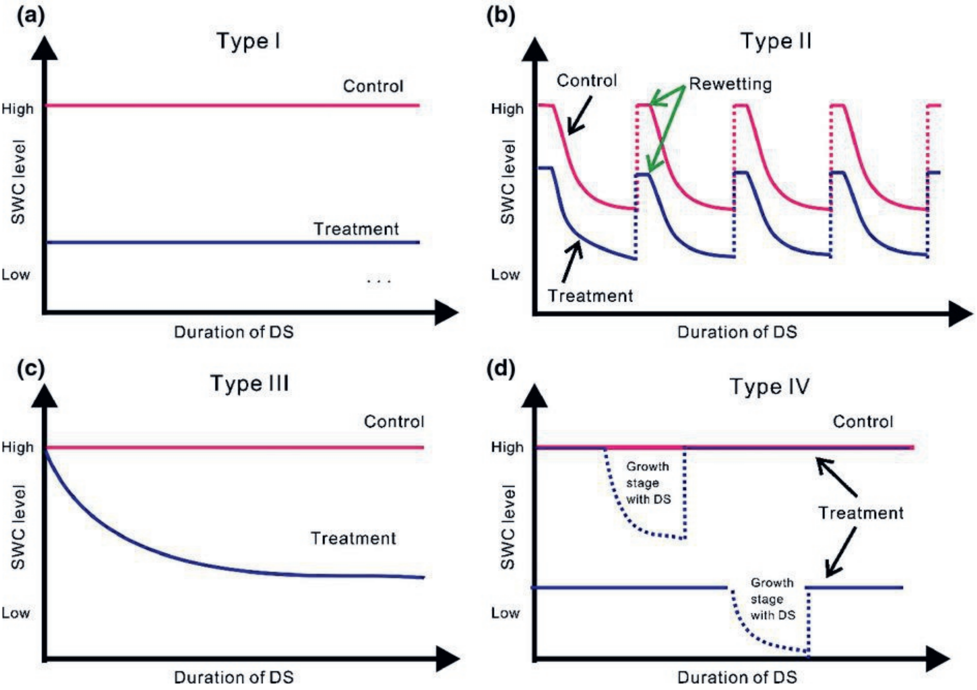

Figure 1.3: Conceptual scheme of different types of drought treatments. Type I: Drought results in a constant lower amount of soil water; Type II: Plants are exposed to different intensities of cycles of drying and rewetting; Type III: Due to less frequent rainfall events, soils dry out gradually to a lower water level; Type IV: Changes in rainfall patterns result in severe drought stress during different stages in the plant cycle (germination, onset of flowering or seed filling). N.B. Changes in frequency could also occur. From He and Dijkstra (2014). (DS = drought stress; SWC = soil water content).

Outline of chapters

Chapter 2: The effect of increased AMF colonization on rice performance under drought

Chapter 2 describes a pot experiment testing the effects of increased AMF colonization on rice growth under drought condition. The first experiment was executed in the rainy season in 2016, and the second experiment in the dry season in 2017. This chapter describes the benefits of increasing AMF colonization on the aspect of nutrient (N and P) uptake, photosynthesis activity (stomatal conductance and photosystem II efficiency), plant hormone regulation (ABA and IAA hormones), and includes data on shoot, root and grain yield. The novelty of these experiments was the comparison between the effects of natural AMF colonization and increased colonization via AMF inoculation, a comparison that is more realistic to the actual farm practice compared with experiments with non-mycorrhizal and mycorrhizal plants.

Chapter 3: Temporal effects of increased AMF colonization on photosynthetic performance

The pot experiment in Chapter 3 is the study of temporal effects of drought on rice plants, and how increasing AMF colonization can retain rice growth under long-term drought of 14 days of withholding water. This chapter focuses on the effects of drought on stomatal conductance and photosystem II efficiency under drought conditions. Moreover, the chapter deals with the recovery of rice plants with lower and higher AMF colonization.

Chapter 4: Achieving mycorrhizal benefits in the field under well-watered and drought conditions

After testing and confirming the benefits of increased AMF colonization on drought tolerance of rice plants in pot experiments, I conducted a field experiment to investigate these benefits in a field trial. The chapter explains the results of addition of AMF inoculum, in order to increase mycorrhizal colonization, in a rice field and effects on rice plant nutrient uptake, photosynthesis activity, and plant biomass. The differences between what I found in pot experiments and field experiments indicate potential limitations of AMF application in rice field.

Chapter 5: Increasing mycorrhizal colonization through varietal mixing

The success of AMF inoculation in pot experiments may not translate to practical management for farmers. This chapter describes the potential practice to increase AMF colonization by mixing two different rice varieties, and the benefits of this practice on rice growth under drought condition. The interaction between varietal mixing, AMF and drought are explained in this chapter.

Chapter 2

Increased arbuscular mycorrhizal fungal colonization reduces yield loss of rice (*Oryza sativa* L.) under drought

Anupol Chareesri, Gerlinde B. De Deyn, Lidiya Sergeeva, Anan Polthanee, Thomas W. Kuyper

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*See also appendix

Abstract

Drought reduces the availability of soil water and the mobility of nutrients, thereby limiting the growth and productivity of rice. Under drought, arbuscular mycorrhizal fungi (AMF) increase P uptake and sustain rice growth. However, we lack knowledge of how the AMF symbiosis contributes to drought tolerance of rice. In the greenhouse, we investigated mechanisms of AMF symbiosis that confer drought tolerance, such as enhanced nutrient uptake, stomatal conductance, chlorophyll fluorescence, and hormonal balance (abscisic acid (ABA) and indole acetic acid (IAA)). Two greenhouse pot experiments comprised three factors in a full factorial design with two AMF treatments (low- and high-AMF colonization), two water treatments (well-watered and drought), and three rice varieties. Soil water potential was maintained at 0 kPa in the well-watered treatment. In the drought treatment, we reduced soil water potential to − 40 kPa in experiment 1 (Expt 1) and to − 80 kPa in experiment 2 (Expt 2). Drought reduced shoot and root dry biomass and grain yield of rice in both experiments. The reduction of grain yield was less with higher AMF colonization. Plants with higher AMF colonization showed higher leaf P concentrations than plants with lower colonization in Expt 1, but not in Expt 2. Plants with higher AMF colonization exhibited higher stomatal conductance and chlorophyll fluorescence than plants with lower colonization, especially under drought. Drought increased the levels of ABA and IAA, and AMF colonization also resulted in higher levels of IAA. The results suggest both nutrient driven and plant hormone driven pathways through which AMF confer drought tolerance to rice.

Keywords: *stomatal conductance, chlorophyll fluorescence, abscisic acid (ABA), indole acetic acid (IAA)*

Introduction

Rice (*Oryza sativa* L.) is a staple food feeding more than half of the world's population. Demand for it is increasing due to an increase in the global population (FAO 2002). More than 75% of global rice production is from lowland rice cultivated under submerged conditions, and the remainder is from upland rice grown under non-submerged conditions (Maclean et al. 2013). Submerging rice paddy fields increases the availability of nutrients in the soil and limits the growth of weeds. However, this practice requires very large amounts of water (Haefele et al. 2008). Whereas, water can be sufficiently supplied in irrigated farming systems, water availability is a problem under rain-fed rice farming. Under those conditions, rainfall is the only water source; therefore, the productivity is highly dependent on the amount of rainfall. Less rainfall leads to water deficit in the soil, inducing drought, which can be a major constraint for producing rice.

Due to global climate change, drought likely will occur more frequently and more severely than in the past, causing problems for crop production in several regions of the world. Compared to other cereals, rice is particularly sensitive to drought. Drought affects growth and grain yield of rice by limiting water and nutrient availability, especially phosphorus (P) (Suriyagoda et al. 2014). In addition, drought reduces stomatal conductance as a mechanism to reduce water loss.

However, lower stomatal conductance decreases gas exchange and photosynthesis efficiency and reduces yields (Lauteri et al. 2014). The effects of drought on rice depend on timing and severity (Prasertsak and Fukai 1997). For instance, drought during vegetative stages has a smaller negative impact on yield than when it occurs during the panicle development stage (Boonjung and Fukai 1996). Drought reduces leaf expansion and delays maturation during the vegetative stage (Lilley and Fukai 1994). Drought can reduce rice yield by more than 60% when it occurs during the panicle development stage (Boonjung and Fukai 1996; Venuprasad et al. 2007). Growth and yield reduction can be mitigated by irrigation. However, such management is not practical for rain-fed farming. Hence, rain-fed farming needs other means to cope with drought. Some drought tolerant rice varieties have been successfully developed through breeding, for instance, Sahbhagi Dhan in India, Sahod Ulan in the Philippines, Sookha Dhan in Nepal, and IR64 in India (Dar et al. 2014). These varieties yield 0.8–1.2 t ha^{-1} more than drought susceptible varieties under drought (Dar et al. 2014).

Plant phenotypic plasticity is important to cope with drought, ideally enabling plants to withstand drought without a yield penalty. Highly adaptable plants can respond to drought by producing more roots, reducing water loss via stomatal closure and early maturation (Jearakongman et al. 1995; Fukai and Cooper 1995). The changes of stomatal conductance under drought also can be related to plant growth hormones, for instance, abscisic acid (ABA) and indole-3- acetic acid (IAA). ABA is the hormone that inhibits shoot growth, especially under drought. Under drought, ABA will be produced in the shoot (Borghi et al. 2015) or transported from the root to the shoot (Ko and Helariutta 2017), inducing stomatal closure. Haider et al. (2018) found a significant increase in leaf ABA content of rice plants under drought. IAA is important for root and shoot development, and it has been reported that IAA can induce the establishment of arbuscular mycorrhizal fungi (AMF) (Lüdwig-Müller and Güther 2007; Fitze et al. 2005). The contribution of AMF to plant levels of IAA has not been well explored.

Apart from these mechanisms, some plants deal with drought through interaction with and assistance by soil micro-organisms. One major group of soil micro-organisms that play an important role in enhancing drought tolerance comprises AMF (Rodriguez and Redman 2008; Ruiz-Lozano and Aroca 2010; Ruiz-Sánchez et al. 2010; Ruiz-Lozano et al. 2016). The symbiosis of AMF and roots increases plant nutrient uptake under drought, especially phosphorus (P) uptake (Augé 2001). In addition, AMF can alter photosynthetic efficiency of plants under drought by maintaining stomatal conductance (Augé 2001; Querejeta et al. 2007; Augé et al. 2015; Ruiz-Lozano et al. 2016) and the efficiency of photosystem II (PS II) (Mirshad and Puthur 2016). Augé et al. (2015) reported that the stomatal conductance of mycorrhizal plants is 24% higher than that of non-mycorrhizal plants. The changes in stomatal conductance also may be related to the effects of AMF regulating plant hormones. According to Estrada-Luna and Davies (2003), the flux of ABA in the shoots of AMF plants is lower than in non-AMF plants, which also results in higher transpiration and leaf water potential.

There are only few studies on AMF symbiosis and rice. This may be because rice is mostly grown in waterlogged conditions, which usually inhibit AMF colonization. Nevertheless, the symbiosis of AMF and rice plants has been reported (Maiti et al. 1995; Wangiyana et al. 2006; Lumini et al. 2011; Watanarojanaporn et al. 2013; Vallino et al. 2014). For instance, the system of rice intensification promotes root colonization and diversity of AMF species in rice roots compared to conventional rain-fed rice cultivation systems (Watanarojanaporn et al. 2013). AMF are more abundant in low input farming and under aerobic conditions than under partly anaerobic and submerged conditions (Lumini et al. 2011; Vallino et al. 2014). Hence, field management that promotes the functioning of AMF symbioses and possibly additional AMF inoculation could increase AMF colonization in rice. Increased AMF colonization may subsequently make rice more tolerant of drought, but the magnitude of this effect is still unknown and therefore needs to be investigated.

We investigated the contribution of AMF to the growth of six different rice varieties with different drought tolerances, under well-watered and drought conditions. Furthermore, we attempted to understand underlying mechanisms of mycorrhiza enhanced drought tolerance, such as higher nutrient uptake, enhanced stomatal conductance, and elevated efficiency of PS II. We also studied the effects of AMF on the regulation of plant hormones in rice without and with drought. In this study, we included the measurement of ABA and IAA hormones. In order to add a level of realism with respect to field conditions, we compared plants with higher and lower colonization by AMF (through inoculum addition), rather than comparing plants with and without mycorrhizas. We hypothesized that

(i) Rice with higher levels of AMF colonization have higher uptake of N and P, and more biomass (shoot, root, grain yield) than plants with lower levels of colonization, and

these AMF benefits are larger under drought than under well-watered conditions.

(ii) Rice with higher levels of AMF colonization have higher stomatal conductance and higher quantum yield of PS II (F_v/F_m) than plants with lower levels of colonization, and these mycorrhizal effects are larger under drought than

under well-watered conditions.

(iii) Rice with higher levels of AMF colonization have lower leaf ABA and higher leaf IAA concentrations than plants with lower levels of AMF colonization.

Material and methods

Experimental setup

Two greenhouse pot experiments were conducted at Khon Kaen University, Thailand. Experiment 1 (Expt 1) was done in the rainy season (August 2016–January 2017) and experiment 2 (Expt 2) in the dry season (December 2017–May 2018). We did not control light and temperature in the green- house. Both experiments were set up as a randomized complete block design with three factors, comprising three rice varieties, two water treatments (well-watered and drought), and two AMF treatments (low- and high-AMF colonization, the latter treatment with inoculum addition) with eight replicates of each treatment.

Soil

The soils used in this experiment were collected from a rice farm in Khon Kaen (16° 29′ 10.9″ N and 102° 34′ 40.5″ E), from the top layer of 0–15 cm. The soil properties were analyzed at the laboratory of the Agriculture Faculty, Khon Kaen University. Soil properties are shown in Supplementary Table 1. We used unsterilized soil in both experiments. The soil was sieved (2-mm sieve) and mixed homogeneously, and then used to fill the pots. Pot size was 0.0066 m^3 (top surface diameter = 0.24 m, bottom surface diameter = 0.17 m and height = 0.19 m). We filled the pots to 0.14 m depth, so each pot contained approximately 5.6 kg soil dry weight. The soil was saturated before planting rice seedlings. Pot weight of the saturated soil at 0 kPa was recorded for calculating the amount of water needed for rewetting the soil after imposing the drought treatment.

Rice varieties

For Expt 1, we used three rice varieties, viz. Khao Dowk Mali 105 (KDML 105), RD6, and Surin 1 (SR1) (Supplementary Table 2). These three rice varieties are the most consumed and economically valuable varieties, especially KDML 105 which is also known as jasmine rice or pandan rice. These varieties are grown during the rainy season. KDML 105 is a lowland rice variety, sensitive to photoperiod, and drought tolerant. RD6 is long grain glutinous rice which was developed from KDML 105. It is also a lowland rice variety, photoperiod sensitive, and moderately drought-tolerant. SR1 was bred from IR61078 and IR46329-SRN-18-2-2-2, it is a long-grain lowland rice variety, not sensitive to photoperiod. SR1 is a highly drought-tolerant variety. Rice seeds were obtained from the Rice Seed Centre of Khon Kaen.

For Expt 2, we used three other lowland rice varieties that are not sensitive to photoperiod. The three rice varieties used were Chainart 1 (CNT1), RD22, and RD33 (Supplementary Table 2). We chose these varieties because the experiment was done during the dry season. These varieties are commonly grown during the dry season in irrigated regions. CNT1 is the most commonly grown rice variety. RD22 is a long-grain sticky rice that is more drought-sensitive than CNT1. RD33 is a variety obtained from breeding KDML 105 and IR70177-76-3-1. It is suggested to be grown in the rain-fed regions in the north and northeast of Thailand. For Expt 2, rice seeds were obtained from the Rice Research Centre of Sakonnakorn, Thailand.

Arbuscular mycorrhizal fungi

The abundance of AMF in the soil was quantified through spore counting after wet-sieving. The wet-sieving was done by extracting 100 g of soil with water, and sieving through a stack of 45- and 35-μm sieves. Then, spores were counted under the microscope. The soil contained approximately 5 AMF spores per 100 g soil. For Expt 2, the soil was collected from the same rice field as for Expt 1; however, spore abundance then had doubled to around 10 AMF spores per 100 g soil. Overall, spore density was low, likely because the rice field is flooded during the rice growing season. We used unsterilized soil in both experiments; therefore, indigenous AMF could colonize the rice roots. As AMF spore density in the soil was low, we inoculated the soil with AMF spores for the high-AMF colonization treatment. We used 3 g of commercial granule AMF inoculum (RootGrow Professional, Kent, UK) which contained *Funneliformis mosseae, F. geosporus, Claroideoglomus claroideum, Glomus microaggregatum, and Rhizophagus irregularis* (Robinson Boyer et al. 2016) in the pots in the planting hole at 5 cm depth during transplanting. The spore density of the AMF inoculum was about 10 spores per g of inoculum.

Planting, water, and nutrient treatments

Rice seedlings were grown for 14 days in plug trays before being transplanted to the pots. One seedling was transplanted to the center of each pot. Rice plants were well-watered during the first 42 days by watering the pots every 2 days. The soil water potential was above − 10 kPa in order to ensure AMF symbiosis establishment and healthy rice plants. The drought treatment was applied at 42 days after planting (DAP), the day we stopped watering the pots in the drought treatment. In Expt 1, the withholding of water in the drought treatment was continued until the soil water potential reached - 40 kPa. This treatment involved no watering for 4 days. After that, the soil in the pots was rewetted to 0 kPa, and then water was withheld again for 4 days. In Expt 2, the withholding of water in the drought treatment was continued until the soil water potential reached - 80 kPa. This treatment involved no watering for 6 days. After that, the soil in the pots was rewetted to 0 kPa, and then water was withheld again for 6 days. The drying and rewetting cycle was done repeatedly until

90 DAP in both experiments. After 90 DAP, the pots remained well-watered, allowing the plants to recover in order to assess final yield and mycorrhizal root colonization. In the well-watered treatment, the soil water potential was maintained at around 0 to − 10 kPa throughout both experiments. Our experiment can thus be classified as type II (drying–rewetting cycle type) in the sub- division of He and Dijkstra (2014). We applied nitrogen (N) fertilizer at the rate of 20 kg N ha^{-1} to all pots 30 DAP. Later, we applied fertilizer which contained N-P_2O_5-K_2O at the rate of 20–20-10 kg ha^{-1} when the rice plants were 60 DAP. The fertilizer was dissolved and added with irrigation water. Harvesting was done after the grain was fully ripened. The harvesting date depended on the physiological age and was somewhat different for each variety (Supplementary table 2). In all experiments, the harvesting date of the rice plants in the drought treatment was about 2 weeks later than in well- watered treatment. The timeline of the experiment is depicted in supplementary figure 2.1.

AMF colonization

After harvest, fresh roots were collected, washed, weighed, and a subsample of approximately 10% taken for quantifying AMF colonization. Roots were stained by clearing in boiling 2.5% KOH solution at 90 °C for 10 min, then left in 1% HCl solution overnight (Koske and Gemma 1989). Finally, they were stained with 0.05% Trypan blue solution (dissolved in glycerin). AMF colonization was quantified by counting vesicles, arbuscules, and intraradical hyphae at × 400 magnification according to Giovannetti and Mosse (1980). The results were expressed as percentage of root length colonized (RLC).

Rice dry biomass (shoot, root, grain)

Rice was harvested when the grains were fully ripened. We harvested shoots, roots, and grain separately. We first harvested the grain by using scissors to cut and separate the panicles from the shoots. We separated filled and unfilled grain from the panicles and assessed the dry weight of filled grain. The shoots were harvested at about 5 cm above the ground. We recorded shoot fresh weight, and then dried the shoots at 80 °C for 48 h to assess dry weight. Roots were collected by washing the roots and subsequent careful removal of soil material and fragments of organic matter in fresh water. The fresh weight of the roots was recorded. Approximately 10% of the roots were taken for AMF colonization measurement, and the rest were dried at 80 °C for 48 h to assess dry weight.

Stomatal conductance and chlorophyll fluorescence

We measured stomatal conductance (g_s) and chlorophyll fluorescence (PS II efficiency) of the rice plants. The measurements were done 54 DAP, hence 12 days after the initiation of the drought treatment. For both measurements, we chose the three youngest fully emerged leaves from each plant and measured at the middle part of the leaf for all selected leaves. Stomatal conductance was measured from 9.30 to 12.00 am by steady AP4 porometer (Delta-T devices, UK). Chlorophyll fluorescence was measured by chlorophyll fluorometer (MINI-PAM, WALZ, Germany). The results of minimum (F_0) and maximum (F_m) fluorescence that the plant leaf can absorb were recorded to quantify the maximum quantum efficiency of photosystem II (PSII) photochemistry (F_v/F_m), with $F_v = F_m\text{-}F_0$ (Murchie and Lawson 2013).

Nitrogen and phosphorus concentration

For the analysis of nutrient concentrations, the third leaf from the apex was chosen. The leaf samples were collected during the panicle development stage. We collected the leaves after the rice was flowering (90 DAP), but in Expt 2, we collected the leaves before the rice was flowering (80 DAP). Five leaves were collected from each pot and dried at 80 °C for 72 h before grinding. The ground plant materials were submitted to the laboratory at Khon Kaen University for N and P analysis. N concentration was quantified by the Kjeldahl method (Bremner 1965), and P concentration was analyzed by wet digestion (nitric-perchloric digestion) and spectrophotometry (Land Development Department (Thailand) 2011). The N:P ratio (based on concentrations) was calculated to assess to what extent plants were limited by N and/or P (Güsewell 2004).

Plant hormone analysis

We analyzed ABA and indole-3-acetic acid (IAA) in Expt 2. The leaves of the rice plants were collected at 48 DAP, hence 6 days after applying drought. From each pot, we collected the second leaf from the apex, three leaves per pot. The leaves were cut, wrapped in aluminum foil, and put directly into liquid nitrogen. The leaf samples were ground in liquid nitrogen and stored in Eppendorf tubes at − 80 °C. The frozen samples were shipped with dry ice from Thailand to the Netherlands for analyses. ABA and IAA were analyzed at the Plant Physiology Laboratory, Wageningen University, the Netherlands. The extraction and analyses of ABA and IAA were done as described in Kolachevskaya et al. (2017) except that the weight of the samples was 4 mg DW.

Statistical analyses

Data are presented as means ± standard error. The results were analyzed in SPSS version 20. Data were first checked for normality (Shapiro-Wilk) and homogeneity of variance (Levene's test). Non-homogeneously distributed data such as N and P concentrations were log-transformed before analysis. Expt 1 and 2 were analyzed separately. Three-way ANOVA was used to determine significant sources of variation at $P < 0.05$. We used Tukey's honestly significant difference test to determine significant differences among treatments.

Results

AMF colonization

In Expt 1, AMF inoculation and water availability were significant sources of variation, whereas rice variety and all two-way and three-way interactions were not. AMF inoculation resulted in significantly higher AMF colonization compared to the low-AMF colonization treatment (fig. 2.1A). Average root length colonization (RLC) was 7 ± 1.6% in the low-AMF colonization (i.e., non-inoculated) treatments, and 16 ± 3.3% in the high-AMF colonization (i.e., inoculated) treatments (fig. 2.1A). Root colonization was higher in plants growing in the soil that was subjected to drought than in plants growing under well-watered conditions (fig. 2.1A). There was no significant difference in AMF colonization between KDML 105, RD6, and SR1 varieties. In Expt 2, AMF inoculation was the only significant source of variation. Colonization was significantly higher in the high- AMF colonization treatment (22 ± 5.1%) than in the low- AMF colonization treatment (11 ± 5.3%; fig. 2.1B).

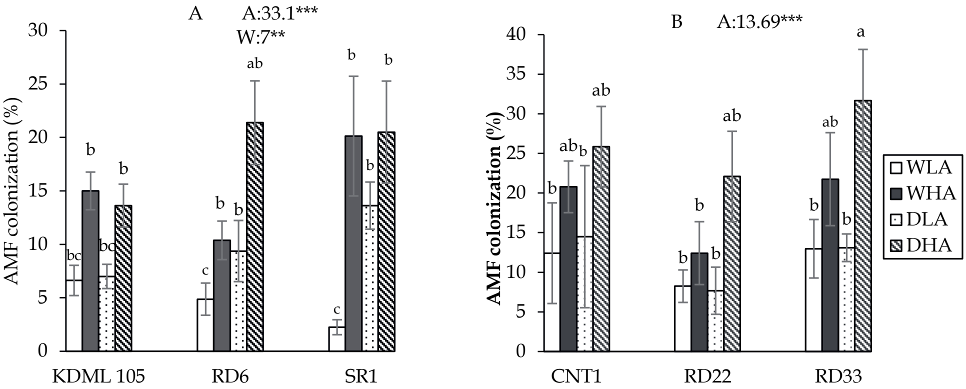


Figure 2.1: Fractional AMF colonization in rice roots of six different rice varieties in relation to drought and AMF inoculation. A= Experiment 1, rice varieties KDML 105, RD6, SR1 and B = Experiment 2, rice varieties CNT1, RD22, RD33. WLA = Well-watered and low AMF, WHA = Well-watered and high AMF, DLA = Drought and low AMF and DHA = Drought and high AMF. The error bar is related to SE. Bars topped by the same letter are not significantly different by Tukey HSD (*P < 0.05*). The significant effects of mycorrhiza (A), Water (W) and variety (V) and their interactions are presented as the F value with test of significance (** P < 0.05; ** P < 0.01; *** P < 0.001*) of three-way ANOVA. Non-significant factors are not shown (see Supplementary table 3 and table 4 for more information).

Shoot and root dry biomass

In Expt 1, water availability and the interaction between water availability and rice variety were significant sources of variation for shoot dry biomass, whereas the other factors and interactions were not. Shoot dry biomass was significantly greater in well-watered conditions compared to drought (fig. 2.2A). Average shoot dry biomass decreased from 13.02 ± 0.31 g in well-watered conditions to 11.36 ± 0.28 g in the drought treatment (fig. 2.2A). The negative effect of drought tended to be stronger in the drought-tolerant variety KDML 105 than in the drought-sensitive variety RD6. For root dry biomass, only rice variety was a significant source of variation, whereas the other main factors and all interactions were not. The drought-tolerant variety SR1 had highest root dry biomass, whereas the equally drought- tolerant KDML 105 had the lowest. Drought had no effect on root dry biomass (fig. 2.2C).

Drought significantly reduced shoot dry biomass in all treatments. The shoot dry biomass decreased from 12.62 ± 0.92 g in the well-watered treatments to 7.78 ± 0.63 g in the drought treatments (fig. 2.2B). For root dry biomass, both water availability and rice variety were significant sources of variation, whereas the mycorrhiza × variety interaction was marginally significant (*P* = 0.051; Supplementary table 2.4). Mycorrhiza and the other interactions were not significant sources of variation. Drought reduced root dry biomass, and the effect was strongest for the drought-tolerant CNT1 (fig. 2.2D). In Expt 2, water was again a significant source of variation for grain yield, whereas the effect of AMF was marginally significant (*P* = 0.054; Supplementary table 2.4). Drought very substantially (more than 40%) reduced grain yield (fig. 2.2F).

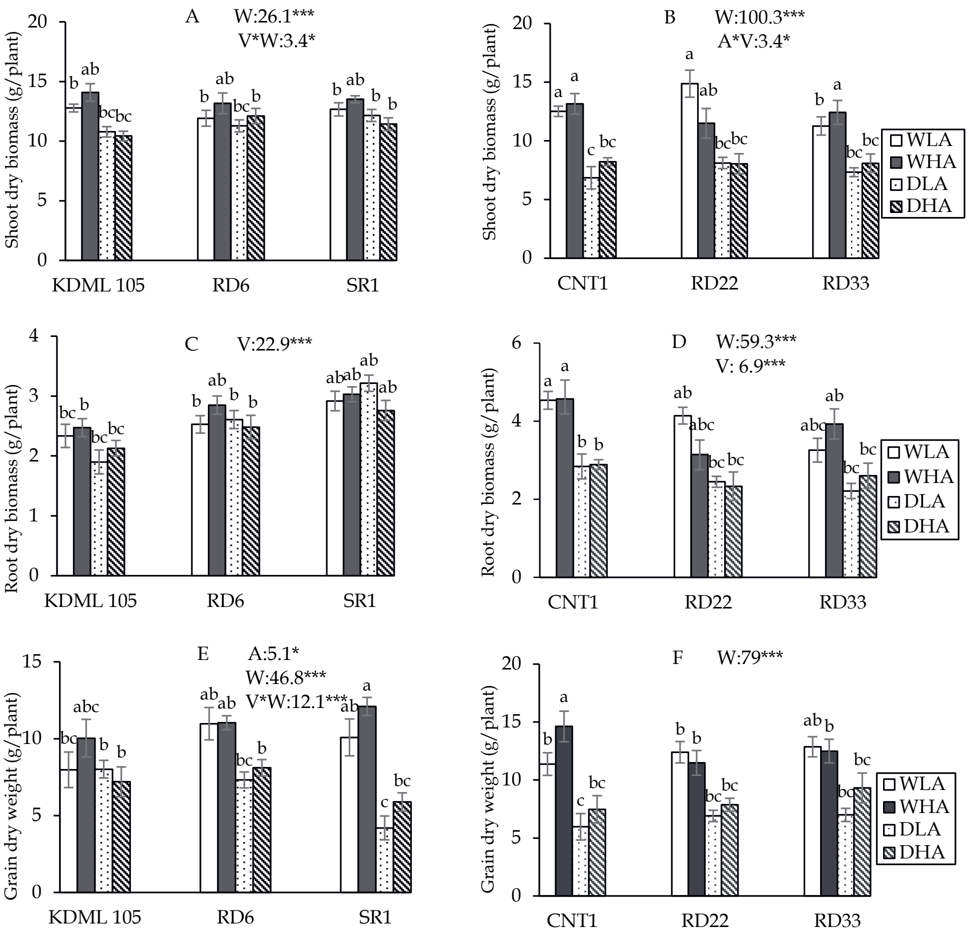


Figure 2.2: The shoot and root biomass and grain yield of six rice varieties in relation to drought and AMF inoculation. A, C, E= Experiment 1 and B, D, F = Experiment 2. WLA = Well-watered and low AMF, WHA = Well-watered and high AMF, DLA = Drought and low AMF and DHA = Drought and high AMF. The error bar is related to SE. Bars topped by the same letter are not significantly different by Tukey HSD ($P < 0.05$). The significant effects of mycorrhiza (A), Water (W) and variety (V) and their interactions are presented as the F value with test of significance (*P< 0.05; ** P < 0.01; *** P < 0.001) of three-way ANOVA. Non-significant factors are not shown (see Supplementary table 3 and table 4 for more information).

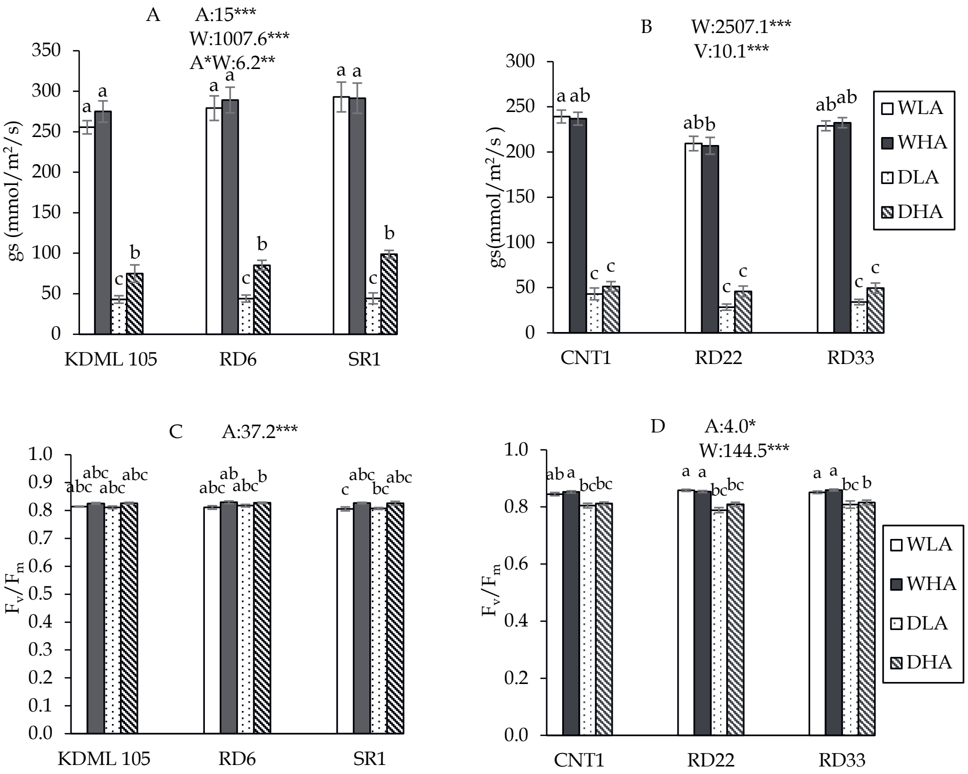


Figure 2.3: The stomatal conductance (g_s) and the maximum quantum efficiency of PS II photochemistry (F_v/F_m). A, C= Experiment 1 and B, D = Experiment 2. WLA = Well-watered and low AMF, WHA = Well-watered and high AMF, DLA = Drought and low AMF and DHA = Drought and high AMF. The error bar is related to SE. Bars topped by the same letter are not significantly different by Tukey HSD ($P < 0.05$). The significant effects of mycorrhiza (A), Water (W) and variety (V) and their interactions are presented as the F value with test of significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) of three-way ANOVA. Non-significant factors are not shown (see Supplementary table 3 and table 4 for more information).

Nitrogen and phosphorus concentrations

In Expt 1, variety was a significant source for variation for both N and P. For P mycorrhiza was also a significant source of variation. The other factors and their interactions were not significant sources of variation (Supplementary table 3). N and P concentrations were highest in KDML 105 and lowest in SR1. Mycorrhiza enhanced P concentration in the leaves of the three varieties. N:P ratios were lowest in KDML 105 and highest in SR1, with higher AMF colonization plants having a lower N:P ratio. N:P ratios were almost always below 10 (except for one treatment where N:P ratio was 11), suggesting that plants were N-limited, whereas the slightly lower N:P ratio in high-colonization treatments indicated either luxury P uptake or more severe N limitation (fig. 2.4A, B, C). The low N concentrations (lower than 10 mg per g) also provided evidence of N-limitation under almost all growing conditions.

In Expt 2, variety again was a significant source of variation for both N and P. Water availability was a significant source of variation for N, whereas it was marginally significant for P (*P* = 0.056; Supplementary table 2.4; fig. 2.4B, D, F). Mycorrhiza was not a significant source of variation for N or P, nor was any interactions. N concentrations were much higher in Expt. 2 than in Expt. 1, whereas P concentrations were much lower. This resulted in much higher N:P ratios of above 20, indicative of P limitation. CNT1 had significantly lower N and P concentrations than the other two varieties. However, the differences in N concentrations between varieties were smaller than differences in P concentrations, and CNT1 had the highest N:P ratio. Drought increased N concentrations and tended to increase P concentrations.

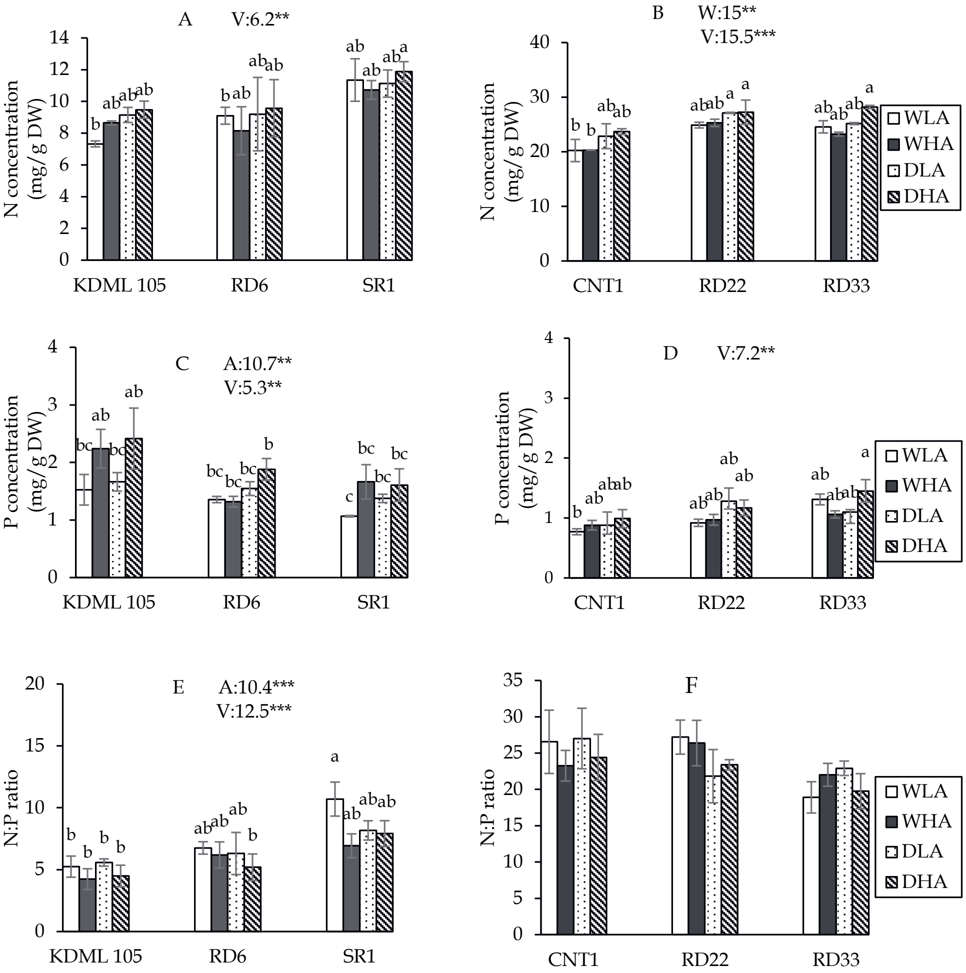


Figure 2.4: N, P concentrations in rice leave and N:P ratio. A, C, E = Experiment 1 and B, D, F = Experiment 2. WLA = Well-watered and low AMF, WHA = Well-watered and high AMF, DLA = Drought and low AMF and DHA = Drought and high AMF. The error bar is related to SE. Bars topped by the same letter are not significantly different by Tukey HSD ($P < 0.05$). The significant effects of mycorrhiza (A), Water (W) and variety (V) and their interactions are presented as the F value with test of significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) of three-way ANOVA. Non-significant factors are not shown (see Supplementary table 3 and table 4 for more information).

Plant hormones (ABA and IAA)

The concentrations of ABA in leaves were significantly different among rice varieties (fig. 2.5A). Rice variety CNT1 had higher ABA level in leaves than RD22 and RD33 varieties. In all varieties, ABA was significantly higher in plants grown under drought than under well- watered conditions (fig. 2.5A). Drought increased ABA approximately by 70% compared to the well-watered treatments. The different levels of AMF colonization did not show significant differences in ABA concentration, although higher AMF colonization tended to decrease ABA in the rice varieties RD22 and RD33 under drought. Drought significantly increased IAA in all three rice varieties (fig. 2.5B). The increase of IAA was more than 35% higher under drought than in well-watered conditions (fig. 2.5B). Plants with higher AMF colonization showed a significantly higher content of IAA in leaves than plants with lower AMF colonization (fig. 2.5B).

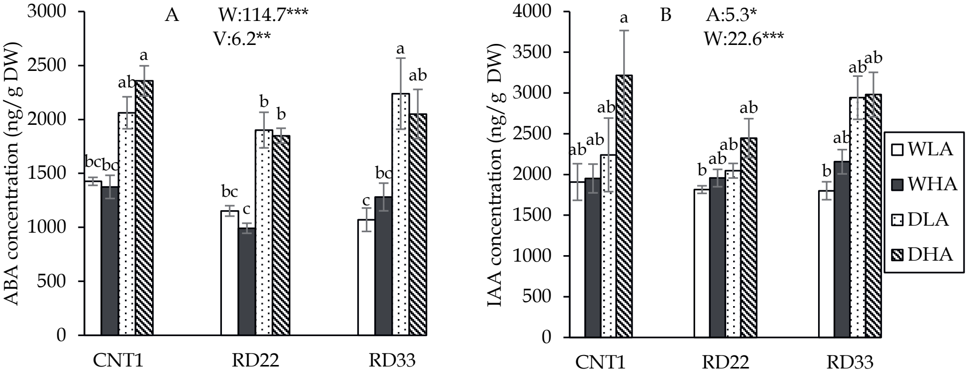


Figure 2.5: Abscisic acid (ABA) and Indole-3-acetic acid (IAA) hormones concentration in rice leaves. WLA = Well-watered and low AMF, WHA = Well-watered and high AMF, DLA = Drought and low AMF and DHA = Drought and high AMF. The error bar is related to SE. Bars topped by the same letter are not significantly different by Tukey HSD ($P < 0.05$). The significant effects of mycorrhiza (A), Water (W) and variety (V) and their interactions are presented as the F value with test of significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) of three-way ANOVA. Non-significant factors are not shown (see Supplementary table 3 and table 4 for more information).

Discussion

Addition of commercial inoculum increased AMF colonization, possibly through both changes in inoculum potential and changes in AMF species composition. We did not assess species composition of the low- and high-AMF treatments, so cannot evaluate the relative importance of possible qualitative changes in the AMF community. Our study showed that inoculum addition resulted in higher grain yields (Expt 1; marginally so in Expt 2), whereas there were no effects on shoot and root dry biomass. There also were no significant mycorrhiza × water availability interactions, except for root dry biomass in Expt. 2, partly supporting our first hypothesis. The second hypothesis also was partly supported by a significant effect of mycorrhizas and the mycorrhiza × water availability interaction for stomatal conductance (Expt 1; marginally so for Expt 2); quantum efficiency was significantly influenced by mycorrhizas in both experiments, but the mycorrhiza × water availability interaction was not significant. There was no effect of mycorrhizas on ABA, whereas the mycorrhizal effect on IAA was significant. The results therefore confirmed our hypotheses on the positive effects of increased AMF colonization on rice under drought, but an interaction between AMF and drought was seldom evident.

AMF colonization

Our experiments showed similar fractional colonization as previous studies. Zhang et al. (2014) reported 2–3% RLC in non-inoculated rice plants and 12–19% RLC in rice roots inoculated with AMF. Wangiyana et al. (2006) found 3–5% RLC in rice roots growing in paddies without AMF inoculation. AMF inoculation increased RLC, suggesting inoculum limitation in the field. The higher root colonization in Expt 2 than in Expt 1 is likely due to the buildup of AMF in the soil between years, as inoculum potential doubled from five to ten AMF spores per 100 g soil between samplings. However, evidence for inoculum limitation, as shown by higher grain yields of plants and higher stomatal conductance and chlorophyll fluorescence with higher levels of colonization, persisted in the second year.

Drought increased mycorrhizal colonization significantly in Expt 1 (but not in Expt 2), in agreement with studies by Lumini et al. (2011) and Vallino et al. (2014), who observed that mycorrhizal colonization was higher in rice roots growing in dry conditions compared to submerged conditions. These studies reflected a change from anaerobic to aerobic conditions and considering the strictly aerobic characteristics of AMF such an increase is not surprising. It is not clear whether our well-watered treatment (0 kPa) created anaerobic conditions. As the effect of drought on mycorrhizal colonization is much smaller than that of the inoculation treatment, our results suggest that a shift from irrigated rice to rain-fed rice will only gradually result in increases in mycorrhizal inoculum potential, and that inoculum addition or management, possibly with the help of cover crops when fields are fallow, could contribute to the buildup and maintenance of sufficient mycorrhizal inoculum, which then has beneficial consequences for yield.

Shoot and root dry biomass and grain yield

Drought reduced rice shoot dry biomass. The moderate drought of Expt 1 had a smaller impact on root dry biomass than the more severe drought of Expt 2. The decrease of root and shoot dry biomass under drought could be both due to the reduced availability of water, which impeded photosynthetic carbon gain, and through drought-induced reduction of nutrient availability, especially of nutrients with low mobility such as P (Prasertsak and Fukai 1997; Suriyagoda et al. 2014). The responses of rice roots under drought contradicted previous reports, which proposed that rice develops increased roots under drought (Yoshida and Hasegawa 1982). Our results did not show that the most drought-tolerant varieties produced more roots than the less drought-tolerant varieties. The marginally significant interaction between mycorrhizas and water in Expt 1 ($P = 0.064$) suggests that under moderate, but not severe drought, AMF can somewhat alleviate this interaction effect.

Drought decreased grain yield of rice in both experiments. Moderate drought of 4 days had a smaller negative effect than strong drought that lasted 6 days. Our result is in line with the study of Venuprasad et al. (2007), who found a reduction of rice yield of more than 60% under drought. Similarly, Ghosh and Singh (2010) found significantly decreased rice yield when soil water potential decreased to − 60 kPa. Both water and nutrients are major limiting factors for rice at the grain filling stage (Fageria 2003). Even though AMF did not increase shoot and root dry biomass, AMF increased rice grain yield in our experiments. In addition, Zhang et al. (2016) stated that AMF plants increase the allocation of N and P to rice panicles compared to non-AMF plants during the grain filling stage, and the grain yield of rice increased about 28%.

Stomatal conductance and chlorophyll fluorescence

Drought substantially decreased stomatal conductance, which can be explained by the plant physiological mechanisms that enabled reduced water loss. When water becomes limiting, stomata are closed to prevent the loss of water via transpiration, which simultaneously reduces the exchange of CO_2. As stated by Hasegawa and Yoshida (1982), the decrease in transpiration rate of upland rice occurs when soil water potential is reduced below − 20 kPa. Under drought, plants with higher AMF colonization exhibited higher stomatal conductance than plants with lower AMF colonization. With more severe drought in Expt 2, the effect was smaller with only a marginally significant effect. The meta-analysis of Augé et al. (2015) showed that, averaged over all studies, stomatal conductance of AMF plants is 24% higher than in non-AMF plants. Higher stomatal conductance might be due to the extension of hyphae to the water and nutrient sources that are inaccessible to plant roots (Smith and Read 2010), but has been hypothesized also to be due to hormonal changes consequent upon mycorrhizal colonization (Birhane et al. 2012). The interaction between AMF and drought in both experiments indicates that AMF can alleviate the negative consequences of stomatal closure under drought. This is in agreement with the meta-analysis of Worchel et al. (2013), who also found greater effects of AMF on the growth of grasses grown under dry than under normal conditions. Li et al. (2014) found a positive effect of AMF (*Rhizophagus intraradices*) on barley, but no effect of drought on stomatal conductance.

Quantum efficiency of PS II was unaffected by the moderate drought of Expt 1, but was significantly reduced by the strong drought of Expt 2. These results are in agreement with the study of Puteh et al. (2013), who reported that the quantum efficiency of rice decreased from 0.78 to 0.60 after 8 days of drought. In our Expt 1, where we did not provide water for 4 days, drought might not have been sufficiently severe to cause a reduction of quantum efficiency. Apparently, chlorophyll fluorescence acts at a different temporal scale than stomatal conductance, in that chlorophyll fluorescence is more resistant to a short drought (Trueba et al. 2019). The values of chlorophyll fluorescence in well-watered plants are close to the theoretical optimum of 0.83 (Björkman and Demmig 1987). Despite values of well-watered plants close to that theoretical maximum, plants with higher AMF colonization had slightly but significantly higher values of chlorophyll fluorescence, possibly due to sink stimulation of photosynthesis by the AMF symbiosis (Kaschuk et al. 2009). Beneficial effects of AMF on chlorophyll fluorescence also have been reported by de Andrade et al. (2015), who found that mycorrhizal rice plants had higher chlorophyll fluorescence under arsenate and arsenite pollution than non-mycorrhizal plants. Mathur et al. (2019), studying the response of wheat to very severe drought, also noted a beneficial effect of AMF on chlorophyll fluorescence. However, Porce et al. (2015) observed that mycorrhizal plants exhibited lower chlorophyll fluorescence than non-mycorrhizal plants, except at high salt levels where fluorescence values increased in mycorrhizal plants compared to a treatment with lower salt levels.

Nutrient uptake: N and P concentration, N:P ratio

Drought limits the availability of nutrients for plant uptake (Suriyagoda et al. 2014). Drought effects were noted for plant biomass, but less so for nutrient concentrations. Mild drought of Expt 1 did not affect leaf N and P concentrations, whereas the more severe drought of Expt 2 did positively affect concentrations of both nutrients. Our results contrast with previous studies that reported reduction of P uptake under drought (Suriyagoda et al. 2014; Sardans and Peñuelas 2004). A meta-analysis by He and Dijkstra (2014) indicated that drought on average reduced plant N and P concentrations by 3.7 and 9.2% respectively. However, their meta-analysis also showed that drought experiments involving a drying–rewetting cycle (their type II, as in our experiment) did not have a negative effect on those concentrations, with rather non-significant positive effect sizes as in our experiment.

Higher AMF colonization did not affect leaf N concentration, suggesting that N immobilization in the mycorrhizal mycelium was not important. N is important for grain filling and ripening. More than 60% of N is finally translocated from shoot to grain during the reproductive stage (Fageria 2003). AMF rice plants allocate more N to the panicle than non-AMF rice plants (Zhang et al. 2016). However, we cannot confirm the effects of AMF on N allocation, because we did not analyze the N concentration in the roots and grain in our experiments. Increasing mycorrhizal colonization increased P concentrations in Expt 1 but did not have an effect in Expt 2. In Expt 1, rice plants were N rather than P limited (to judge from N:P ratios below 10), so a major part of the additionally acquired P could not be translated into biomass increase but rather showed as higher P concentrations. In Expt 2, plants were P limited, as N:P ratios were above 20, but it is unclear why plants did not show a biomass response to higher AMF colonization.

Plant hormones (ABA and IAA)

ABA has been considered the abiotic stress hormone by Bahadur et al. (2019), and the increase of ABA under drought agrees with their review. Dobra et al. (2010) found that ABA increased about 50–80 times in tobacco leaves grown under drought. Ludwig-Müller (2010) and Bahadur et al. (2019) also included data on changes in ABA levels due to mycorrhizal colonization, and both decreases in root ABA (in tomato and the legume (*Glycyrrhiza*) and increases in ABA (in maize) have been noted. Reduction of ABA in AMF plants has also been reported by Estrada-Luna and Davies (2003) in mycorrhizal *Capsicum annuum* compared to non-inoculated plants. Higher levels of ABA would result in stomatal closure and a lowering of stomatal conductance, so we cannot explain a mycorrhiza effect on stomatal conductance without an effect on ABA levels in our study. According to Borghi et al. (2015), plants may produce or transport more ABA to the leaves to regulate stomatal closure when subjected to drought. However, earlier studies referred to ABA levels in roots, and therefore may not be comparable to our study where we assessed ABA levels in shoots as data on root–shoot signaling through hormones remain scarce (Ludwig-Müller 2010).

Both drought and AMF resulted in increased IAA levels. The increase of IAA in response to drought is not consistent with observations by Dobra et al. (2010) on tobacco, who observed decreases in IAA levels in young leaves (we collected the second leaf for hormone analysis), but increases in IAA levels in middle and lower leaves and also in roots. Literature data indicate both cases where IAA levels were upregulated by AMF and cases where hormone levels were unchanged, but most of the available data refer to changes in hormone levels in roots (Ludwig-Müller 2010). Some plant species produce IAA also to stimulate the symbiosis with soil micro-organisms under stress conditions. That could be the reason that IAA content in plants with higher AMF colonization was higher than in plants with lower AMF colonization. AMF inoculation also may increase the level of IAA in plant leaves. Fitze et al. (2005) found an increase of IAA in maize after 20 and 30 days after AMF inoculation. However, there are other studies that reported that IAA does not change with AMF inoculation (Torelli et al. 2000; Shaul-Keinan et al. 2002).

Conclusion

AMF colonization in rice fields is usually low, but it may be possible to enhance colonization by adding AMF inoculum. Increased RLC improves rice plant performance through uptake of nutrients such as N and P, resulting in higher grain yields (hence a higher harvest index), without much effect on total plant biomass. Moreover, AMF increase photosynthesis, especially under drought, via a smaller reduction in stomatal closure and by maintaining higher levels of chlorophyll fluorescence. These effects are likely mediated both through nutrients and through regulation of plant hormones, especially IAA. AMF therefore contribute to a better recovery after drought resulting in higher rice grain yields. The outcomes of our study may be relevant under climate change where drought is becoming a major factor restricting rice production. Increasing AMF colonization may be important for water savings.

Supplementary data

Table S2.1: Soil properties of the soil used in expt 1 and expt 2

Properties	expt 1	expt 2
Soil texture [a]	Sandy loam	Sandy loam
Bulk density (g cm^{-3})	1.3	1.4
Water holding capacity (%)	25.5	30.6
pH (H_2O)	7.5	7.3
Organic matter (%)[b]	0.62	0.57
Total N (mg/kg)[c]	270	310
Extractable P (mg/kg)[d]	19.5	23.5
Total K (mg/kg)[e]	995	964

[a] Gee and Bauder (1986), Raynolds (1993), [b] Walkley and Black (1987), [c] Bremner (1965), [d] Bray and Kurtz (1945), [e] Land Development Department (Thailand) (2011).

Table S2.2: Rice varieties used in both experiments. (Source: http://kkn-rsc.ricethailand.go.th/index.php/e-library/varieties)

	Expt 1			Expt 2		
	KDML 105	RD6	SR1	CNT1	RD22	RD33
Photoperiod sensitivity	Yes	Yes	No	No	No	No
Growing period	May-November	May – November	Year round	Year round	Year round	Year round
Harvesting (days after sowing)	25-Nov*	21-Nov*	138	130	130	130
Average grain yield (kg/ ha)	2251	4129	4452	4588	4241	3057
Average height (cm)	140	154	122	113	120	**
Drought tolerance	High	Moderate	High	High	Low	High

* The estimated harvesting date (because the harvest of photoperiod-sensitive varieties depends on the day length)

** No data

Table S2.3: ANOVA table for Experiment 1 (2016). RCL = fractional root colonization; SDW=Shoot dry weight, RDW = Root dry weight, GDW = Grain dry weight, F_v/F_m = The maximum quantum efficiency of PS II photochemistry, gs = Stomatal conductance (g_s), N = Nitrogen and P = Phosphorus mass-fraction. Bold numbers indicate significant results at $P < 0.05$.

Independent		RCL		SDW		RDW		GDW		F_v/F_m		gs		N mass-fraction		P mass-fraction	
variables	df	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value
AMF (A)	1	33.1	**<0.001**	2.6	0.109	0.2	0.698	5.1	**0.027**	37.2	**0.000**	15.0	**<0.001**	0.2	0.675	10.7	**0.002**
Water (W)	1	7.0	**0.010**	26.1	**<0.001**	3.5	0.066	46.8	**<0.001**	0.1	0.785	1007.6	**<0.001**	1.4	0.241	2.4	0.130
Variety (V)	2	1.7	0.197	0.6	0.545	22.9	**<0.001**	1.5	0.236	1.1	0.343	2.9	0.063	6.2	**0.005**	5.3	**0.009**
A*W	1	0.4	0.531	3.5	0.064	2.7	0.102	0.2	0.619	0.2	0.642	6.2	**0.015**	0.1	0.750	0.03	0.854
A*V	2	0.8	0.463	0.8	0.471	1.3	0.270	1.4	0.262	0.7	0.518	0.0	0.998	0.2	0.834	1.0	0.379
V*W	2	2.3	0.110	3.4	**0.038**	1.6	0.209	12.1	**<0.001**	0.2	0.854	0.5	0.613	0.2	0.836	0.3	0.736
A*V*W	2	2.3	0.104	0.4	0.684	1.2	0.311	0.4	0.677	0.4	0.670	0.9	0.430	0.3	0.736	0.3	0.723

Table S2.4 : ANOVA table for Experiment 2 (2018). RCL = fractional root colonization SDW=Shoot dry weight, RDW = Root dry weight, GDW = Grain dry weight, F_v/F_m = The maximum quantum efficiency of PS II photochemistry, gs = Stomatal conductance, N = Nitrogen and P = Phosphorus mass-fraction. Bold numbers indicate significant results $P < 0.05$.

Independent variables	df	RCL		SDW		RDW		GDW		F_v/F_m		gs		N mass-fraction		P mass-fraction	
		F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value
AMF (A)	1	13.69	**<0.001**	0.03	0.865	0.001	0.974	3.8	0.054	4.0	**0.048**	3.3	0.073	0.6	0.450	0.3	0.578
Water (W)	1	2.1	0.147	100.3	**<0.001**	59.3	**<0.001**	79.0	**<0.001**	144.5	**<0.001**	2507.6	**<0.001**	15.0	**0.002**	4.5	0.056
Varieties (V)	2	2.2	0.114	1.0	0.360	6.9	**0.002**	0.6	0.543	1.0	0.385	10.1	**<0.001**	15.5	**<0.001**	7.2	**0.009**
A*W	1	1.7	0.197	1.6	0.212	0.3	0.567	0.7	0.412	1.0	0.329	3.7	0.057	1.4	0.256	0.9	0.352
A*V	2	0.2	0.796	3.4	**0.037**	3.1	0.051	1.4	0.252	0.0	0.998	0.3	0.741	0.1	0.939	0.3	0.755
V*W	2	0.0	0.981	0.6	0.576	0.8	0.458	1.0	0.366	1.7	0.192	3.0	0.058	0.2	0.853	0.6	0.547
A*V*W	2	0.2	0.845	1.3	0.288	0.9	0.391	1.4	0.250	1.1	0.332	0.2	0.862	1.1	0.372	2.3	0.139

Table S2.5: ANOVA table for Abscisic acid (ABA) and Indole-3-acetic acid (IAA) hormones from Experiment 2 (2018). Bold numbers indicate significant results *P* < 0.05.

Independent variables	df	ABA		IAA	
		F	P-value	F	P-value
AMF (A)	1	0.0	0.916	5.3	**0.027**
Water (W)	1	114.7	**<0.001**	22.6	**<0.001**
Varieties (V)	2	6.2	**0.005**	2.3	0.118
A*W	1	0.1	0.831	0.6	0.459
A*V	2	0.8	0.452	0.3	0.753
V*W	2	0.7	0.498	1.3	0.300
A*V*W	2	1.9	0.168	1.5	0.245

Chapter 3

Arbuscular Mycorrhizal Fungi (AMF) mitigate negative drought impacts on stomatal conductance and photosystem II activity of rice plants

Anupol Chareesri, Gerlinde B. De Deyn, Anan Polthanee, Sophon Boonlue, Thomas W. Kuyper

Abstract

Rice productivity decreases under drought because drought hampers photosynthesis due to reduced stomatal conductance and photosystem II (PSII) activity. Arbuscular mycorrhizal fungi (AMF) can alleviate drought impacts by promoting stomatal conductance and efficiency of PSII. However, the temporal patterns of both processes are poorly known. We conducted a three-factorial greenhouse pot experiment with two rice varieties, high and low levels of AMF colonization, and under well-watered and drought treatment. We measured stomatal conductance and PSII activity every two days over 14 days since the onset of the drought treatment. From all rice plants subjected to drought, we re-watered half of the replicates to assess potential recovery after 12 days. Drought reduced stomatal conductance after 4-6 days, depending on variety, and PSII activity after 8-10 days. Drought-induced reductions were less severe in plants with high AMF colonization. Shoot biomass was also significantly greater in plants with high AMF colonization. Furthermore, plants with higher levels of AMF colonization tended to have a higher yield. Overall, higher AMF colonization delayed or reduced a decline in stomatal conductance and PSII activity, thereby expanding the window of opportunity of rice to escape from drought impacts that will be more common under global climate change.

Keywords: *arbuscular mycorrhiza, photosynthesis, stomatal conductance, chlorophyll fluorescence, photosystem II*

Introduction

Rice (*Oryza sativa* L.) is one of the major food crops consumed globally. Rice is mostly grown under rain-fed conditions. Under those conditions its productivity is highly dependent on rainfall. Over the last decade, rainfall patterns have become more variable in duration and intensity with more intensive rainfall events, often causing serious flooding, and prolonged periods of drought. These changes have occurred more frequently and more severely in many parts of the world. Drought directly affects the growth and productivity of crops, including a staple food like rice. Rice grain yield can decrease up to 60% when exposed to drought (Venuprasad et al. 2007). A modelling study of rice production indicated potential increases in yield loss risks of 20% at the end of this century (Leng and Hall, 2019). Thailand was mentioned as a very vulnerable country in that study. For Thailand, Prabnakorn et al. (2018) noted that past climatic change (1984-2013) had only a limited impact on rice yields (3% loss per decade), but future trends of climate change suggest more serious yield losses in the future.

Drought limits water and nutrient availability for plant uptake (Suriyagoda et al. 2014). When soil water becomes limiting, plants respond by reducing transpiration via regulating stomatal behavior and ultimately stomatal closure. However, stomatal closure also reduces stomatal conductance, which regulates CO2 exchange between plant leaves and the atmosphere, and thereby the amount of carbon that is available for photosynthesis (Lauteri et al. 2014). Cha-um et al. (2006) and Sikuku et al. (2010) reported that the reduction of stomatal conductance can also lead to a decrease of photosystem II (PSII) activity, the efficiency of PSII. Hence, drought may reduce the efficiency of the photosynthetic process via reduced carbon uptake and reduced efficiency of the photosystem. The effects of drought on stomatal conductance and PSII activity depend on the intensity and duration of drought. Withholding water for 14 days decreased stomatal conductance of basmati rice up to 48% (Akram et al. 2013). Sikuku et al. (2010) reported no effects of drought on PSII activity of rice plants that were subjected to drought for six days. In contrast, a 14-day drought in rice reduced PSII activity with more than 50% (Yooyongwech et al. 2016).

Rice is less effective in regulating its transpiration compared with other cereal crops (Austin 1989; Duursma et al. 2019). Therefore, mechanisms of rice to overcome the adverse effects of drought may be different from other cereal crops. One possible mechanism by which rice may promote its drought tolerance is via the association with soil microorganisms or beneficial root endophytes (Rodrigues and Redman 2008; Redman et al. 2011; Andreo Jimenez et al. 2019; Tisarum et al. 2019; Mathur and Roy 2021). Arbuscular Mycorrhizal Fungi (AMF) are amongst the plant-symbiotic soil microorganisms that can increase plant drought tolerance (Augé et al. 2015; Ruiz-Lozano and Aroca 2010; Ruiz-Sánchez et al. 2010; Chareesri et al. 2020; Das et al. 2021; Etesami et al. 2022; Kuyper and Jansa 2023). The hyphae of AMF are able to reach to water and nutrient sources where plant roots do not have access to (Smith and Read 2010; Ruiz-Lozano and Aroca 2010; Ruiz-Sanchez et al. 2010). When a plant can obtain additional water and nutrients via AMF hyphae, its photosynthetic activity will also remain higher (Augé 2001). Furthermore, AMF may regulate plant growth hormones that can maintain the photosynthetic activity of the rice plant. In a previous study we showed that AMF increased Indole-3-acetic acid (IAA) and tended to decrease Abscisic acid (ABA), associated with less yield reduction of rice subjected to drought (Chareesri et al. 2020).

The mycorrhizal role in conferring tolerance or resistance to drought (including salt-stress induced drought) through effects on stomatal conductance have been summarized in a meta-analysis (Augé et al. 2015). The study concluded that AMF ameliorate stomatal conductance and the positive effect was larger under drought than under well-watered conditions. A meta-analysis on the effect of salinity on the performance of PSII indicated a beneficial effect of the AMF symbiosis on that performance, with a significantly larger effect for monocotyledonous plants (like rice) than for dicotyledonous plants (Wang et al. 2019). An exception to this pattern was reported by Porcel et al. (2015), who observed that under normal and mildly saline conditions PSII activity of mycorrhizal rice plants was lower than that of non-mycorrhizal plants; under more saline conditions there was no mycorrhizal effect on PSII activity. The authors also reported that under those conditions the actual quantum yield of PSII was higher in mycorrhizal than in non-mycorrhizal plants. No explanation for this divergent pattern was provided.

Studies that simultaneously investigated the effects of AMF on both stomatal conductance and PSII activity are rare. Zhu et al. (2012) reported that under drought mycorrhizal maize (*Zea mays* L.) exhibited higher stomatal conductance and photosystem II activity than non-mycorrhizal plants, whereas Chareesri et al. (2020) reported the same effects for rice, when comparing plants with higher levels of mycorrhizal colonization with plants with lower colonization. Both studies measured these parameters only once, and it is still unclear to what extent and on what temporal scale the mycorrhizal regulation of both parameters differs after the onset of drought. Trueba et al. (2019) compared both parameters across ten perennial plant species and noted that decline of stomatal conductance precedes decline of PSII activity, and that when the PSII is substantially damaged, its damage is irreversible, whereas stomatal conductance can be recovered if plants are watered in time. Studying the temporal dynamics of both parameters is especially important for rain-fed rice cultivation when there is a period without rain as the mycorrhizal status might affect the chances of tolerating the drought and of recovery after subsequent rainfall events. The reduction of rice productivity due to drought periods would be less, if AMF reduce the effect of drought on the photosynthetic capacities of rice plants until the rain comes. Especially beneficial effects by the AMF symbiosis on PSII could be critical as that could extend the period over which recovery can take place.

AMF occur in rice fields, but their abundance is often low to very low (Chareesri et al. 2020; Lumini et al. 2011; Vallino et al. 2014; Wangiyama et al. 2006). While adding AMF inoculum to rice fields is challenging, farmer management could be modified such that it becomes more conducive for AMF. However, whereas previous studies showed mycorrhizal benefits compared with non-mycorrhizal plants, it is unclear whether increased AMF colonization can equally benefit rice plants. The benefits of AMF on enhancing plant performance differ between plant species but also within plant species and this latter effect could be substantial (Stahlhut et al. 2023). Several studies have shown such variety-dependent mycorrhizal benefits in rice. Gao et al. (2007) noted a trade-off between mycorrhizal benefit and the ability of the plant to acquire nutrients under the non-mycorrhizal condition, due to varietal differences in root morphological and physiological traits. Davidson et al. (2019) equally noted variation in root colonization and plant performance in 334 rice varieties and identified putative genetic markers that could be linked to variation in plant responsiveness. Such studies indicate that plant breeding with intended and unintended effects on root traits impact on mycorrhizal benefits (Kuyper et al. 2021). The extent to which plant breeding for drought tolerance might impact mycorrhizal responsiveness is currently unknown but likely. Chareesri et al. (2020) showed a marginally significant interaction between AMF and rice variety on root biomass. The more drought-tolerant variety (SR1) had higher root mass than the less drought-tolerant variety (KDML105), but in that experiment the grain yield of SR1 was reduced more than KDML105 under drought. The stomatal conductance of SR1 tended to be higher than KDML105.

This study aimed to investigate the contribution of augmented levels of AMF in rice field soil through AMF inoculum addition to the growth of two rice varieties (KDML105 and SR1) under both well-watered and drought conditions, and to understand underlying changes in photosynthetic activity based on changes in stomatal conductance and PSII activity over time. We hypothesized that:

(i) Plants with higher AMF colonization will exhibit higher stomatal conductance and PSII activity than plants with lower AMF colonization, especially in plants subjected to drought.

(ii) Plants with higher AMF colonization will have higher N and P concentration in leaves than plants with lower AMF colonization, especially in plants subjected to drought.

(iii) Plants with higher AMF colonization will produce more biomass (shoot, root, grain yield) than plants with lower levels of colonization, especially in plants subjected to drought.

(iv) Plants with higher AMF colonization will recover from drought faster than plants with lower levels of colonization.

(v) Under drought, the more drought-tolerant variety (SR1) will retain higher stomatal conductance and PSII activity than the less drought-tolerant variety (KDML105), especially with higher levels of mycorrhizal colonization.

Materials and methods

Experimental setup

A greenhouse pot experiment using (non-sterilized) field soil was conducted from July 2017 to December 2017 at Khon Kaen University, Khon Kaen, Thailand. We set up a full, three-factorial design comprising two rice varieties (different in drought tolerance), two AMF treatments (low and high AMF colonization) and two water treatments (well-watered and drought). Each treatment was replicated eight times. We applied drought by withholding water 42 days after transplanting (DAP) the rice seedlings. Four replicates of the drought treatments were maintained without watering till the rice plants died (and time for collapse was noted), while the other four replicates were re-watered 12 days after starting the drought treatment (DAD) to assess potential recovery of the rice plants. The rice plants were harvested at 107 days after transplanting.

Rice varieties

We used two rice varieties: Khao Dowk Mali 105 (KDML105) and Surin 1 (SR1). These two rice varieties are the most consumed and economically important varieties in Thailand, especially KDML105. KDML105 is long-grain lowland rice that is sensitive to photoperiod and possesses moderate drought tolerance. SR1 is also a long-grain lowland rice, bred from IR61078 and IR46329-SRN-18-2-2-2. This variety is more tolerant to drought and not sensitive to photoperiod. We obtained rice seed of both varieties from the Rice Seed Centre of Khon Kaen, Thailand.

Soil

We used soil collected from the 0-15 cm soil layer from a rice farm in Khon Kaen (6°29'10.9"N 102°34'40.5"E). The soil was a sandy loam soil containing 60% sand, 31% silt, 8% clay and 1% organic matter. The pH of the soil was 7.5. Bulk density was 1.29 g cm-3 with 0.52% porosity and 25.5% water holding capacity. We sieved the dry soil through a 2 mm sieve and then mixed it homogeneously. We filled approximately 5.6 kg dry soil equivalent around 7.2 kg fresh soil saturated status per pot (pot volume was 6.6 dm3; top diameter = 24 cm, bottom diameter = 17 cm and height = 19 cm). The soil was saturated (soil water potential 0 kPa) before planting the rice seedlings and pot weight was recorded to calculate the amount of water needed for rewetting.

Arbuscular Mycorrhizal Fungi

To increase the levels of AMF in the field soil and create the 'high AMF colonization' treatment, we used the commercial granule inoculum (RootGrow Professional, Kent, UK) that contained *Funneliformis mosseae*, *F. geosporus*, *Claroideoglomus claroideum*, *Glomus microaggregatum*, and *Rhizophagus irregularis* (Robinson et al. 2017). We inoculated the pots during rice seedling transplanting by adding six grams of the AMF inoculum to the bottom of the planting hole at five cm depth. One gram of inoculum contained approximately 10 AMF spores. Therefore, each pot received about 60 AMF spores. The natural levels of AMF inoculum in the field soil served as the 'low AMF colonization' treatment. We did not add the microbial wash because we did not sterilize the field soil in both treatments.

Planting, water treatments and fertilization

We grew rice seedlings in plug trays for 21 days before transplanting them to the pots. We planted one seedling per pot. The rice plants were well-watered by maintaining soil water potential above - 10 kPa during the 42 DAP to ensure AMF symbiosis establishment and the growth of the rice plants. We applied drought according to a drying-rewetting cycle (type III) as defined by He and Dijkstra (2014). We applied the drought treatment by stopping watering the plants for 12 days (four replicates) or till the plants died (the other four replicates of the drought treatment). We re-watered the half of the pots of the drought treatment after 12 days of drought to allow recovery of the rice plants. Each pot received water based on pot weight to bring the soil water potential back to 0 kPa. Soil water potential increased to 0 kPa on the day we re-watered the pots. Soil water potential in the drought treatment was recorded in one pot every two days until day 14 since the start of the drought treatment (fig. 3.1). Soil water potential was always maintained at 0 kPa in the well-watered treatment.

We applied nitrogen (N) fertilizer at the rate of 20 kg N ha-1 to all pots 30 DAP. Later we applied fertilizer which contained N-P-K at the rates of 20 - 4.6 - 8.3 kg ha-1 respectively when the rice plants were 60 DAP.

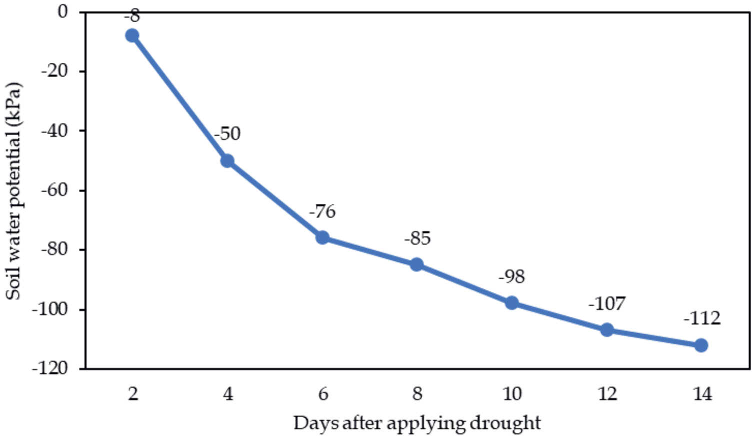


Figure 3.1: Soil water potential in the drought treatment during the period of 14 days of no watering.

Measurements

AMF colonization

After harvesting the rice plants, we collected and washed the roots, then dried them at 80 °C for 48 hours. We took 10% of the roots from the total root mass for assessment of mycorrhizal colonization. These roots were cut into small fragments of about 2 cm long. We placed the roots in plastic tissue cassettes. Roots were stained by clearing in 10% KOH solution at 90 °C for 15 minutes. The roots were then rinsed and left in 1% HCl solution for 30 minutes. Finally, we stained the roots by leaving them in 0.05% Trypan blue solution over 24 hours (500 ml glycerin: 450 distilled water: 50 ml of 1%

HCl and 0.5 g Trypan blue). The stained roots were de-stained (2:1 v/v of distilled water: glycerin) for 48 hours. After de-staining, subsamples of the stained roots were placed on microscope slides, and then AMF colonization was quantified by microscopic observations at 400 x magnification (Giovannetti and Mosse 1980). The results were calculated as percentage of root length colonized (RLC) according to

$$RLC\ (\%) = (\text{Number of root intersections with mycorrhiza} / N) * 100$$

RLC = percentage of root length colonization by AMF (%), N = total number of intersections per slide which was 100.

Stomatal conductance

Stomatal conductance was always measured in the morning (from 9.30-12.00 am) using a AP4 porometer (Delta-T devices, UK). We measured stomatal conductance on the two youngest, fully emerged leaves of each rice plant, and measured at the middle part of the leaf for all selected leaves. We measured stomatal conductance of all plants at day 2, 4, 6 and 10 after applying drought. We could not measure stomatal conductance at day 8 and day 12 because it was raining and cloudy. These conditions made it difficult to measure and calibrate the AP4 porometer. In addition, stomatal conductance is sensitive to variation of environment, therefore data measured on a cloudy day cannot be compared with data measured on a clear day (Meinzer et al. 1997). In the recovery pots, we measured stomatal conductance two days after re-watering, that is day 14 after the start of the drought treatment.

Photosystem II (PSII) activity

We measured PSII activity of the plants in all pots at day 2, 4, 6, 8, 10, and 12 after the start of the drought treatment. We also measured PSII activity in the rice plants grown in the recovery pots two days after re-watering. The PSII activity was measured with a dark – light adapted chlorophyll fluorometer (MINI-PAM, WALZ, Germany). We measured PSII activity on two youngest fully emerged leaves of each rice plant. We recorded the results of minimum (F_0) and maximum (F_m) fluorescence that the plant leaf could absorb to quantify the maximum quantum yield of PSII photochemistry (F_v/F_m) (Murchie and Lawson 2013).

$$F_v/F_m = (F_m - F_0) / F_m$$

Leaf nitrogen and phosphorus concentration

Leaf N and P concentration was assessed. For the analysis, the third leaf from the apex was collected during the panicle development stage. Five leaves were collected from each pot and dried at 80 °C for 72 hrs before grinding. Due to limitations of the available plant material, we had to combine plant material from two pots of the same treatment as one composite sample. The ground plant materials were submitted to the laboratory at Khon Kaen University for N and P analysis. Leaf N was quantified by Kjeldahl method (Bremner 1965), and leaf P was analyzed by wet digestion (Nitric-perchloric digestion), Spectrophotometry (Land Development Department Thailand 2011).

Rice biomass

Rice plants were harvested when the grain was fully ripened at 107 days after transplanting. Shoot biomass was harvested at about five cm above the soil. We collected the roots by removing soil, followed by root washing in fresh water. Shoot and root material were dried at 80 °C for 48 hrs and then weighed.

We could harvest grain yield only from the well-watered plants and from the four recovery replicates of the drought treatment as the plants from the full drought treatment had died 14 days after the start of the drought treatment and did not produce grain. We harvested the grain by using scissors and took out only the panicle part from the straw. Then we separated filled and unfilled grains from the panicles and dried the grains under sunlight for 5 days before measuring dry weight of the grain.

Statistical analysis

The data were analyzed in SPSS version 20. Repeated-measures analysis of variance (ANOVA) was done to determine the effect of AMF, water treatment and rice variety on stomatal conductance and PSII activity over time. Non-homogeneously distributed data were log-transformed before analysis. We analyzed stomatal conductance and PSII activity from eight replicates for the non-recovery treatment until day 12 since the start of the experiment. To test the impacts of AMF, rice variety and their interaction on the recovery of rice upon re-watering after a drought period, the data from recovery replicates and of the controls were analyzed using three-way ANOVA (treatment factors AMF, drought, rice variety and their interactions), with four replicates of the drought treatment. The results of N concentration, P concentration, shoot dry weight, root dry weight and grain yield were analyzed from the data obtained from the control and recovery treatment.

Results

AMF colonization

AMF inoculation resulted in a significant increase of average level of root length colonized (RLC) by AMF, compared with the low-AMF treatment with only field soil inherent AMF. The RLC was 31 ± 5 % in the high-AMF treatment and 20 ± 5 % in the low-AMF treatment (fig. 3.2). Drought and rice variety did not have a significant effect on AMF colonization (table 3.1).

Stomatal conductance

The drought treatment caused a significant decrease in stomatal conductance 4 days after the last watering, with a very substantial drop between 4 (for SR1) and 6 (for KDML105) days (table 3.2; fig. 3.3). At 6 days after applying the drought treatment (DAD), stomatal conductance was more than threefold lower in rice plants subjected to drought compared with those in the well-watered treatment. The stomatal conductance of the plants in the drought treatment was around 50 mmol s-1 m-2 at 10 DAD for SR1 and 20 mmol s-1 m-2 for KDML105, close to the minimum stomatal conductance reported for rice (Duursma et al. 2019). The decline of stomatal conductance during the drought treatment occurred earlier in the more drought tolerant SR1 compared with KDML105; however, its final (minimum) values were higher in SR1 than in KDML105. At 4 DAD plants of KDML105 had higher stomatal conductance than those of SR1, but after 6 DAD and subsequent days, variety SR1 subjected to drought had significantly higher stomatal conductance than KDML105. Stomatal conductance of rice plants with high AMF colonization tended to be higher than those with low AMF colonization, yet the magnitude of this effect differed between rice varieties, with the mycorrhizal effect in alleviating decline in stomatal conductance tending to be more favorable in SR1 than in KDML105 (fig. 3.3). Repeated-measures ANOVA indicated that variety, water, AMF and the interactions variety × water and variety × AMF were significant sources of variation (table 3.4).

Rice plants that received water 12 DAD showed rapid recovery of stomatal conductance; at 14 DAD the stomatal conductance was not different from the rice plants grown in the well-watered treatments (fig. 3.5A). Stomatal conductance of the plants grown in the drought treatment without recovery could not be measured 14 DAD, because the leaves were almost completely dry.

Photosystem II activity

In plants subjected to drought PSII activity remained unchanged till 6 days DAD (table 3.3). There was a significant negative effect of drought on PSII activity after 8 days, even though differences were still very small 8 DAD and small 10 DAD. There was a very drastic decline in PSII activity in plants subjected to drought compared with the well-watered plants at 12 and 14 DAD (fig. 3.4). At 14 DAD, drought resulted in a decrease of PSII activity by approximately 75% in both rice varieties. The effect of AMF on PSII activity was significant at 14 DAD as the decrease of PSII activity due to drought was less under high AMF colonization than under low AMF colonization. The figure suggests also some possible mycorrhizal effect at 10 and 12 DAD, but the large variation between replicates, with some showing declines in PSII activity, while other did not (yet) show a decline, resulted in differences not being significant. The significant interaction effect of AMF x water treatment was due to a stronger effect of

AMF on PSII activity in the drought treatment compared with the mycorrhizal effects in rice plants grown in well-watered soil. Repeated-measures ANOVA indicated that water, AMF and the interaction water × AMF were significant sources of variation (table 3.4). After re-watering, the PSII activity at 14 DAD was not significantly different between well-watered and drought treatment (fig. 3.5B), again indicating rapid recovery. Rice variety and AMF inoculation did not affect PSII activity on 14 DAD of rice plants that were re-watered.

N and P concentrations

There were no significant effects of drought, AMF inoculation or variety, and their interactions, on leaf N and P concentrations (table 3.1). There were also no treatment effects on the N:P ratio either. The N:P ratio of all treatments was high, ranging about 19 – 23, showing P-limitation.

Shoot, root biomass, and grain yield

The variety x water interaction showed that KDML105 and SR1 responded differently to drought. Despite its claimed higher drought tolerance, shoot biomass of SR1 was reduced when subjected to drought, whereas there was no effect of drought on biomass of KDML105. High AMF colonization consistently increased shoot biomass compared with low AMF colonization (table 3.1, fig. 3.6A). Root biomass was significantly different between both rice varieties (table 3.1), with SR1 producing more root biomass than KDML105. Root biomass under well-watered conditions was higher than in the drought condition, irrespective of rice variety or AMF inoculation. Drought decreased root biomass of the rice plants by approximately 25% (fig. 3.6B). AMF did not show a significant effect on root biomass. Rice grain yield differed between rice varieties with SR1 producing significantly higher grain yield than KDML105 (fig. 3.6C). Drought significantly reduced grain yield of both varieties, but the impact of drought on yield differed between both varieties (table 3.1), with the more drought tolerant SR1 producing more grain than KDML105 under drought. Drought decreased grain yield of KDML105 by approximately 80%, and about 65 % of SR1. AMF did not affect the grain yield of the rice plants.

Table 3.1: ANOVA table testing the effects of variety, water and AMF treatment on the rice response variables RLC = root length colonization, SDW = Shoot dry weight, RDW = Root dry weight, GDW = Grain dry weight, N = Nitrogen concentration in leaves and P = Phosphorus concentration in leaves. SWD, RWD and GWD are from well-watered and drought recovery treatments, n = 4. Significant effects ($p < 0.05$) are indicated in bold.

Independent variables		RLC		SDW		RDW		GDW		N		P		N/P ratio	
	df	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value
Variety (V)	1	0.06	0.940	0.0	0.945	9.8	**0.005**	9.3	**0.006**	0.1	0.735	2.2	0.175	2.3	0.167
Water (W)	1	2.0	0.170	6.8	**0.016**	9.6	**0.005**	365.1	**0.000**	2.9	0.127	2.4	0.162	0.6	0.444
AMF (A)	1	7.9	**0.010**	7.2	**0.013**	2.0	0.171	0.8	0.366	1.3	0.292	2.4	0.162	0.11	0.752
V×W	1	0.2	0.663	5.1	**0.033**	0.1	0.773	4.8	**0.038**	0.4	0.535	1.4	0.271	0.1	0.824
V×A	1	0.8	0.775	0.2	0.683	0.4	0.540	0.1	0.716	0.1	0.781	1.7	0.235	0.2	0.675
W×A	1	0.9	0.363	0.0	0.945	0.0	0.934	0.1	0.712	0.0	1.000	0.0	0.921	0.0	0.969
V×W×A	1	0.9	0.363	0.0	0.889	0.7	0.397	0.7	0.419	0.2	0.689	0.0	0.960	0.2	0.654

Table 3.2: ANOVA table; Stomatal conductance of rice plants in response to varieties, water and AMF treatments. D-x = x days after applying drought, and R = recovery treatment. From D-2 to D- 10 n = 8, and D-14R n = 4. Significant effects ($p < 0.05$) are indicated in bold.

		D-2		D-4		D-6		D-10		D-14-R	
	Df	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value
Varieties (V)	1	0.0	0.836	1.3	0.264	14.2	**0.001**	1.5	0.232	0.0	0.958
Water (W)	1	0.3	0.595	35.5	**0.000**	356.1	**0.000**	399.6	**0.000**	0.8	0.384
AMF (A)	1	0.0	0.992	3.9	0.060	0.6	0.447	0.3	0.563	0.9	0.349
V×W	1	0.6	0.433	12.2	**0.002**	11.6	**0.002**	0.0	0.876	0.1	0.697
V×A	1	0.3	0.602	0.8	0.368	0.1	0.808	0.3	0.599	1.1	0.303
W×A	1	1.0	0.338	0.7	0.406	0.8	0.380	0.0	0.976	0.2	0.627
V×W×A	1	0.0	0.984	0.0	0.906	0.8	0.380	0.1	0.761	0.0	0.857

Table 3.3: The photosystem II activity of rice plants of different varieties in response to varieties, water and AMF treatments. D-x = x days after applying drought, NR = non recovery treatment, and R = recovery treatment. From D-2 to D- 10 n = 8, for D-x-NR and D-x-R n = 4. Significant effects ($p < 0.05$) are indicated in bold.

		D-2		D-4		D-6		D-8		D-10		D-12-NR		D-14 -NR		D-12-R		D-14-R	
	df	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value
Variety (V)	1	1.9	0.174	1.2	0.278	2.6	0.116	1.4	0.242	0.0	1.000	0.3	0.600	0.3	0.599	2.4	0.134	0.0	1.000
Water (W)	1	0.1	0.732	0.8	0.385	1.6	0.219	5.6	**0.021**	16.6	**<0.001**	24.4	**<0.001**	939.3	**<0.001**	12.0	**0.002**	0.7	0.408
AMF (A)	1	0.1	0.732	0.0	0.827	0.5	0.480	1.0	0.315	1.6	0.213	1.2	0.277	9.6	**0.005**	0.1	0.715	0.3	0.579
V×W	1	1.1	0.306	2.3	0.131	1.6	0.219	0.7	0.402	0.0	0.962	0.3	0.600	0.1	0.734	2.4	0.134	0.1	0.781
V×A	1	1.1	0.306	0.2	0.663	2.0	0.161	1.4	0.242	0.1	0.810	0.6	0.448	1.3	0.258	0.1	0.715	0.0	1.000
W×A	1	0.5	0.494	0.4	0.514	2.0	0.161	0.0	1.000	1.2	0.270	1.3	0.261	9.2	**0.006**	0.1	0.715	0.7	0.408
V×W×A	1	0.0	1.000	0.2	0.663	1.1	0.291	1.4	0.242	0.1	0.772	0.6	0.448	1.8	0.191	0.1	0.715	2.0	0.173

Table 3.4: The repeated measure analysis of the stomatal conductance and photosystem II activity and over the first 10 days of drought, n = 8. Significant effects ($p < 0.05$) are indicated in bold.

		Stomal conductance		Photosystem II activity	
Treatment	df	F	P-value	F	P-value
Varieties (V)	1	17.1	**<0.001**	0.1	0.916
Water (W)	1	1725.7	**<0.001**	154.6	**<0.001**
AMF (A)	1	8.4	**0.005**	5.6	**0.026**
V×W	1	18.2	**<0.001**	0.0	0.844
V×A	1	4.8	**0.032**	1.2	0.282
W×A	1	0.6	0.437	4.5	**0.044**
V×W×A	1	0.4	0.528	1.4	0.245

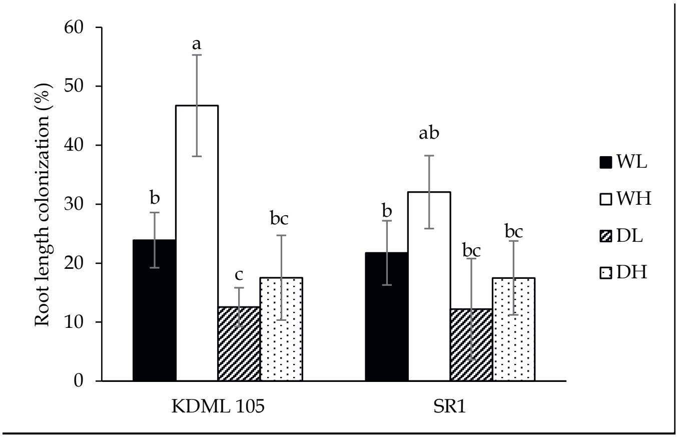


Figure 3.2: AMF root length colonization (RLC) of KDML105 and SR1 variety rice plants. WL = well-watered with low AMF, WH = well-watered with high AMF, DL = drought with low AMF, DH = drought with high AMF; bars are means ± 1 SE, n = 8. Bars with completely different letters indicate significant differences between treatments (P-value < 0.05)

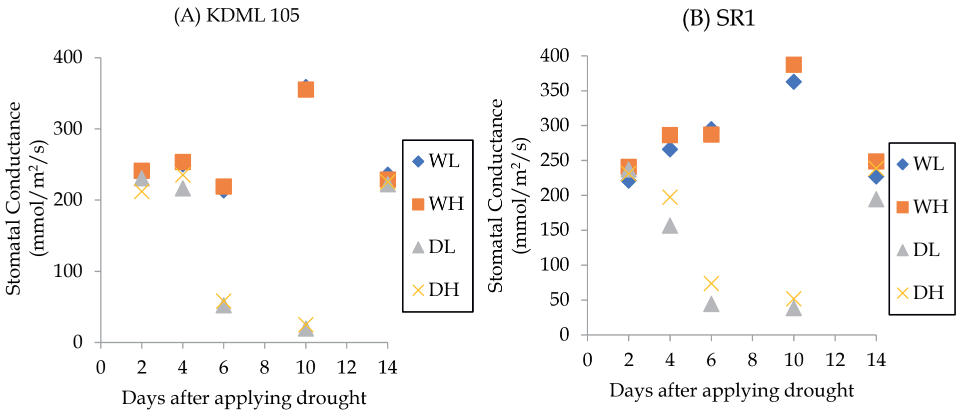


Figure 3.3: Stomatal conductance of KDML105 (A) and SR1 (B) rice plants over time. WL = well-watered with low AMF, WH = well-watered with high AMF, DL = drought with low AMF and DH = drought with high AMF. Symbols indicate the mean value across 8 replicates for day 0-12, and 4 replicates from recovery treatment for day 14.

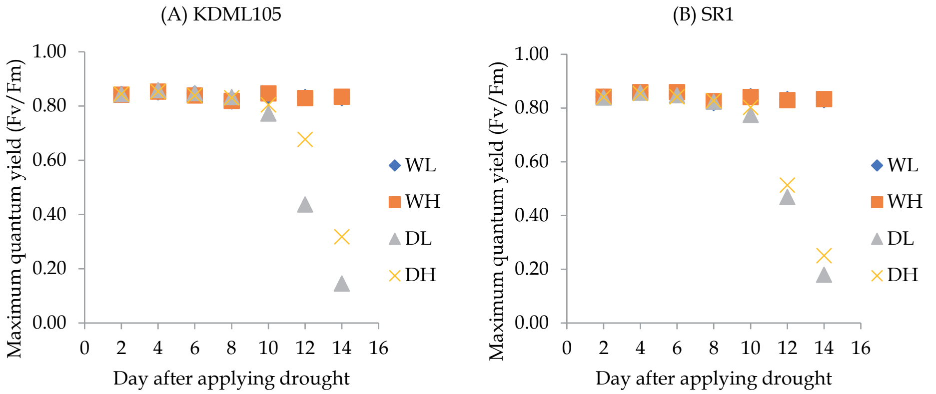


Figure 3.4: The photosystem II activity of KDML105 (A) and SR1 (B) over time. WL = well-watered with low AMF, WH = well-watered with high AMF, DL = drought with low AMF and DH = drought with high AMF. Symbols indicate the mean value across 8 replicates.

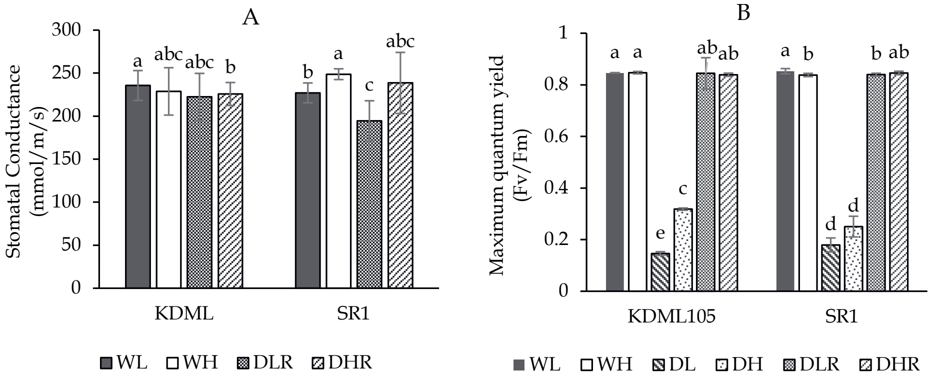


Figure 3.5: The drought recovery of stomatal conductance (A) and photosystem II activity (B) at 14 DAD for both rice varieties KDML105 and SR1. Bars are means ± 1SE, n = 4. The stomatal conductance of the plants subjected to drought was not measured for DL and DH treatment and only for the rewatered plants, because the rice plants collapsed due to drought stress. WL = well-watered with low AMF, WH = well-watered with high AMF, DL = drought with low AMF and DH = drought with high AMF, DLR = drought with low AMF+rewater for recovery and DHR = drought with high AMF+rewater for recovery. Bars with completely different letters indicate significant differences between treatments (P-value < 0.05)

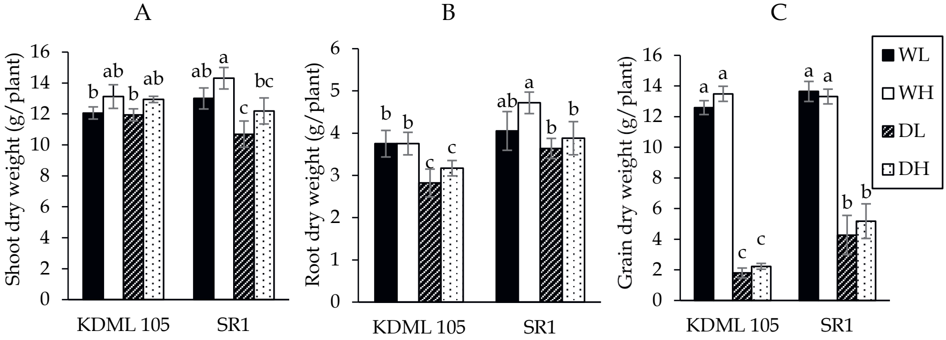


Figure 3.6: The shoot (A), root (B) and grain yield (C) dry weight of KDML105 and SR1 rice plants in relation to drought and AMF inoculation. WL = well-watered with low AMF, WH = well-watered with high AMF, DL = drought with low AMF and DH = drought with high AMF. Bars are means + 1SE, n = 4. Bars with completely different letters indicate significant differences between treatments (P-value < 0.05)

Discussion

As expected, inoculation of AMF in non-sterile field soil increased AMF colonization in rice roots, therefore supporting the expectation of inoculum limitation in rice fields in our study area. Increasing mycorrhizal colonization due to inoculum addition had a positive effect in alleviating the negative effects of drought on stomatal conductance and most notably on PSII activity. Rice plants when subjected to drought decreased stomatal conductance as an early response and decreased PSII activity as a response that started later. The magnitude of the responses depended on AMF colonization level. A higher AMF colonization resulted in a weaker or delayed decline in stomatal conductance and PSII activity compared with the low-colonization treatment. High AMF colonization increased shoot biomass but did not affect grain yield or N and P concentration in leaves. Our results show a contribution of AMF to drought tolerance of rice but not to recovery from drought. This may in part be attributed to the timing of the recovery, as the window of opportunity for recovery after a severe decline of PSII activity was small. Whereas plants recovered if they were watered again 12 DAD, they had all collapsed 14 DAD. It requires further experiments to verify the potential role of drought intensity for the impact of AMF on drought recovery, especially in conditions where the soil water potential is not restored as rapidly as in our study.

AMF colonization

The roots of rice plants from all treatments were colonized by AMF, and AMF inoculation significantly increased colonization. Therefore, our AMF inoculation was a successful treatment and significant mycorrhizal effects on stomatal conductance and PSII activity can be caused by variation in mycorrhizal root colonization. Drought did not affect the levels of AMF colonization. Earlier studies have found decreases as well as increases in AMF root colonization when subjected to drought and it is not clear why these differential responses occur. Vallino et al. (2014) found an increase of mycorrhizal colonization of rice under drought, but their study compared flooding with potential anoxic conditions with a treatment without flooding. Ryan and Ash (1996) reported a decrease of colonization in wheat roots from 40-70% to 5-16% when the soil was subjected to drought. One explanation may be that the response of mycorrhizal colonization of rice to drought is unimodal, with strong effects both at the very wet (anoxic, due to flooding) and very dry end of the moisture gradient, and minor responses at less extreme conditions. Mycorrhizal colonization was measured at the end of the experiments, that is after the pants recovered from drought, and this late assessment could also underestimate effects. Our results are like findings in our previous pot experiment, where no effect of drought on AMF root colonization of rice was observed (Chareesri et al. 2020).

Stomatal conductance

Stomatal closure started 4-6 DAD, supporting earlier studies that stomatal closure exhibits a fast response to drought (Bartlett et al. 2016; Trueba et al. 2019). When plants take up less water, and stem and leaf water potentials decline, they close their stomata to prevent the loss of water via transpiration, which also reduces the stomatal conductance. In our study, drought reduced stomatal conductance significantly 4 DAD or at soil potential - 50 kPa. This reduction is comparable to our previous study (Chareesri et al. 2020), where we found a decrease of stomatal conductance when soil water potential was reduced to below - 40kPa.

Increased colonization by AMF alleviated this negative effect of the drought treatment, and plants with higher levels of colonization retained higher levels of stomatal conductance. The high AMF colonization treatment tended to have higher stomatal conductance at 4 DAD. This mycorrhizal effect was more pronounced in the more drought tolerant SR1 than in KDML105. Our results are in line with previous studies by Augé et al. (2015), and Ruiz-Lozano et al. (2016) that found higher stomatal conductance in plants inoculated with AMF under drought. In our study stomatal closure occurred earlier in the drought-tolerant rice variety SR1 than in the somewhat more less drought tolerant KDML105 (after 4 and 6 days respectively), as indicated by the significant variety effect at 6 DAD, and variety × water treatment interaction at 4 and 6 DAD. Possibly early stomatal closure after sensing that the soil is drying out is part of the adaptive response of the more drought-tolerant variety. However, SR1 maintained higher levels of stomatal conductance at the end of the drought treatment

(50 mmol $s^{-1}m^{-2}$ for SR1, 20 mmol $s^{-1}m^{-2}$ for KDML105), suggesting less complete stomatal closure during drought. We cannot explain why SR1 did not fully close its stomata, but one possibility is that low levels of transpiration contribute to transpiration cooling that could delay damage to the photosystem. The results are in contrast with our previous pot experiment (Chareesri et al. 2020) that showed no significant varietal differences in stomatal conductance of SR1 and KDML105 variety at - 40 kPa soil water potential. In this experiment, variety effects on stomatal conductance were significant at 6 DAD when soil water potential was around - 70 kPa.

Stomatal conductance could show potential rapid recovery after the termination of the drought treatment. In our experiment, stomatal conductance increased rapidly after the soil was re-watered. Unfortunately, due to unfavorable weather conditions on 12 DAD we could not more accurately determine the recovery rate for stomatal conductance.

Photosystem II activity

In contrast to stomatal conductance, PSII activity responded more slowly after the onset of drought, with significant differences between both water treatments being noted only from 8 DAD onwards. At that time differences with the well-watered treatment were significant, even though they were still quite small. This differential temporal response, compared with stomatal conductance, is in line with other studies that measured both stomatal conductance and PSII activity over time during drought (Trueba et al. 2019). After re-watering, PSII activity also recovered and achieved pre-drought values, suggesting that our drought treatment did not result in irreversible damage to PSII. Our finding that PSII activity in rice decreased under drought is in line with previous studies (Cha-um et al. 2010; Sikuku et al. 2010). In our previous study (Chareesri et al. 2020), PSII activity of KDML105 and SR1 did not significantly decrease after four days of drought and declined shortly after, yet in that study we did not extend the drought period as long as in the current study. A 14-day drought reduced PSII activity of Homnil rice plants more than 50% (Yooyongwech et al. 2016).

From the results, higher PSII activity in rice plants with high AMF colonization under drought showed the contribution of AMF in retaining photosynthesis activity by maintaining high quantum yield. The ability of AMF to mitigate effects of drought, through enhanced water and nutrient acquisition and / or changes in the hormonal status of the plant have been discussed previously (Kuyper et al. 2021; Kuyper and Jansa 2023). The beneficial effect of AMF on PSII activity was also found before (Ruiz-Sánchez et al. 2010; de Andrade et al. 2015). However, these studies compared plants with and without AMF colonization, whereas our study focused on plants with different levels of colonization, again suggesting that under realistic field conditions, where AMF are (almost) always present, though sometimes in limiting amounts, increases in AMF inoculum potential might have benefits. There was no difference between the rice varieties in PSII activity, in contrast with varietal effects on stomatal conductance during the onset of drought.

Nutrients (N, P)

N concentration of the rice leaves remained unchanged in rice plants after the drought treatment compared with rice plants in the well-watered treatment. However, total N acquisition must have been substantially higher under well-watered treatments because of treatment effects on biomass (especially of SR1) and large drought effects on grain yield (and hence grain N content). The lack of effects of drought on leaf N concentration may be due to differences in reallocation of N between the well-watered plants and the plants subjected to drought. More N might have been allocated from the leaves to the strong nutrient sink formed by the panicles and grain in well-watered treatments, whereas in the drought treatment there were fewer grains so that N may have remained mostly in the leaves. In rice N allocation under well-watered conditions has been summarized previously (Fageria 2007), where more than 70% of N was allocated from shoot to grain during the reproductive stage. In addition, in our experiment N was not limiting in all treatments, based on the high N:P ratio which was (much) higher than 14, indicating P limitation (Koerselman and Meuleman 1996). Therefore, drought could have limited P-uptake, resulting in smaller plants, while the rice plants downregulated N uptake to maintain a favorable N:P balance. The absence of a significant effect of AMF on N concentration might furthermore be because plants also benefitted from AMF for N uptake even with low colonization.

Similar to the results of N concentration, the P concentration of the rice plants in the drought treatment did not decrease. Usually, P becomes limiting under water limitation, resulting in low P uptake by plants (Suriyagoda et al. 2014; Sardans and Peñuelas 2004). However, a meta-analysis stated that drought experiments with drying-rewetting cycle (type III) did not have a negative effect on P concentration (He and Dijkstra 2014).

Several studies have reported the benefits of AMF on uptake under P deficiency conditions (Smith and Read 2010; Ruiz-Lozano and Aroca 2010), and in environmental stress conditions (Zhang et al. 2018; Zhang et al. 2016). Most studies have been conducted by comparing non-AMF and AMF plants, which might show a more pronounced effect of AMF on P concentrations. Our experiment compared low and high AMF colonization, which might not have significant effects on P concentrations because all plants benefitted from AMF. However, the beneficial effect of inoculum addition on plant biomass implies that inoculum addition improved total P uptake. Benefits of AMF inoculum may not be significant in case soil P is high or indigenous inoculum potential is adequate (Lekberg and Koide 2005).

Shoot and root biomass, and grain yield

As expected, shoot and root biomass were lower of plants subjected to drought compared with the well-watered condition. The negative effect of drought on shoot biomass was only noted for SR1 but not for KDML105, while both varieties were equally negatively affected for root biomass. At first sight, the differential effect on shoot biomass might suggest a higher tolerance to drought for KDML105 than for SR1, contrary to the literature. The lower levels of stomatal conductance under drought for KDML105 than for SR1 also suggest that the former variety is better able to reduce water losses. However, differential shifts in the root:shoot ratio of both varieties under drought and the higher grain yield under drought for SR1 than for KDML105 would not support that conclusion. Under drought, AMF hyphae may play a role in foraging for nutrients (Augé 2001; Ruiz-Lozano and Aroca 2010). The hyphae can extend to the areas that are not accessible to roots (Smith and Read 2010). This AMF benefit could have resulted in an increasing shoot biomass. The negative effect of drought on shoot biomass of SR1 and the absence of such an effect for KDML105 (as shown by the significant water × variety interaction in table 3.1) and a somewhat smaller negative effect of drought on root biomass of SR1 compared with KDML105 could suggest a stronger plastic response on the functional equilibrium in SR1 that contributes to its higher tolerance to drought.

Under optimal conditions KDML105 and SR1 produce different grain yields, with SR1 yielding more than KDML105 as reported from rice fields in Thailand (Bureau of Rice Research and Development, Thailand accessed on 28th June 2020). In our experiment under well-watered conditions, the productivity of SR1 was similar to KDML105. However, there was a significant difference in productivity under drought (as shown by the significant variety × water interaction), where SR1 produced almost double amount of grain yield compared with KDML105. Whereas AMF stimulated shoot biomass, this did not result in increased grain yield. Grain yield was severely negatively affected by drought, even though the plants after 12 days of drought were allowed to fully recover and achieved the same shoot and root biomass. Apparently, the period that we applied drought (42 DAP) was a very sensitive period for flowering, fertilization, and seed formation. Daryanto et al. (2017) stated that yield reductions in maize and rice can be very substantial when drought occurs during flowering.

Differential rice sensitivity to drought during the various growth stages was investigated by Sarvestani et al. (2008), who reported drought-induced grain yield reductions of 21%, 50% and 21% respectively, when water deficit occurred during vegetative, flowering and grain-filling stage. Experiments in which the timing of drought is investigated are therefore important to provide a better understanding of the recovery of photosynthesis after drought and the effects of drought on grain production. Other studies equally reported a significant reduction of rice grain yield under severe drought (Ghosh and Singh 2010; Venuprasard et al. 2007). Consistent with its reported higher drought tolerance, SR1 suffered somewhat less from drought than KDML105. The SR1 plants closed their stomata earlier than KDML105 (decline in stomatal conductance noted 4 and 6 DAD respectively) but exhibited higher stomatal conductance during drought. Lauteri et al. (2014) established a very significant correlation between stomatal conductance and grain yield, and an even higher correlation between mesophyll conductance and grain yield in rice. These mechanistic links between stomatal behavior, both during drought and during subsequent recovery, and final grain yield demand further study.

Conclusion

The growth of rice plants is limited by drought, which affects its photosynthetic performance. Stomatal conductance and PSII activity showed different responses to drought with respect to the onset of the effect and possibly its subsequent recovery after the termination of severe drought. Stomatal conductance showed a relatively rapid response and also showed significant differences between the rice varieties, whereas PSII activity responded more slowly and did not show varietal effects. Both parameters for photosynthetic performance exhibited fast recovery upon re-watering the soil. Plants with a higher degree of AMF colonization showed a lower reduction in stomatal conductance and a lower decline in PSII activity in response to drought. While possibly the levels of colonization of the plants without inoculum addition were already enough to provide some benefits to the rice plants in terms of drought tolerance, the data confirm that mycorrhizal management, resulting in plants with higher levels of mycorrhizal colonization, can result in further mitigation of drought impacts. This mitigation may translate in an enhanced window of opportunity in coping with drought, contributing to higher survival till the next rains come. This extended window of opportunity, even if small as our data suggest, could still be increasingly important with current human-induced climate change

Chapter 4

Challenges of field inoculation with arbuscular mycorrhizal fungi to mitigate drought effects on rice performance

Anupol Chareesri, Gerlinde B. De Deyn, Thomas W. Kuyper

Abstract

Insufficient rainfall causes drought stress, which is one of the major constraints for rice productivity in rain-fed cultivation systems. Better water management, for instance through investing in irrigation facilities, involves high costs for the farmers and may therefore regularly be prohibitive. The use of drought-tolerant varieties may be limited by the fact that currently known varieties still remain sensitive to drought. An alternative measure to reduce the negative impact of drought is to use beneficial soil microbes. Arbuscular mycorrhizal fungi (AMF) improve plant nutrient uptake and photosynthesis, and increase plant growth under various forms of abiotic and biotic stress. Benefits of the mycorrhizal symbiosis are often larger under dry than under well-watered conditions. Earlier pot experiments have demonstrated benefits of AMF on rice growth under drought, but the effects have less frequently been studied in the field. We conducted a factorial field experiment with three rice varieties that differed in their reported drought tolerance to compare the effects of field inoculum and inoculum addition to increase AMF colonization in plots that were well-watered or subjected to drought. For logistic reasons the well-watered and drought treatment could not be replicated, and for that reason the factors mycorrhiza and rice variety were tested independently in both plots which each contained 24 subplots. Drought significantly reduced the biomass of rice plants and grain yield, across all varieties and mycorrhizal treatments. Inoculum addition significantly increased mycorrhizal colonization in the drought plot, but not in the well-watered plot. However, the increase in colonization did not translate into a significant effect on rice performance. Different rice varieties responded differently to drought in terms of P uptake, number of grains per panicle, and percentage of filled grain; however, these differences could not be related to their described drought tolerance. The complications of executing field experiments in which water levels are manipulated are discussed. Legacy effects of previous field management practices, resulting in a relatively high mycorrhizal inoculum potential even without inoculum addition could be a further reason why this study did not show a significant mycorrhizal effect on rice performance and grain yield.

Key words: *arbuscular mycorrhizal fungi, rice, field inoculation, drought*

Introduction

Rice (*Oryza sativa* L.) is the main food crop in Thailand. It is mostly grown under rain-fed conditions, where water availability is strongly dependent on rainfall. Rice is tolerant to flooding conditions, but not to drought. Drought significantly reduces the growth of rice via reducing water and soil nutrient availability (Mumtaz et al. 2020). Drought at the vegetative stage reduces the development of tillers, resulting in lower shoot biomass. Drought during panicle development affects yield components such as number of panicles, number of grains and grain filling, which together lead to a considerable reduction in grain yield (Kumar et al. 2020; Suriyagoda et al. 2014).

Global climatic change will likely affect water availability for rice production, both as a consequence of higher temperatures and as a consequence of changes in precipitation. For Thailand, Prabnakorn et al. (2018) noted that past climatic change (1984-2013) had only a limited impact on rice yields (3% loss per decade), future trends of climate change suggest more serious yield losses in the future. Climate change scenarios for Thailand show large uncertainties. A study commissioned by the World Bank and Asia Development Bank (2021), based on a modelling study by Naumann et al. (2018) indicated that Thailand is less likely to experience extreme increases in drought intensity compared with countries in West and Central Asia. Nevertheless, more prolonged drought periods could be expected. A study by Shrestha (2014) suggested increases in precipitation in the order of 15-40%, dependent on the specific climate change scenario. The study by Kiguchi et al. (2021) also indicated intensification of heavy rainfall events over Thailand and hence an increased flooding risk. A more recent modelling study to predict extreme precipitation in the period 2020-2029 indicated increased likelihood for such events in eastern Thailand, but reduced likelihoods for northern Thailand, whereas the number of rainy days was predicted to decline all over Thailand. The number of consecutive wet days was also predicted to decline over Thailand (Amnuaylojaroen 2021).

Most scenarios therefore imply both increases in extreme precipitation events, leading to flooding, and periods of drought which could hamper the productivity of upland rice. Babel et al. (2011) predicted an 18% decline in grain yield for the 2020s compared with the period 1997-2006. A future projection (for the 2080s) of yields of Thai jasmine rice (variety KDML105) suggested declines in the order of 35-40% (Shrestha et al. 2017) and an even more pessimistic estimate of a yield decline of 45% in the 2080s was published by Chun et al. (2016). These large negative effects are due to higher temperatures and especially higher night temperatures which negatively impact flowering and subsequent seed development. As country-wide or province-wide studies mostly focus on paddy rice where heath stress is more likely to occur than drought stress, the impact of drought on rice performance, which could have a major impact especially on rain-fed upland rice, has received less attention.

Although rice plants are vulnerable to drought, they possess adaptive mechanisms to maintain growth and productivity under not too severe drought conditions (Fukai and Cooper 1995). Under drought, plants respond by shifting the functional equilibrium through producing more roots to obtain more available water (Comas et al. 2013). Plants will produce more abscisic acid (ABA) when exposed to drought (Haider et al. 2018), which stimulates stomatal closure (Hasegawa and Yoshida 1982; Dash et al. 2018) and thereby reduces the loss of water. However, stomatal closure has a trade-off as it reduces photosynthetic activity (Lauteri et al. 2014).

Researchers have investigated various ways to improve drought tolerance of rice. One of the major methods is to breed drought-tolerant varieties (Nahar et al. 2018). At genetic level, quantitative trait loci (QTLs) for drought tolerance have been studied and used in molecular breeding, for instance, *qDTY2.1* (Venuprasad et al. 2009) for enhancing grain yield under drought, and *qDTY12.1* (Bernier et al. 2007) for improving lateral-root formation. From a physiological perspective, drought-tolerant varieties possess a better root morphology with increased root length or a deeper root system (Yoshida and Hasegawa 1982; Comas et al. 2013), a higher leaf area index, and flag leaf area (Mishra and Panda 2017; Farooq et al. 2009).

Plants can also establish mutualistic symbioses with beneficial soil micro-organisms that confer drought tolerance. Both the use of root endophytic fungi (Redman et al. 2011; Mbodj et al. 2018; Andreo Jimenez et al. 2019; Mathur and Roy 2021) and arbuscular mycorrhizal fungi (AMF) (Ruiz-Sánchez et al. 2010; Chareesri et al. 2020; Das et al. 2021; Etesami et al. 2022) have been suggested as ways of conferring drought tolerance to rice. Rice is a mycorrhizal plant, although it is mostly grown under flooding conditions that create unfavorable environments for AMF due to anoxia. Nevertheless, several studies have reported the presence of AMF in rice fields (Watanarojanaporn et al. 2013; Vallino et al. 2014) and temporally drier soil conditions as during a drought can promote AMF establishment in rice (Chareesri et al. 2020). Various mechanisms contribute to enhanced drought tolerance conferred by the mycorrhizal symbiosis (Kuyper et al. 2021; Kuyper and Jansa 2023). While there is not much evidence that AMF increase water uptake and transport to the plant, because of physical constraints of transport of water through small hyphae, they can improve nitrogen (N), phosphorus (P), and potassium (K) uptake and plants with a better nutritional status are generally more tolerant of drought. AMF can additionally regulate rice plant hormones, with cascading effects on stomatal conductance and photosynthetic activities under drought (Ruiz-Lozano et al. 2016; Chareesri et al. 2020). These benefits of being mycorrhizal could reduce yield losses when rice plant is exposed to drought. Benefits of the AMF symbiosis could be achieved through either including the mycorrhizal symbiosis in rice breeding programs (Huang et al. 2022; Mitra et al. 2023) or by increasing AMF abundance, through inoculation or judicious plant and soil management.

Genetic variation within species in the benefits derived from mycorrhiza have been reported for many wild plant species and crops (Kuyper et al. 2021; Stahlhut et al. 2023). Campo et al. (2020) investigated 12 rice varieties, with 11 of European origin and one from Taiwan, in the non-mycorrhizal condition and when mycorrhizal with *Funneliformis mosseae* or *Rhizophagus intraradices*. They observed positive, neutral or negative effects on plant height due to inoculation. They also noted fungal species-specific effects, with *Funneliformis* generally being more beneficial than *Rhizophagus*.

Effects on grain yield were only investigated for two cultivars and only *Rhizophagus* was used as the source of mycorrhizal inoculum. Both cultivars showed higher grain yield when mycorrhizal than when non-mycorrhizal. No information was provided whether these varieties differ in drought tolerance.

While the benefits of AMF on drought tolerance of rice have been mainly demonstrated in pot experiments under controlled conditions, there are only a few field studies on mycorrhizal rice. Zhang et al. (2015) inoculated paddy rice with a AMF strain of *Funneliformis mosseae* and observed increased mycorrhizal root colonization from 2.5% in non-inoculated rice to 12-20% after inoculation. Furthermore, inoculation reduced root biomass, while it had no effect on shoot biomass. Inoculated rice plants showed increased N and P uptake, shoot N and P content and grain yield, with the largest mycorrhizal effects under conditions of lowest nutrient supply. A study on upland rice in Madagascar (Rakotoarivelo Njaramanana et al. 2022) showed that inoculation with *Rhizophagus irregularis* increased grain yield with 28% in the absence of P fertilizer. Addition of 9 kg P ha^{-1} as mineral fertilizer resulted in substantially higher yield increases (+85%), but a combination of P fertilizer and inoculation showed no additional benefits. These studies demonstrate the challenge of matching beneficial effects of AMF with the farmers' fertilization management.

Usually, mycorrhizal benefits in field experiments are lower than those in laboratory experiments under more controlled conditions. A meta-analysis by Zhang et al. (2019) showed that the beneficial effect of AMF on cereal grain yield (of seven cereal species) was significantly higher in lab studies than in field studies. For rice Zhang et al. (2019) reported a beneficial mycorrhizal effect on grain yield (+17%) and a larger effect was also observed for lab studies than for field studies, however due to large variability and relatively small sample size, the difference was not significant. Two main factors explain the discrepancy between field and laboratory studies. Usually field studies exhibit larger variability, as the researcher cannot easily control for environmental variation due to unexpected weather events and for site heterogeneity, and increased variability together with limitations in manageable numbers of replicates reduces statistical power. Moreover, often laboratory studies compared the effect between non-mycorrhizal and mycorrhizal plants, however, in the field rice is normally mycorrhizal, even if levels of root colonization are low. It is likely that also a low colonization has (some) beneficial effects and laboratory studies therefore tend to overestimate effects sizes from an agronomic perspective. Few studies have investigated the benefits of increasing mycorrhizal colonization for the plant, based on comparisons of effects of plants with low and high mycorrhizal colonization.

In mycorrhizal studies effects of inoculation are usually assessed on the basis of performance parameters such as aboveground or belowground biomass or yield. Additionally measuring physiological parameters during the growth period but before the final harvest can provide additional insights in mycorrhizal functioning. For mycorrhizal functioning under drought both stomatal conductance and the efficiency of photosystem II, as assessed through chlorophyll fluorescence, have proven to be good indicators. Augé et al. (2015) concluded that AMF ameliorate stomatal conductance with a larger positive effect under drought than under well-watered conditions. A meta-analysis by Wang et al. (2019) on the effect of salinity, which causes physiological drought, on the efficiency of photosystem II indicated a beneficial effect of the AMF symbiosis, with a significantly larger effect for monocotyledonous plants (like rice) than for dicotyledonous plants. For rice that was exposed to drought, Chareesri et al. (2020) reported higher values of stomatal conductance and efficiency of PSII under conditions of higher mycorrhizal colonization compared with lower colonization. In addition, considering rice genetic variation in drought tolerance, it is relevant to study the effects of AMF inoculum addition to rice varieties with reported differential drought tolerance under well-watered and drought conditions in the field. The application of AMF and its benefits on improving drought tolerant of rice in field trial is still one of the main knowledge gaps, therefore we hypothesized that:

(i) Field-grown plants with AMF inoculation will have higher AMF colonization than plants without AMF inoculation.

(ii) Plants with higher AMF colonization will have higher stomatal conductance and efficiency of photosystem II than plants with low AMF colonization.

(iii) Plants with higher AMF colonization will have higher N and P uptake, and higher plant biomass (shoot, root, and grain yield) than plants with low AMF colonization.

(iv) Inoculum addition will increase mycorrhizal colonization more under drought than under well-watered conditions.

(v) Inoculum addition will have a larger beneficial effect under drought than under well-watered conditions.

(vi) Rice varieties that are more drought tolerant will benefit more from inoculum addition than rice varieties that are (somewhat) less drought tolerant, especially under drought.

Materials and methods

Experimental setup

The field experiment was conducted from November 2018 – March 2019 in Ban Fang district (6°29'10.9"N 102°34'40.5"E), Khon Kaen province, Thailand. We used a split-plot design by having water treatments as the main plot (but unreplicated), while the subplots (within the well-watered and drought conditions) comprised two AMF treatments and three rice variety treatments in a factorial design. Each treatment had four replicates, arranged in blocks in a complete randomized block design (CRBD), which made a total of 24 subplots in both the well-watered and drought treatment. The two main plots were separated by a ridge of 30 cm height. The size of the subplots was 2 m x 2 m, and the distance between the subplots was 1.5 m. Distance between rice plants within a subplot was about 25 cm. A subplot had 9 rows with 9 plants per row, therefore each subplot contained 81 rice plants.

Soil properties and land preparation

The soil at the field was a sandy loam soil containing 60% sand, 31% silt, 8% clay and 1% organic matter. The pH of the soil was 7.5. Bulk density was 1.29 g cm^{-3} with 0.52%

porosity and 25.5% water holding capacity. Prior to the field experiment, the field was used for rice and maize cultivation with intensive use of synthetic fertilizer.

Rice plants

We planted three non-photosensitive rice varieties, viz. Chainart 1 (CNT1), RD22, and RD33. All three rice varieties are commonly grown by local farmers during the dry season. The three varieties had also been used in a pot experiment in 2018 for studying the benefit of AMF on rice under drought (Chareesri et al. 2020). CNT1 is reportedly a drought-tolerant variety (Sawatdikarn and Kansomtob 2012). RD22 is responsive to N fertilizer, but we were unable to find reports on its drought tolerance. As it is suggested for growth in irrigated areas, we assume its drought tolerance is somewhat less than CNT1. RD33 is stated to be least tolerant to drought (Anugoolprasert 2016). However, the pot experiments result of Chareesri et al. (2020) showed that CNT1 is more affected by drought than RD33 and RD22 in terms of grain yield reduction. The average yield potential under well-watered condition is 4500 kg ha^{-1} for CNT1, 4300 kg ha^{-1} for RD22, and 3100 kg ha^{-1} for RD33 (Thai Rice Department 2016). We received the rice seeds from Sakonnakorn Rice Research Center, Thailand. Seeds were untreated with any fungicide of insecticide.

We soaked the rice seeds overnight to stimulate germination. Then we sowed the rice seeds in square trays that were filled with soil from the experimental field where this study was executed. We grew the seedlings for 15 days before transplanting, provided them with adequate watering, and did not apply fertilizer. We transplanted individual seedlings by pulling them from the tray, and then planted them in holes that we had have made in each subplot. Each planting hole contained only one rice seedling.

Water treatments

The water treatments included a well-watered and drought treatment. The experiment was done in dry season, so we used a water pump to pump water from the shallow well to the field when needed to maintain the well-watered condition. For logistic reasons we could not replicate the water treatments. All plots were well-watered during the first 14 days after transplanting (DAT). The drought treatment was applied 15 DAT, by draining all water and by not watering for 14 days, simulating an early-season dry spell. All the subplots in the well-watered treatment plot received water every 5 days. Re-watering was done in repeated cycles by pumping in the water and flooding the field at five cm above soil surface. The drought treatment cycle was carried out until the grain filling stage which is around 69 DAT. After that, all the subplots in the drought treatment plot were watered similar to the subplots in the well-watered treatment plot. During the experiment, there were rainy days, which caused the drought treatment to be incomplete. After rainfall showers, we drained the water and restarted the drought cycle in the subplots of the drought treatment plot.

Arbuscular mycorrhizal fungi

To increase inoculum potential of AMF we added to the rice plants a commercial granule inoculum (RootGrow Professional, Kent, UK) which contained *Claroideoglomus etunicatum*, *Funneliformis mosseae*, *F. geosporus*, *Glomus microaggregatum* and *Rhizophagus irregularis*. The same inoculum was used in the pot experiment by Chareesri et al. (2020). We added the inoculum to the high AMF treatment at the moment that we planted the rice seedlings in the field by making holes in the soil and putting the inoculum into the hole. Then we transplanted the rice seedling in the hole, we tried to ensure good root contact with the inoculum as much as possible. We added 10 g of inoculum in every planting hole of high AMF treatment. The field was inoculated with the same AMF inoculum once in a pilot experiment in 2016 before the experiment, where we applied 24 g of inoculum per 1 m^2.

Measurements

AMF colonization

After harvesting, the roots were collected and dried at 80 °C for 48 hours. The we took 10% of the root sample based on root dry weight. Roots were cut into small fragments of about 2 cm long. We placed the roots in plastic tissue cassettes. Roots were stained by clearing in boiled 10% KOH solution at 90 °C for 15 minutes, then rinsed, and left

in 1% HCl solution for 30 minutes (INVAM 2014). Finally, we stained the root by leaving the roots in 0.05% Trypan blue solution over 24 hours (500 ml glycerin: 450 ml distilled water: 50 ml of 1% HCl and 0.5 g Trypan blue). The stained roots were de-stained in the de-staining solution (2:1 v/v of distilled water: glycerin) for 48 hours. After de-staining, subsamples of the stained roots were placed on microscope slides, and then AMF colonization was quantified by microscopic observations at 400 x magnification according to Giovannetti and Mosse (1980). The results were calculated as percentage of root length colonized (RLC) according to
RLC (%) = (Number of root intersections with mycorrhiza / N) *100

RLC = percentage of root length colonization by AMF (%), N = total number of intersections per slide.

Shoot and root biomass, and grain yield

We harvested the rice plants by leaving out the outer three rows to avoid border effects. We made the 0.25 m^2 square in the middle of the subplot, therefore we harvested nine plants per subplot. The plants were cut at about five cm above the soil in every subplot. We counted the tillers and panicles of each plant and quantified the percentage of productive tillers. The shoots were chopped, dried at 80 °C for 48 hours, and then dry weight assessed. Roots were collected at 0-20 cm depth, at 10 cm distance from the center of the rice plants. In each plot, we collected root samples of three rice plants that we then bulked. Roots were washed and afterwards dried at 80 °C for 48 hours. After that we assessed root dry weight.

Grain yield was determined at harvest when the plants had fully ripened. We collected the grain by cutting the shoots, and then separating the panicles from the stems and leaves. Then we dried the grain and measured the filled-grain dry weight. In addition, we randomly picked five panicles from each plot to count number of filled and unfilled grains in order to identify the average number of grains per panicle and the percentage of filled grain.

Nitrogen and phosphorus mass fraction

The leaves for N and P mass fraction analysis were collected before the flowering stage (60 DAT). We collected the third leaf from the apex of each plant, and collected nine plants per plot. We dried the leaves at 80 ˚C for 72 h. After that we ground the dry leaves and kept them in zip bags. We combined the samples of individual treatment from block one with those from block two, and block three with those of block four. The ground plant materials were submitted for N and P analysis at Khon Kaen University. N concentration was quantified by the Kjeldahl method (Bremner 1965), and P concentration was analyzed by wet digestion (nitric-perchloric digestion) and spectrophotometry (Land Development Department Thailand 2011). The N:P mass ratio was calculated to assess to what extent plants were limited by N and/or P (Güsewell 2004).

Stomatal conductance

Stomatal conductance of the rice plants was measured in the field on 21 DAT, hence 7 days after starting the drought treatment. Thus, the plants in well-watered treatment were watered two days before the measurement, while the plants in drought treatment had been exposed to drought for 7 days. Stomatal conductance was measured in the morning (from 9.30-12.00 am) using a AP4 porometer (Delta-T devices, UK). We measured stomatal conductance on the two youngest fully emerged leaves of each rice plant, and measured at the middle part of the leaf for all selected leaves. The measurement was done on the growing plants without destructively harvesting plants.

Chlorophyll fluorescence

We measured chlorophyll fluorescence of the rice plants in the field also on 21 DAT. Chlorophyll fluorescence was measured with a dark – light adapted chlorophyll fluorometer (MINI-PAM, WALZ, Germany). We measured chlorophyll fluorescence on two youngest fully emerged leaves of each rice plant. The measurement was done on the growing plants without destructively harvested plants. We recorded the results of minimum (F_0) and maximum (F_m) fluorescence that the plant leaf could absorb to quantify the maximum quantum yield of photosystem II (PSII) photochemistry (F_m-F_0/F_m) (Murchie and Lawson 2013).

$$F_v/F_m = (F_m - F_0) / F_m$$

Statistical analysis

Data were analyzed in SPSS version 22. As the watering treatment itself was not replicated at plot level, an analysis of variance (ANOVA) would commit the sin of pseudoreplication by using the subplots as replicates (Hurlbert 1984). As our main research questions were focused on the effect of AMF inoculation and varietal choice and not on the effects of drought (as it is well-known that drought negatively affects rice growth and grain yield), we calculated the confidence limits for a number of parameters in both plots, based on n = 24 (2 levels for inoculation × 3 varieties × 4 replicates). We indicated in bold when the confidence limits did not overlap as a basis for judging the effect of the drought treatment. Technically it would have been almost impossible to include the irrigation treatment as a separate factor in a three-factorial experiment, as it would have necessitated the ridging over each and every plot and randomization of the water treatment across the field. Because the whole field had been previously used by the farmer for cropping, we assumed that the field was sufficiently homogeneous and not creating a bias between the plots.

Next, we analyzed the well-watered and drought plots separately, using two-way ANOVA for testing the effects of AMF inoculum addition and rice variety on the plant response variables. Block effects were included in the analysis of AMF inoculation and rice variety effects in both water treatments as the fields might not have been 100% levelled and hence there could have been heterogeneity in the field subplots from north to south i.e., the orientation in which we designed the blocks based on a priory observation.

Results

Comparison of main treatment plots

The part of the field where we applied the well-watered treatment and the part where we applied the drought treatment were different for nearly all plant response parameters (table 4.1).

Table 4.1. Average values and confidence limits of plant parameters in the well-watered and drought plot (n = 24 for both). Values in bold indicate non-overlapping confidence limits.

Parameter	Well-watered	Drought
Mycorrhizal colonization (%)	13.1 ± 2.5	13.3 ± 2.5
Shoot dry weight (g)	94.3 ± 9.3	79.8 ± 6.3
Root dry weight (g)	**8.63 ± 0.26**	**8.11 ± 0.18**
Grain dry weight (g)	**102.8 ± 14.0**	**71.8 ± 6.8**
Productive tillers (%)	82.9 ± 2.8	79.9 ± 4.3
Nr. Grain/ panicle	100.9 ± 13.2	94.6 ± 11.3
Filled grain (%)	**86.53 ± 2.78**	**78.62 ± 4.83**
Stomatal conductance (mmol m^{-2} s^{-1})	**157.7 ± 6.0**	**144.4 ± 4.5**
Photosystem II efficiency	**0.85 ± 0.007**	**0.83 ± 0.007**
N (mg g^{-1})	14.3 ± 0.9	15.7 ± 0.9
P (mg g^{-1})	1.02 ± 0.06	1.08 ± 0.06
N/P mass ratio	14.1 ± 1.1	14.9 ± 1.0

These data provide strong suggestive evidence that the drought treatment was successful. In the part of the field where we withheld water, root (-6%), and grain dry weight (-30%) were lower than in the part of the field that was well-watered, consistent with the negative effect of withholding water on plant productivity and especially yield. Stomatal conductance and chlorophyll fluorescence also were lower in the part of the field where we applied the drought treatment, again consistent with the negative effect of withholding water. Average stomatal was above 158 mmol m^{-2} s^{-1} in the well-watered treatment and below 144 mmol m^{-2} s^{-1} in the drought treatment plot. Chlorophyll fluorescence was 0.85 in the well-watered treatment and

0.83 in the drought treatment. Both parameters indicate that there was a drought stress response yet that the impact of the drought treatment was relatively mild after one week. Rice leaf N and P mass fractions and the N:P ratio was not different. Mycorrhizal colonization of the rice roots was also not different between the well-watered and drought treatments (table 4.1). Considering the sensitivity of AMF to anoxic conditions that may have still occurred in the drought treatment during the time the soil was drying out and after rain events and rewatering, and that the field soil had low inherent AMF inoculum potential, lack of effects may not be surprising. Based on the nature of the differences between both plots, we interpret the effects as caused by drought and therefore refer to these plots as the well-watered and drought treatment plots, although our design cannot exclude that there were other differences between the plots between different parts of the field.

AMF root colonization

Addition of AMF inoculum increased root colonization significantly under drought ($P< 0.05$), but not under well-watered conditions (table 4.2). Due to the lower sensitivity of statistical tests for the individual varieties, the small increases in root colonization after inoculum addition in the drought treatment across all varieties (increases with 3-6%) were not statistically significant per variety (fig. 4.1). In the well-watered treatment, there was also no effect of rice variety on AMF colonization.

Shoot and root biomass, grain yield

Inoculum addition, variety, and their interaction were not significant sources of variation for shoot and root biomass, and grain yield, both under well-watered and drought conditions (fig. 4.2). Under well-watered conditions, CNT1 produced about 4500 kg ha^{-1}, RD22 produced 3700 kg ha^{-1}, and RD33 produced 4160 kg ha^{-1}, however these differences were not statistically significant. In both the well-watered and drought treatment, variety was a significant source of variation for the number of grains per panicle and the fraction filled grains, whereas mycorrhiza and the interaction were not (table 4.2). Under well-watered conditions RD33 had the highest number of grains per panicle; under drought conditions CNT1 had the lowest number of grains per panicle (fig. 4.2E). The fraction of filled grains was always highest in CNT1, irrespective of water availability or AMF inoculation.

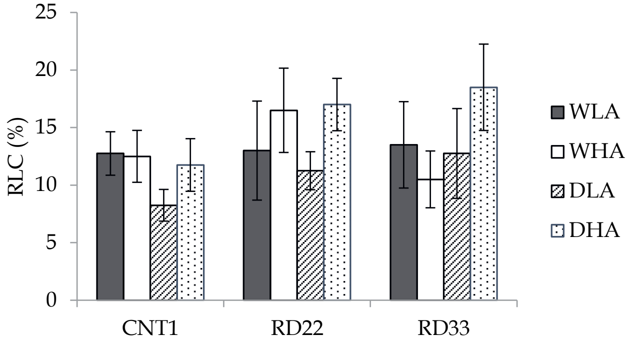


Figure 4.1: Root length colonization of AMF in three rice varieties (CNT1, RD22 and RD33 variety) growing under well-water and drought treatments. Error bars represent the SE of the treatments.

Leaf N and P mass fractions

There were no significant effects of AMF inoculation, variety or their interaction on plant N concentration in both the well-watered and drought treatment. For P concentration rice variety was a significant source of variation in the well-watered condition (table 4.3; fig. 4.3). In the well-watered treatment, RD33 had higher leaf P concentration than RD22 and CNT1 respectively. There was a marginally significant effect of variety on plant N:P mass ratio in the well-watered treatment. N:P ratios ranged between 15 and 18, suggesting P limitation.

Stomatal conductance and efficiency of photosystem II

Neither inoculation, nor variety, nor the inoculation × variety interaction were significant sources of variation for both parameters of photosynthetic performance, in both well-watered and drought treatment (table 4.2). As noted above, the values of both parameters (fig. 4.4) indicate that the impact of the drought treatment was relatively mild after one week, and for that reason the likelihood of finding significant effects of inoculation of variety were small.

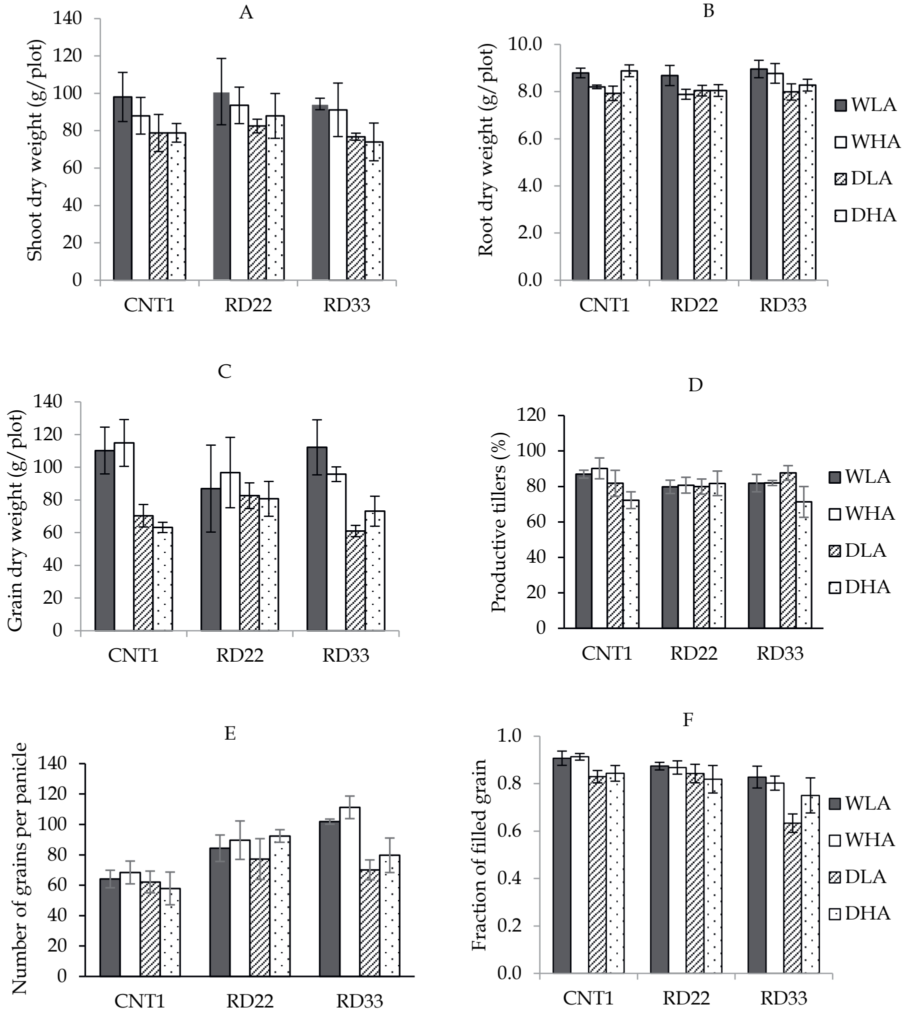


Figure 4.2. Plant performance parameters of rice in response to drought and AMF colonization. A: shoot dry weight; B: root dry weight; C: grain yield (mass per 0.25 m^2); D: productive tillers per plant; E: number of grains per panicle; F: fraction of filled grain. Error bars represent the SE of the treatments (n = 4).

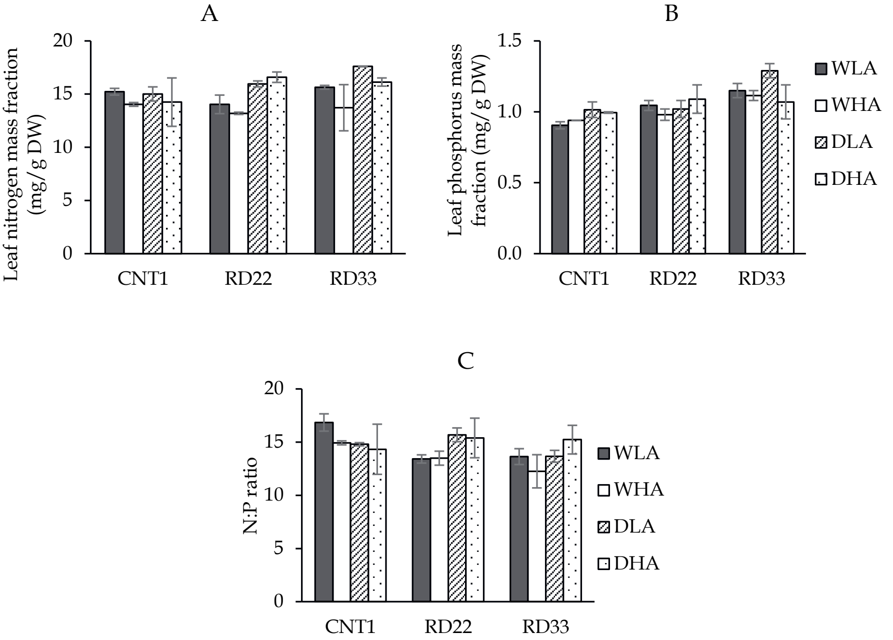


Figure 4.3. Leaf N (A) and P (B) and leaf N:P mass ratio (C). Error bars represent the SE of the treatments (n = 4).

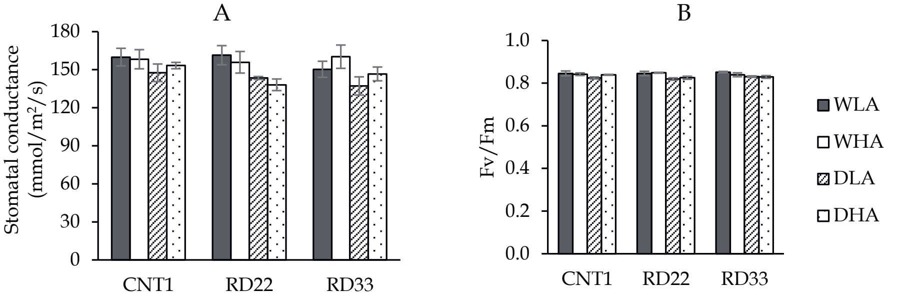


Figure 4.4. Photosynthetic performance as assessed through stomatal conductance (A) and chlorophyll fluorescence as proxy for photosystem II efficiency (B). Error bars represent the SE of the treatments (n = 4).

Table 4.2: Two-way ANOVA table testing the effects of AMF, rice varieties and their interaction, including block as random factor. The analysis was done separately for the well-watered and drought treatment plot. Numbers in bold are significant result ($P < 0.05$), numbers underlined are marginally significant effects ($0.05 < P < 0.10$). RLC = root length colonized, SWD = shoot dry weight, RDW = root dry weight, GDW = grain dry weight.

Two-Way ANOVA		RLC		SDW		RDW		GDW		Productive tillers (%)		nr. grain/ panicle		filled grain (%)		Stomatal conductance.		Chlorophyll fluorescence	
	Df	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Well- watered																			
Block	1	5.0	**0.04**	26.7	**0.00**	0.2	0.63	13.8	**0.00**	0.5	0.47	3.7	0.07	4.2	0.06	0.0	0.90	0.2	0.65
AMF	1	0.0	0.97	1.1	0.30	1.9	0.18	0.0	0.95	0.0	0.99	0.7	0.43	0.1	0.70	0.0	0.87	0.1	0.80
Variety	2	0.5	0.61	0.2	0.82	0.8	0.45	1.2	0.33	1.8	0.20	9.0	**0.00**	6.3	**0.01**	0.1	0.87	0.1	0.89
AMF*Variety	2	0.6	0.54	0.1	0.91	0.2	0.80	0.5	0.60	0.0	0.97	0.0	0.96	0.2	0.84	0.5	0.61	0.3	0.74
Drought																			
Block	1	0.2	0.68	3.6	0.08	1.3	0.27	1.5	0.24	0.8	0.38	0.3	0.57	0.5	0.47	1.3	0.28	9.7	**0.01**
AMF	1	4.8	**0.04**	0.0	0.88	1.9	0.19	0.0	0.87	0.5	0.50	0.8	0.40	0.8	0.38	0.6	0.47	2.2	0.15
Variety	2	2.2	0.14	0.9	0.43	0.1	0.87	2.7	0.10	0.4	0.65	3.4	0.06	5.7	**0.01**	2.2	0.15	1.9	0.18
AMF*Variety	2	0.1	0.90	0.2	0.86	0.5	0.60	0.9	0.41	2.4	0.12	0.5	0.59	1.2	0.32	0.6	0.57	1.1	0.35

Table 4.3: Two-way ANOVA table testing the effect of AMF and rice varieties on leaf nitrogen (N) and phosphorus (P) mass fraction, and N:P mass ratio. The analysis was done separately for the well-watered and drought treatment. As analysis of plant nutrient mass fractions was based of combining plants of block 1 with block 2, and block 3 with block 4, we could not test for block effects. P-values in bold are significant (*P* < 0.05).

2-Way ANOVA/ water treatment		N		P		N:P	
	df	F	P-value	F	P-value	F	P-value
Well-watered							
AMF	1	2.8	0.15	0.6	0.47	2.4	0.17
Variety	2	0.8	0.50	18.6	**0.00**	6.9	**0.03**
AMF*Variety	2	0.2	0.85	1.1	0.39	0.7	0.52
Drought							
AMF	1	0.0	0.97	0.0	0.92	0.0	0.92
Variety	2	1.2	0.37	0.7	0.52	0.3	0.75
AMF*Variety	2	0.2	0.84	0.4	0.71	0.2	0.82

Discussion

Our study found very little significant effects with respect to effects of AMF inoculum addition and variety on rice performance under well-watered and drought conditions. In fact, inoculum addition resulted in increased mycorrhiza colonization under drought, but not under well-watered conditions. While this outcome partly supports our hypothesis 4, the fact that this effect only occurred under drought, also led us to reject hypotheses 1-3. We also did not find support for hypothesis 5, as apart from enhanced colonization under drought, inoculum addition had no effect on plant performance. There were also only small differences between the three varieties under the drought treatment, and for that reason there was only (very) limited support for hypothesis 6.

Under ideal conditions one would prefer to set up such experiments as a true factorial experiment with three factors, viz. drought, inoculum addition, and rice variety.

However, logistics precluded a full factorial design, as arranging water availability on a subplot level through a pumping system while ridging each and every individual subplot is impossible. We therefore had to execute separate experiments in the field and first analyze the effect of treatment. Technically this constitutes pseudoreplication (Hurlbert 1984), the unwarranted inflation of degrees of freedom which generates overconfidence in significant outcomes. Even though the field has always been under uniform management, we cannot exclude variation within the field. We therefore only used the main treatment (drought versus well-watered) to assess the success of that treatment, while subsequently focusing on the effect of inoculation and varietal choice within the drought and within the well-watered treatment. This strategy can also be justified because negative effects of drought on rice performance, and especially yield and photosynthetic performance, are well-known. For that reason, we only briefly discuss drought effects to evaluate its severeness.

Table 4.1 demonstrates that the treatment of withholding water was successful, and grain yield was reduced by close to 35%. At the same time, the drought treatment was apparently fairly mild, as could be judged from lower values of stomatal conductance and photosystem II efficiency. Stomatal conductance in the drought treatment ranged between 137 - 153 mmol m^{-2} s^{-1}, compared to 150 - 161 mmol m^{-2} s^{-1} in the well-watered treatment. In an earlier experiment by Chareesri et al. (2020) the drought treatment resulted in a much stronger, significant decline of stomatal conductance 12 days after the drought treatment of around 80%. In a subsequent experiment (Chapter 3) a decline in stomatal conductance of around 80% was noted 6 days after the drought treatment. Photosystem II efficiency responds more slowly to drought (Trueba et al. 2019), and small difference (0.85 in the well-watered treatment and 0.83 in the drought treatment) is therefore less surprising. The study by Chareesri et al. (2020) did not indicate a significant effect of drought 12 days after the treatment started, and the subsequent study (Chapter 3) indicated a considerable decline only 12 days after the drought treatment started.

A further problem with this kind of field experiments is that one cannot control rainfall, and so heavy rain showers could have had effects on actual, rather than intended, water availability in the drought treatment. Also, within treatments field experiments can pose difficulties. For both the drought and the well-watered treatment, table 4.2 showed significant block effects. These block effects are very likely due to the fact that the field was not completely level after land preparation, and that some subplots may have experienced higher or lower availability of water than is typical for the intended treatment. As such effects occur patchily, and patches do not necessarily coincide with blocks, the sensitivity of our design may have been limited, which could have contributed to the fact that most hypotheses were not confirmed. However, the lack of effects, or only the relatively small effects, where they occurred, cannot only be attributed to the complexity of field experimentation. Earlier studies with rice have also shown only small, and not always significant, mycorrhizal effects on yield, nutrient uptake and photosynthesis (Zhang et al. 2019; Chareesri et al. 2020; Chapter 3).

AMF colonization

Inoculum addition did increase mycorrhizal root colonization under drought but not under well-watered conditions. These results are contrary to the earlier study under controlled conditions by Chareesri et al. (2020), where inoculum addition resulted in a significant increase in mycorrhizal colonization, independent of water availability. The increase in mycorrhizal root colonization after inoculum under drought, from 10% to 15%, occurred with all three varieties; however, the relatively large variation in root colonization resulted in the effect not being significant for each variety. Levels of mycorrhizal colonization for these varieties were somewhat higher in the drought treatment in the earlier study by Chareesri et al. (2020), where inoculum addition resulted in colonization levels between 20% and 30%. In a paddy-rice soil Zhang et al. (2015) reported an increase in mycorrhizal root colonization after inoculation from 2.5% to 12-20%. However, paddy soils experience regular anoxia, which is unfavorable for AMF, and hence a low mycorrhizal inoculum potential could be expected. The study by Rakotoarivelo Njaramanana et al. (2022) reported no effect of mycorrhizal inoculation in upland-rice fields in Madagascar, with colonization levels around 10%. A study by Solaiman and Hirata (1997) reported much larger effects of inoculation in the nursery stage, where mycorrhizal colonization after 6 weeks increased from 22% to 55%. Such high colonization levels have not been reported in other studies.

Apparently, field inoculation does not always guarantee success and the reasons for lack of response include lack of adaptation of the introduced strains to the local environmental conditions, which include temporary flooding the field, and sufficiency of indigenous mycorrhizal inoculum that outcompeted the introduced inoculum. Ryan and Graham (2018) mentioned in their review that it may not be worth to apply AMF to field crops. This was not in agreement with the meta-analysis of Zhang et al. (2019), who stated that AMF increased grain yield by 16% in field experiments with inoculum addition.

A final factor that may have caused differences in effects of inoculum addition could be a legacy effect. While for both pot and field experiment, we had collected soil from the same field, spore density was around 5 spores per 100 grams of soil when we collected soil for the pot experiment, and around 10 spores per 100 grams of soil when we assessed inoculum potential before the start of the field experiment (A. Chareesri, unpublished). Legacy effects might have reduced differences in AMF colonization between inoculated plots and in non-inoculated plots. In addition, this field experiment had higher RLC in non-inoculated plots than what has been reported in previous studies. Legacy effects seem also possible, considering earlier reports of mycorrhizal colonization of rice of around 2-3% in Zhang et al. (2015) and 3-5% in Wangiyana et al. (2006) in non-inoculated rice plants growing in the field.

Shoot and root biomass, and grain yield

The effects of AMF on rice performance were not significant, both under well-watered and drought conditions. In our earlier study with the same varieties, Chareesri et al. (2020) also found no significant effect of inoculum addition on grain yield, whereas with three other varieties the positive effect of inoculum addition was significant

(Chareesri, Chapter 2). A relatively small effect of inoculum addition (and increased mycorrhizal colonization) was also reported in the meta-analysis of Zhang et al. (2019), who reported that the effect of AMF in field experiments is less promising than in pot experiments. In pot experiments, we can manipulate experimental conditions better than in field experiments. Furthermore, and likely more importantly so, most mycorrhizal studies are done by comparing non-mycorrhizal to mycorrhizal plants. In our field experiment, we allowed natural colonization and therefore assessed only the effects of inoculum addition. And as effects of inoculum addition were absent or small, lack of inoculum effects on plant performance is not surprising. The productivity of CNT1, RD22 and RD33 under well-watered conditions were comparable to the data of the Thai Rice Department (2016). The lack of varietal effects in the drought treatment suggests that the varieties exhibited only small differences in drought tolerance.

Rice variety effects

CNT1 has been reported as a drought-tolerant variety. There have been several proposals how to operationalize drought tolerance. Adhikari et al. (2019) mentioned seven different indices of drought tolerance. For this study we used YSI, Yield Stability Index, initially proposed by Bouslama and Schapaugh (1984). It is defined as the ratio of rice yield under drought compared with biomass under well-watered conditions (Sawatdikarn and Kansomtob 2012). For CNT1 these authors reported the YSI (or DTI, the term used by them) as 0.89. We were unable to find DTI values for the two other varieties. In our previous pot study (Chareesri et al. 2020) the YSI of CNT1, RD22, and RD33 were reported as 0.52, 0.63 and 0.66 respectively, suggesting that the three varieties had a comparable drought index. Considering both the fact that the drought treatment was mild and the relatively small differences between these varieties, it is not surprising that the factor variety was almost always not significant in our study.

Conclusion

Yield and photosynthetic performance were lower in the drought treatment than in the well-watered treatment. Because the mycorrhizal symbiosis has been reported to confer drought tolerance and to increase plant performance and yield, also in the case of rice, one would therefore predict that increasing mycorrhizal inoculum and mycorrhizal root colonization of rice plants would expand the window of opportunity to grow rice under increasing risks of dry spells under climate change. However, our field experiment failed to provide support for these beneficial effects. While lack of effect could partly be due to the inherent difficulties of field experimentation, such as limited control over the actual rather than the intended water availability, we cannot exclude the possibility that mycorrhizal benefits through inoculation, if and when they occur, might be fairly limited and not be of prime relevance for Thailand's farmers. However, that conclusion should not be interpreted as a statement that management of mycorrhizal symbiosis is not very relevant, as other forms of management, for instance through mixed cropping or varietal mixing, could be more effective to raise mycorrhizal inoculum potential and hence to generate mycorrhizal benefits under drought (Chapter 5).

Chapter 5

A mixture of rice varieties enhances arbuscular mycorrhizal fungal colonization and grain yield

Anupol Chareesri, Gerlinde B. De Deyn, Thomas W. Kuyper

Abstract

Arbuscular mycorrhizal fungi (AMF) form a mutually beneficial symbiosis with plant roots, often resulting in increased plant biomass and fitness. Colonization by AMF in rice roots can also improve drought tolerance of plants. However, increasing root length colonization in the field is challenging. Adding AMF inoculum can be one of the methods, but it requires a huge amount of inoculum and increases the cost for farmers, making this method often prohibitive. Intercropping between different species or mixing of varieties of the same species can be a promising alternative method to promote AMF colonization. We investigated whether mixtures of two rice varieties can increase root length colonization by AMF and grain yield, under normal water availability and drought. We used field soil and conducted a full factorial greenhouse experiment with 2 water treatments (well-watered and drought), 2 AMF treatments (inoculated and non-inoculated), and 3 varietal mixing treatments (monoculture and mixture). Varietal mixing enhanced root length colonized, and increased rice shoot dry weight, root dry weight and grain dry weight. The benefits of varietal mixtures occurred independent of inoculum addition and the water treatment. The relative benefit through varietal mixing was larger under drought than under well-watered conditions. Relative yield total (RYT), based on mycorrhizal colonization, was positively correlated with RYT for grain weight. The varietal-mixture effect was largely due to the complementarity, whereas the small negative selection effect indicated that the less-productive rice variety benefitted somewhat more from mixing than the more productive variety. Drought reduced shoot, root, and grain dry weight, but had no effect on mycorrhizal root length. The addition of AMF inoculum increased AMF root length colonization but had no effect on root, shoot or grain dry weight. Mycorrhizal colonization can be enhanced both by AMF inoculum addition and by mixtures of different rice varieties. Increases in root colonization through varietal mixtures translated into higher grain yield. Our results indicate that varietal mixing could be a potential means for increasing AMF colonization and grain yield in field crops, both under sufficient and limited water supply.

Keywords: *rice intercropping, varietal mixture, arbuscular mycorrhizal fungi, rice, drought*

Introduction

Arbuscular mycorrhizal fungi (AMF) are known for their benefit on promoting plant growth. AMF can increase plant nutrient and water uptake, especially under nutrient and water limiting conditions (Smith and Read 2010; Kuyper et al. 2021; Kuyper and Jansa 2023). In nature, AMF can be found in the majority of species of plants and vegetation types and also in all major soil classes, but the benefit to plants varies according to plant root traits, soil properties, and the abundance and species composition of the AMF community (Hoeksema et al. 2010; Yang et al. 2015; Delavaux et al. 2017). The presence of AMF depends on environmental conditions, for instance, we may find low abundance of AMF under anerobic or saline conditions. Agricultural management equally affects the abundance of AMF, with soil tillage, use of high amounts of mineral fertilizer, especially of phosphorus, and the use of fungicides, especially copper-based fungicides, reducing AMF abundance, and hence the benefits that farmers could derive from AMF. Conversely other practices, including cover crops and intercrops that host AMF, have a beneficial effect on AMF (Verbruggen and Kiers 2010; Vukicevich et al. 2016; Millar and Bennett 2016).

Rice (*Oryza sativa* L.) is one of the main staple food crops globally. The largest rice production is from lowland rice which is usually grown under submerged conditions. Flooded rice fields are not favorable for AMF occurrence because of widespread anoxia; however, several studies revealed the occurrence of AMF and their benefits in flooded rice fields (Watanarojanaporn et al. 2013; Vallino et al. 2014). Nevertheless, Lumini et al. (2011) and Vallino et al. (2014) found higher AMF colonization in rice roots growing in drier as compared with submerged soil. Rice is very susceptible to drought, and especially drought during flower initiation and flowering can results in yield reductions of 50% (Sarvestani et al. 2008). In non-irrigated areas, lowland rice productivity is therefore often limited by drought due to unpredictable rainfall. Low yields due to drought are likely to be exacerbated in the future due to the effects of climate change that predict increased variability with both increasing severity of rainfall events and a lower number of consecutive days with rain (Amnuaylojaroen 2021).

Under these conditions AMF may promote rice yield given the benefit of AMF on drought tolerance and resilience of plants. Various mechanisms have been proposed that cause this enhanced drought tolerance of plants induced by AMF, such as access to water-filled pores that are too small for roots or root hairs to enter, increased uptake of phosphorus and potassium that results in a better nutritional status that confers tolerance, and hormonal changes that impact photosynthetic behavior, such as increases in stomatal conductance and in efficiency of photosystem II (Augé et al. 2015; Wang et al. 2019; Chareesri et al. 2020; Chapter 3). Such mycorrhizal effects are most easily demonstrated when comparing plants with and without AMF, but can also be observed, although to a smaller extent, when plants with higher and lower levels of mycorrhizal root colonization are observed (Chareesri et al. 2020). These latter observations suggest, then, that there could be inoculum limitation to harness such mycorrhizal benefits for drought tolerance. Beneficial mycorrhizal effects with increased root colonization are important when scaling up the results from mycorrhizal studies in the lab and greenhouse, because in the field there is almost always mycorrhizal inoculum, although its abundance could be limiting.

In our field experiment (Chapter 4) we did not find beneficial impacts on rice grain yield by mycorrhizal inoculum addition under drought, even though root colonization was significantly increased. However, the increases were rather small and it may have been possible that the amount of inoculum added was (too) low. Another possible explanation is competition between the native AMF inoculum residing in the soil and the commercial inoculum that is applied (Verbruggen et al. 2013; Janouškova et al. 2013; Berruti et al. 2016). Increased fungal competition could even reduce, rather than enhance, plant productivity (Janouškova et al. 2013; Engelmoer et al. 2014). An alternative solution would therefore be to increase the inoculum potential of native AMF. However, raising site- and crop-specific inoculum in sufficiently large amounts is likely to be prohibitively expensive to farmers, especially those under resource-poor conditions. Thus, we should look for alternative means to increase AMF colonization without a big investment and that is easily feasible and compatible with (resource-poor) farmer practices.

Intercropping is the farming practice of growing mixtures of plant species or varieties, which is usually aimed at providing benefits for increasing plant biomass and biodiversity (Malézieux et al. 2009; Li et al. 2020). Three mechanisms underlie the beneficial effect of intercropping: complementarity, the increased use of resources through niche differentiation; facilitation, the abiotic amelioration of the physical and chemical environment; and biotic feedbacks, where plant species or variety-specific pathogens may be diluted through increased distance between sensitive plants as a consequence of crop mixtures (Barry et al. 2019). Pathogen dilution has been shown to explain increased yield of glutinous rice and reduced disease severity of rice blast when grown in mixtures compared with the growth in monocultures (Zhu et al. 2000). A meta-analysis by Zhang et al. (2019) showed decreased disease severity of both wheat (*Triticum aestivum* L.) and faba bean (*Vicia faba* L.) in intercropping compared with monocultures. Niche differentiation, the cause for complementarity, has frequently been demonstrated for intercrops consisting of different species. A few studies have also shown benefits similar to those by intercropping if two varieties of the same species were mixed. In a pot experiment Wang et al. (2020) demonstrated increase maize (*Zea mays* L.) yield through varietal mixing. Their study specifically reported yield increases when maize was mycorrhizal, but no varietal-mixture benefits when maize was non-mycorrhizal. In this study the use of varietal mixing resulted in increased mycorrhizal root colonization and / or extraradical hyphal length, and it is plausible that increased AMF performance was the underlying mechanism for yield increases.

Intercropping studies with rice and mungbean (*Vigna radiata* L.) and rice and peanut (*Arachis hypogaea* L.) equally showed enhanced mycorrhizal colonization and yield increase by intercropping (Li et al. 2009; Kusnarta et al. 2022). Studies on varietal mixing of mycorrhizal rice are rare, especially under drought conditions. Hence, we conducted a pot experiment using field soil to investigate the effects of mixtures of two different rice cultivars on AMF colonization, with and without additional AMF inoculation, and their benefits to root and shoot biomass and grain yield. We also addressed the mycorrhizal role in varietal mixtures under well-watered and drought conditions. In our study we hypothesized that:

(i) Varietal mixing increases AMF colonization compared with monocropping.
(ii) Varietal mixing enhances the effectiveness of AMF inoculation compared with monocropping.
(iii) Varietal mixing increases rice plant biomass (shoot and root biomass, and grain yield) under both well-watered and drought conditions, with a relatively larger effect under drought.
(iv) Increases in AMF colonization due to varietal mixing are positively correlated with yield increases in rice grain yield under well-watered and drought condition.

Methods

Experimental design

A greenhouse pot experiment was set up in a full factorial design, comprising 2 water treatments (well-watered and drought), 2 AMF treatments (inoculated and non-inoculated), and 3 varietal-mixture treatments (mono-KDML105, mono-SR1, and the KDML105+SR1 mixture) treatments. Each treatment was replicated 8 times, resulting in 96 pots in total and these were positioned in a random block design of 8 blocks.

The pots were filled with soil collected from a rice farm in Khon Kaen (6°29'10.9"N 102°34'40.5"E) from the 0-15 cm soil layer and were planted with 2 rice plants per pot (see below). The soil was a sandy loam soil, and contained 60% sand, 31% silt, 8% clay, 1% organic matter, and the pH of the soil was 7.5. Bulk density was 1.29 g cm^{-1} with 0.52% porosity and 25.5% water-holding capacity. After collecting the soil from the field, we sieved the soil through a 2 mm sieve and then mixed the soil homogenously. We filled the pots with approximately 5.6 dm^3 dry soil (around 7 kg) per pot (the pot volume was 6.4 dm^3; top surface diameter = 24 cm, bottom surface diameter = 17 cm and height = 19 cm).

Rice varieties

We used two rice varieties: Khao Dawk Mali 105 (KDML105) and Surin 1 (SR 1). These varieties were selected because these varieties are commonly grown in Thailand and showed different responses to AMF inoculation and drought in our previous pot experiment (Chareesri et al. 2020). In that experiment AMF colonization in SR1 tended to be higher than in KDML105 and when subjected to drought the grain yield of SR1 was reduced more than of KDML105.

The rice seeds were germinated and grown in trays with field soil till they were large enough to be transplanted into the pots (after 14 days). Each pot was planted with two rice seedlings. For the monoculture treatments we planted two seedlings of the same variety in one pot. For the varietal-mixture treatment, we planted one KDML 105 and one SR1 seedling.

Mycorrhizal treatment

The field soil was not sterilized. The density of field soil inherent AMF spores was 10 spores per 100 grams of soil. For the AMF inoculation treatment, we inoculated 3 g of commercial AMF inoculum per plant (or 6 g per pot, ± 60 spores) (RootGrow Professional, Kent, UK) which contained 5 species of AMF: *Funneliformis mosseae, F. geosporus, Claroideoglomus claroideum, Glomus microaggregatum,* and *Rhizophagus irregularis* (Robinson Boyer et al. 2016). The inoculation was done by making a planting hole and addition of the inoculum, after which the rice seedling was planted into the planting hole.

Water treatment

All pots received water to the level of being well-watered (saturated) during the first two weeks to ensure plant growth and AMF establishment. The drought treatment started after that period (14 days after transplanting), and was carried on till the flowering stage (66 days after transplanting). The drought treatment was done by imposing a drying–rewetting cycle, similar to the drought treatment in our previous experiments (Chareesri et al. 2020; Chapter 3) and in line with the type III drought as presented in He and Dijkstra (2014). The drying cycle comprised withholding water for 6 days, then rewatering and withholding the water again for another 6 days. This created a drought level of about -40 kPa soil water potential.

Assessment of shoot and root biomass and grain yield

We harvested the plants when the rice grain was fully ripened (after about 105 days since transplanting). We used scissors to cut the shoot at about five centimeter above soil surface. Panicles were separated from the straw, then sun-dried for 48 h. Then straw and grains of each plant were separated and each kept in a separate paper bag, and put in the oven at 80 °C for 48 h. We determined dry weight of straw and grains of each plant separately.

For root mass assessment, we took the roots and the soil out of the pot. We separated the soil from the stubble and the roots of each plant by first gently shaking the soil and subsequently washing the roots. We cut the stubble, and collected only rice roots by carefully washing the roots in water and separating the roots per individual plant. Individual roots were put in a paper bag and dried at 80 °C for 48 h for root dry weight measurement.

Root length colonization by AMF

We took 10% dry roots from the total root dry weight of each plant for analysis of mycorrhizal colonization. The dried roots were cut into small fragments of about 2 cm long. We put the roots in plastic tissue cassettes, one per sample. We stained the roots by clearing the roots in 10% KOH solution at 90 ˚C for 15 minutes. Then, roots were rinsed and left in 1% HCl solution for 30 minutes (INVAM 2017). After that, we stained the root by leaving the roots in 0.05% Trypan blue solution over 24 hours (500 ml glycerin: 450 ml distilled water: 50 ml of 1% HCL and 0.5 g Trypan blue). The stained roots were de-stained in the de-staining solution (2:1 v/v of distilled water: glycerin) for 48 hours. After de-staining, the subsamples of the stained roots were placed on microscopy slides, and the AMF colonization was quantified by microscopic observations at 400 x magnification according to Giovannetti and Mosse (1980). The results were calculated as percentage of root length colonized (RLC) according to

$$RLC\ (\%) = (\text{Number of root intersection with mycorrhiza} / N)*100$$

With RLC = percentage of root length colonization by AMF (%), N = total number of intersections per slide, which is 100.

Data analysis

To quantify intercropping and varietal-mixing effects, we calculated both relative and absolute overyielding of the mixture in relation to the monocrops. Relative yield total (RYT; also known as LER, Land Equivalence Ratio, the amount of land needed to raise the same yield as the intercrop or varietal mixture when plants are grown separately):

$$RYT = \frac{Y1}{M1} + \frac{Y2}{M2}$$

Where Y1 and Y2 are the biomass of KDML105 and SR1 respectively when grown as mixture and M1 and M2 the biomass of KDML105 and SR1 respectively when grown as single crop. A mixture overyields if RYT is significantly larger than 1, calculated by the 95% confidence limits of the eight blocks. We also calculated overyielding for mycorrhizal root colonization.

As absolute overyielding (expressed in biomass) is much more relevant to the farmer than relative overyielding (a dimensionless number), we also calculated absolute overyielding, following the methods by Loreau and Hector (2001):

$$\Delta Y = Y_O - Y_E = \overline{N\Delta RY}\ \overline{M} + N_{COV}\ (\Delta RY, M)$$

Where ΔY refers to the difference of observed yield of the mixture and the expected yield, the yield under the assumption that the yield per plant in the mixture would be the same as the yield per plant in the monocropping treatment. We determined the two constituents of ΔY, viz. the complementarity effect $\overline{N\Delta RY}\ \overline{M}$ (which assesses the degree to which niche differentiation by both species contributes to overyielding) and the selection effect N_{COV} *(ΔRY, M)* (which assesses the extent to which the more productive species causes an increase in yield of the mixture; note than a negative selection effect indicates that the less performing species as monocrop contributed more strongly to overyielding than the more productive species). We finally calculated Pearson correlations between RYT, based on colonization, and RYT of root, shoot and grain weight.

The data were statistically analyzed in IBM SPSS Statistic version 29.0.0.0 (241). Three-way analysis of variance (ANOVA) was used for testing of significant sources of variation of to the three imposed treatment factors (AMF inoculation, varietal mixing, drought) and their interactions.

Results

Mycorrhizal root colonization

Both cropping system and AMF inoculation were significant sources of variation, whereas water was not. Varietal mixing and AMF inoculation significantly increased mycorrhizal root colonization (fig. 5.1). The AMF inoculation increased root colonization with about 4% compared with the non-inoculated treatment, yet this increase was larger in plants subjected to drought. The significant interaction between water × inoculation indicates that the effectiveness of AMF inoculation to promote root colonization was dependent on the watering regime (table 5.1). There was also a marginally significant interaction between inoculation × cropping system whereby AMF inoculation effects tended to be larger in the mixture than in the monocultures.

Shoot and root biomass and grain yield in response to drought, varietal mixing and AMF

Drought significantly affected plant biomass and grain yield of rice, whereas varietal mixing and AMF inoculation did not affect plant biomass or grain yield (table 5.1). Drought significantly reduced shoot dry weight (fig. 5.2A) and grain yield (fig. 5.2C) and marginally suppressed root biomass (table 5.1, fig. 5.2B). Drought severely affected grain yield, resulted in a decline of 75% grain yield (fig. 5.2C). KDML 105 in monoculture produced highest shoot biomasses under well-watered treatment, but under drought its biomass reduction was more than SR1 monoculture and mixture treatment (fig. 5.2A). Mixing resulted in an increase in root dry weight (fig. 5.2B) and grain yield (fig. 5.2C) compared with the monoculture treatments. To investigate the effects of mixing further we analyzed relative yield and overyielding of root colonization and plant biomass and grain yield and how these may be affected by drought and AMF inoculation.

Relative and absolute overyielding

There was a significantly positive correlation between RYT for mycorrhizal root colonization and RYT for grain yield ($r = 0.38$; $P = 0.03$; $n = 32$), whereas RYT for mycorrhizal colonization and RYT for root and shoot biomass were not significantly correlated ($P > 0.05$ in both cases).

For root colonization there was a positive complementarity effect (an increase of 2-4% in root colonization) in three out of four treatments; only in the case of drought without AMF inoculation no complementarity effect was observed. The selection effect was always not significantly different from zero, indicating that both varieties benefitted to a similar extent from the mixture by increasing root colonization (fig. 5.4A). Consistent with the lack of significance for cropping system, shoot dry weight did not show clear evidence for complementarity or selection effects, except in the case of inoculation in the drought treatment when there was a positive complementarity effect (fig. 5.4B). Root dry weight showed more evidence of complementarity, with three out of four cases exhibiting a positive complementarity effect; only under drought in the absence of AMF inoculation was there no complementarity effect. There was either no or a negative selection effect, indicating that the cultivar that produced the lower amount of root biomass benefitted more from the mixture (fig. 5.4C). For grain yield all four cases resulted in a positive complementarity and a negative selection effect. The magnitude of the complementarity effect was independent of the water treatment (fig. 5.4D).

Table 5.1: The results of 3-Way ANOVA testing the effects of Water, AMF and Varietal mixing on mycorrhizal root colonization (RLC), Shoot dry weight (SDW), Root dry weight (RDW), and Grain dry weight (GDW). P-values in bold are significant (*P* < 0.05), P-values underlined are marginally significant (0.05 < *P* < 0.10), n = 8.

3-Ways ANOVA		**RLC**		**SDW**		**RDW**		**GDW**	
	Df	F	P	F	P	F	P	F	P
Water (W)	1	0.3	0.60	9.9	**0.00**	3.6	0.06	1139	**0.00**
AMF (A)	1	17.1	**0.00**	1.5	0.22	0.2	0.68	2.3	0.13
Varietal mixing (I)	2	9.3	**0.00**	1.0	0.36	3.7	**0.03**	5.7	**0.00**
W × A	1	4.9	**0.03**	0.5	0.47	1.6	0.21	0.1	0.76
W × I	2	0.1	0.88	1.0	0.37	0.1	0.91	0.8	0.43
A × I	1	2.7	0.08	0.5	0.61	0.8	0.46	2.3	0.11
W × A × I	2	0.2	0.80	0.1	0.93	1.4	0.26	0.0	0.95

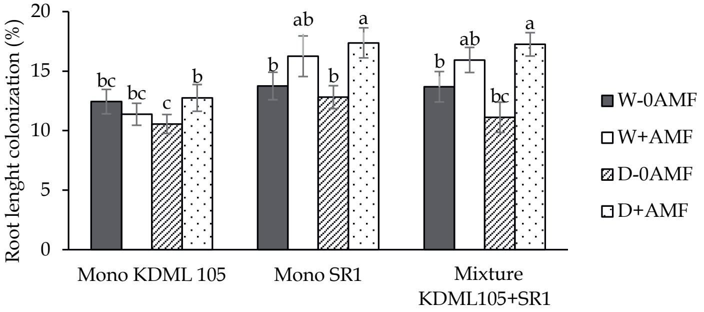


Figure 5.1: The percentage of root length colonization of AMF on rice root in monoculture and mixtures in response to AMF inoculation and watering treatment. KDML105 = Khao Dowk Mali 105 and SR1 = Surin 1 variety. W-0AMF = well-watered treatment without AMF inoculation, W+AMF = well-watered treatment with AMF inoculation, D-0AMF = drought treatment without AMF inoculation, and D+AMF = drought treatment with AMF inoculation. Bars represent mean values per treatment and error bars represent the SE, N= 8. Bars with completely different letters indicate significant differences between treatments (P-value < 0.05).

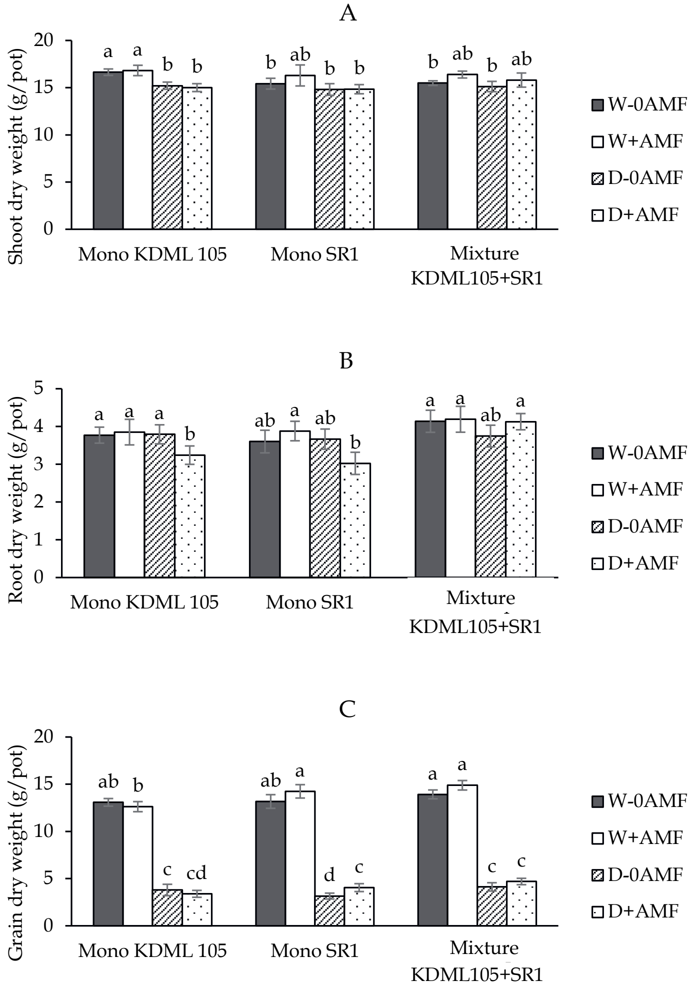


Figure 5.2: Rice shoot (A) and root (B) dry weight, and grain biomass (C) biomass in response to AMF inoculation and watering treatment. KDML105 = Khao Dowk Mali 105 and SR1 = Surin 1 variety. W-0AMF = well-watered treatment without AMF inoculation, W+AMF = well-watered treatment with AMF inoculation, D-0AMF = drought treatment without AMF inoculation, and D+AMF = drought treatment with AMF inoculation. The error bars represent the SE of the treatments, n = 8. Bars with completely different letters indicate significant differences between treatments (P-value < 0.05).

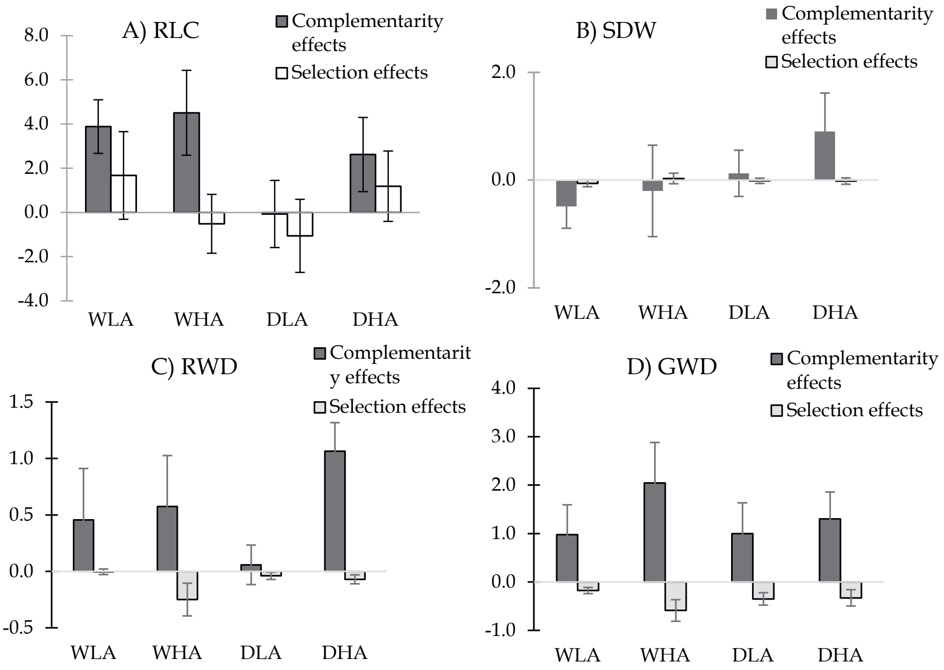


Figure 5.3: The complementarity and selection effects on mycorrhizal root colonization (A), shoot dry weight (B), root dry weight (C), and grain yield (D) in relation to AMF inoculation (L vs H) and watering treatment (W vs D). WLA = well-watered, without inoculation; WHA = Well-watered, with inoculation; DLA = Drought treatment, without inoculation; DHA = Drought treatment, with inoculation. The error bars represent the 95% confidence interval, n = 8.

Discussion

Varietal mixing resulted in increased mycorrhizal colonization compared with monocropping and also resulted in increased grain yield, but not in higher shoot and root biomass. The relative increase in colonization was positively correlated with the relative increase in grain yield. These beneficial effects of varietal mixing on grain yield occurred both under well-watered and drought conditions. Partitioning of overyielding in grain yield suggested a major role for complementarity and a much smaller, and often non-significant, negative selection effect, implying that mixing varieties somewhat more benefitted the rice variety (KDML105) with lower grain yield.

Impact of varietal mixtures on mycorrhizal root colonization

There are two mechanisms to explain the effect of intercropping (incl. varietal mixing) on increased root colonization (and also on enhanced plant performance). Bainard et al. (2012) stated that intercropping could change diversity and community composition of AMF. While we have no information on the species composition of the indigenous inoculum, it is plausible that some species of the exotic inoculum did not naturally occur in the field. Inoculation likely increased AMF species diversity and several studies have reported the positive correlation between AMF species richness and productivity of plant communities, because the increased species diversity results in increased functional-trait diversity which may be the underlying mechanism for increased productivity (Powell and Rillig 2018). This mechanism may explain the larger effect after inoculum addition than when plants were only colonized by the indigenous inoculum; however, it would not explain the positive varietal-mixture effect by the indigenous inoculum. An additional mechanism could be that the presence of different plant species or varieties induces niche differentiation among the AMF and reduce competition as different AMF species might associate with different species or varieties and potentially induce different benefits to these plant species or varieties (Montesinos-Navarro et al. 2012; Wang et al. 2020; Zhang et al. 2020). This mechanism also assumes alignment between AMF fitness and plant fitness, but such an alignment is not always evident (Bever et al. 2009; Wang et al. 2019). Further studies on the species composition of AMF in plants grown as monocrop and in mixtures, with and without inoculum addition are needed to evaluate this hypothesis.

Other intercropping studies with rice equally showed an increase in root colonization when intercropped rice was compared with monocropped rice. In the study of Xiao et al. (2010), the mixed cultivation of upland rice and mung bean together with AMF inoculation significantly increased root colonization from 10% to 14%. Both the absolute fraction of rice roots colonized and the increase in colonization due to intercropping and inoculum addition were low and did not reach values above 20%. With the larger fraction of the root system not colonized, it is unlikely that in our experiment competition for space between AMF in the rice roots occurred, as has been reported in previous studies. Evidence for competition with indigenous AMF was also obtained in the study by Köhl et al. (2016) where the authors added inoculum of *Rhizophagus irregularis* to eight soils in which a mixture of the grass *Lolium multiflorum* Lam. and the legume *Trifolium pratense* L. was grown. Without inoculum addition the legume achieved colonization levels of 50-75% and inoculation increased colonization in four out of eight soils, whereas the grass achieved colonization levels of 25% and inoculum addition achieved increases in colonization in all eight soils.

Impact of intercropping or varietal mixing on plant biomass

Intercropping in many studies increased plant yield compared to monocultures (Li et al. 2020; Beillouin et al. 2022; but see Li et al. 2023 for a specific analysis of transgressive overyielding, the comparison with the most productive single crop). Most studies pertained to intercropping different species, but meta-analysis of cultivar mixtures also showed a small but significant yield increase (Reiss and Drinkwater 2018). Reiss and Drinkwater (2022) and Schöb et al. (2023) noted that overyielding was more common in stressful environments. The latter authors mentioned both complementarity and changes in the composition of beneficial microbes as underlying mechanisms, but did not specifically refer to the role of the mycorrhizal symbiosis. However, Brooker et al. (2021) in a study of barley (*Hordeum vulgare* L.) noted no additional beneficial effects of varietal mixing on overyielding under drought, but also referred to additional benefits of varietal mixing such as weed and pathogen suppression. Our study also did not show a significant cropping system × water interaction. A mycorrhizal role for overyielding, as in this study, was earlier reported by Wang et al. (2020), but the underlying mechanisms remain elusive. Apart from an increase in root colonization and extraradical hyphal length, Wang et al. (2020) found an increase of phosphatase activity and an increase in P content, suggesting that a nutritional effect was as the basis for overyielding.

In our study effects of varietal mixing on shoot biomass were not observed, but effects were obvious in root biomass and grain yield. The positive complementarity effect on rice root biomass (especially after inoculation in the drought treatment; fig. 5.3B) indicated that there was no increased inter-varietal competition relative to the intra-varietal. An investigation where black rice was intercropped or not with soybean (*Glycine max* (L.) Merrill) and with or without AMF inoculum addition showed increased grain yield both as a consequence of intercropping and addition of mycorrhizal inoculum, but no data on mycorrhizal colonization were provided (Wangiyana et al. 2021). Xiao et al. (2010) reported that intercropping rice with mung bean in the presence of mycorrhizal fungi significantly increased rice shoot biomass (+57%) and significantly reduced root biomass (-77%), both effects not observed in our study. Unfortunately, no data on rice yield were provided in Xiao et al. (2019).

Our study comprised the comparison between two rice varieties under controlled conditions. Upscaling to field conditions remains a necessary subsequent step. Increasing the portfolio of suitable rice varieties for mixtures is an essential step in order to harness this cropping system for climate-resilient rice production. Varietal selection has often not been based on systematic principles of underlying mechanisms, so there is a clear need to use ecological principles for guidance in selection for varietal mixing (Bourke et al. 2021; Wuest et al. 2021; Kopp et al. 2023). Next to ecological principles, farmer preferences should not be neglected (Neupane et al. 2023).

Conclusion

Varietal mixing and AMF inoculation resulted in increased root colonization of rice roots. Varietal mixing also resulted in increased root biomass and grain yield, whereas AMF inoculation did not impact plant performance. Considering limitations of using AMF inoculum, such as costs for obtaining the inoculum, additional labor costs for inoculating rice seedlings before transplanting, and the possible lack of adaptation of introduced inoculum to the prevailing environmental conditions, alternatives to increasing mycorrhizal root colonization should be sought. Varietal mixing achieved increased colonization and increased grain yield in this pot experiment. The next challenges will be too upscale this outcome to the field, while also testing different rice varieties for compatibility. Considering benefits of intercropping such as the delivery of other ecosystem services such pathogen protection and weed suppression, a focus on varietal diversification in rice production systems seems desirable.

Chapter 6

General discussion

Main findings

The aims of my thesis were to study the benefits of the AMF symbiosis for rice plants under drought conditions. I conducted my experiments in Thailand where rice is the economically most important crop. The rice cultivation area covers more than 1/3rd of Thai agricultural land (Thai OAE 2023). Most rice cultivation areas are lowland and rain-fed, and in these areas, rainfall is the main source of water. Recently, climate change has caused rising temperatures, and unpredictable rainfall which results in more frequent flooding and drought in many regions. These changes are becoming the major problems for stable food crop production such as rice. High night temperature can reduce grain yield and quality of milling rate of rice (Impa et al. 2021), flooding causes oxygen shortage (Bouman et al. 2007), lack of rainfall induces drought resulting in water deficiency and limiting nutrients for rice growth (Suriyagoda et al. 2014).

I conducted pot and field experiments using natural and commercial AMF inoculum and several rice genotypes, subjected them to different levels of drought stress, and collected important variables that are related to the role of AMF in conferring drought tolerance of rice. One novelty of my thesis is the comparison of the effects of lower and higher AMF colonization rather than comparing plants in the mycorrhizal and non-mycorrhizal condition. Such studies provide mechanistic insight in the direct and indirect effects of the AMF symbiosis on plant performance, but as mycorrhizal inoculum occurs ubiquitously in most agricultural fields, such studies at the same time lack relevance for the agronomist. Such studies are also prone to overestimating the mycorrhizal benefit under realistic conditions, which is one of the reasons that AMF benefits are more prominent in lab experiments than field experiments (Zhang et al. 2019). As various forms of management can result in different amounts of mycorrhizal inoculum potential in the field, studies with plants with different levels of mycorrhizal colonization might have more practical relevance for farmers as they provide indications how field management can be optimized for mycorrhizal functioning. The meta-analysis of Lekberg and Koide (2005) showed that AMF colonization can be increased by inoculation, followed by shortened fallow and reduced soil disturbance respectively. I have achieved different levels of AMF root colonization by inoculating AMF into non-sterilized field soil to increase AMF colonization. In rice fields, indigenous AMF are present, but the diversity and abundance may not be sufficient to produce benefits on the growth of rice plants. Hence, I was interested in knowing whether the increase of AMF colonization can also produce more benefits to host plants that can be of relevance for the farmers' practice. Furthermore, I studied the temporal effects of drought and AMF colonization on rice growth, drought recovery, nutrient uptake, photosynthetic activity related to stomatal conductance and efficiency of photosystem II (measured through chlorophyll fluorescence), and phytohormones such as abscisic acid (ABA) and indole-3-acetic acid (IAA). Lastly, I also investigated the potential to increase AMF colonization in rice via mixing two rice varieties to see more benefits of increasing AMF colonization. Intercropping and varietal mixing may be more efficient for increasing AMF in field crops because AMF inoculation can be costly for farmers.

In this final chapter, I first summarize the main findings of my studies (fig. 6.1) and discuss the relations between (i) drought and rice growth (Chapter 2, 3 and 4), (ii) AMF and drought tolerance of rice (Chapter 2, 3 and 4), (iii) increasing AMF colonization by AMF inoculation and varietal mixing (Chapter 5). Finally, I give recommendations for further research.

The main findings of this thesis are:

1) Drought reduces rice plant growth, and the effects are more severe when the soil water potential is reduced to below -40 kPa. The effects of drought are critical for rice grain yield when long severe drought occurs during tillering and the reproductive stage (Chapter 2 and 3).

2) Drought reduces rice plant stomatal conductance and chlorophyll fluorescence. These effects occur at different temporal scales (Chapter 2 and 3).

3) AMF colonization in rice grown in non-sterilized field soil with indigenous AMF increases with added commercial AMF inoculum.

4) The efficacy of AMF inoculation in relation to stomatal conductance, chlorophyll fluorescence and rice grain yield are greater in pot experiments than in field experiments (Chapter 2, 3 and 4).

5) Different rice varieties, selected for their drought tolerance show different levels of AMF colonization and differential effects of AMF-induced drought tolerance. The high and moderate drought tolerant variety SR1 had higher AMF colonization than KDML105, but the grain yield reduction under drought was lower in KDML105 than SR1 (Chapter 2 and 5).

6) The increase of AMF colonization is associated with the regulation of rice plant hormones such as IAA, retention of stomatal conductance, chlorophyll fluorescence, and increased leaf P concentration. These effects result in higher grain yield in rice plants with higher AMF colonization (Chapter 2 and 3).

7) Mixing two rice varieties increases AMF colonization and rice grain yield (Chapter 5).

In summary, drought affects rice growth and grain production. The increase of AMF colonization via AMF inoculation and varietal mixing can mitigate drought effects by maintaining photosynthetic activity, increasing nutrient uptake, and improving the capacity to recover. These benefits consequently result in less grain yield loss under drought (fig. 6.1). Although the effects of AMF are not grandiosely remarkable in this study, it demonstrates that even a small increase in AMF colonization can produce benefits for drought tolerance of rice plants, benefits that are likely to become more important under climate change.

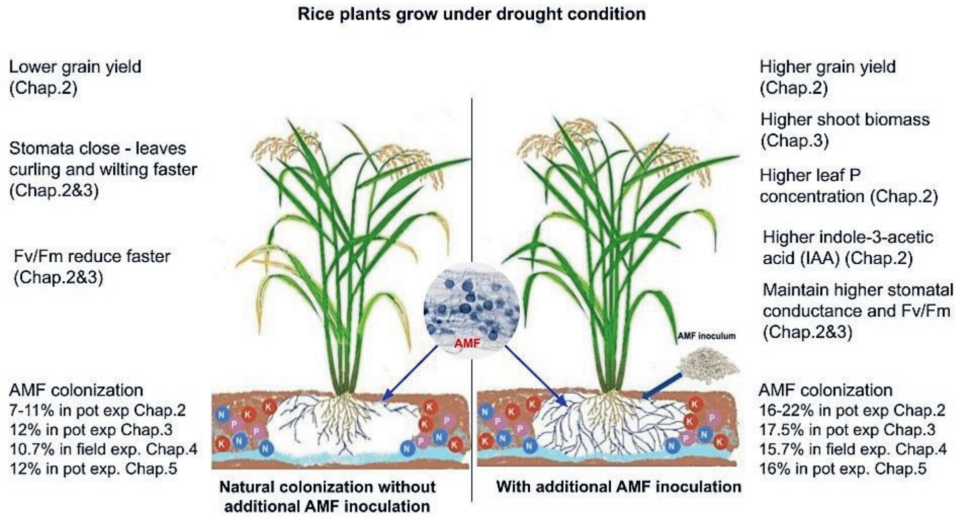


Figure 6.1: The benefits of increasing AMF colonization in rice plants grown in field soil under drought, as established in my thesis (with pertinent chapters in brackets) in which rice plant responses to drought under conditions without (left panel) and with AMF inoculation (right panel) are shown.

Drought and adaptation mechanisms in rice

Climate change and drought

Climate change affects precipitation because rising temperatures increase evaporation and at higher temperatures more humidity is stored in the air. This can result in more frequent or heavy rainfall in some areas, but also less rainfall in some other areas. The increase of weather variability can both cause extreme flooding and drought in many regions. Drought occurs more often, and results in decreasing crop productivity. Rice is one of the crops that is most affected by drought, having a much lower drought tolerance than the other major cereals like maize, wheat, sorghum, or barley. In the rain-fed rice cultivation areas in Thailand, farmers rely on rainfall for rice cultivation. In El Niño years, the drought period tends to be longer than in the past (WBG 2019). Thailand faced a major drought during 2015-2016, primarily affecting the northern and northeastern regions. Several provinces experienced water shortages, affecting agricultural activities and causing crop failures. Drought was even more severe in 2018-2020, to the extent that the government declared a state of emergency in some provinces. In 2023 El Niño impacted rice quality and productivity and even international rice trade. Rice quality in Vietnam has been affected by variation of rainfall, which caused too much rainfall during harvesting period in 2023. India and USA reduced the export of rice in 2023, and Thailand rice productivity may be reduced with about 2.5% due to drought. The reduction of productivity and export has already made the rice price increase to the highest level in 12 years (FAO Rice Price Update 2023).

The rice planting season in northeastern Thailand begins in May. In July till August there is usually a long period without rain, and the rainy season starts in September. Climate change may cause changes of drought which compromises the optimization of the rice planting scheme. For example, drought in Thailand occurs in late June-July, and mid-October-May. These changes may increase the risk of drought for rice productivity. In June and July rice is typically in its vegetative stage, and in October rice plants are in the grain-filling stage which is the most critical period for yield reduction. From December to May is the planting season for off-season rice which can be grown only in irrigated areas.

Drought effects on rice plant growth

Rice is a C_3 crop like wheat, but more susceptible to drought compared to other cereal crops like wheat (Kebede et al. 2019). It is known that rice has a small root system, and the stomatal closure is very sensitive to drought (Hirasawa et al. 1999). Insufficient water availability leads to lower uptake of nutrients, and results in stunted growth, reduced tillering, and grain filling, and ultimately reducing grain yield and yield quality. The level of soil water potential that I found to be critical for rice plant growth begins at -40 kPa. As of this level of water deficit drought can affect various physiological processes within rice plants such as stomatal conductance and chlorophyll fluorescence, which resulted in reduced photosynthesis.

Planting time is of key importance in the context of weather patterns because drought exerts different impacts at different growing stages of rice (fig. 6.2). During the vegetative stage, drought reduces the expansion of leaves and tillers. However, rice can withstand drought duration for around 14 days, after which they can recover and continue growing after receiving water again. When the drought period is longer than 14 days, the rice plants may collapse and not be able to recover. The most critical period for drought impacts on rice yield is the reproductive and grain filling stage (Farooq et al. 2009). Rice plants should have sufficient water to maintain the nutrients uptake and to allow grain filling. When due to drought the availability of N and P in the soil decreases with the reduction of soil moisture, this will impact grain filling and grain quality. In addition, drought can delay or shorten the flowering period, leading to poor pollination and fewer grains formed. These factors result in reduction of grain production.

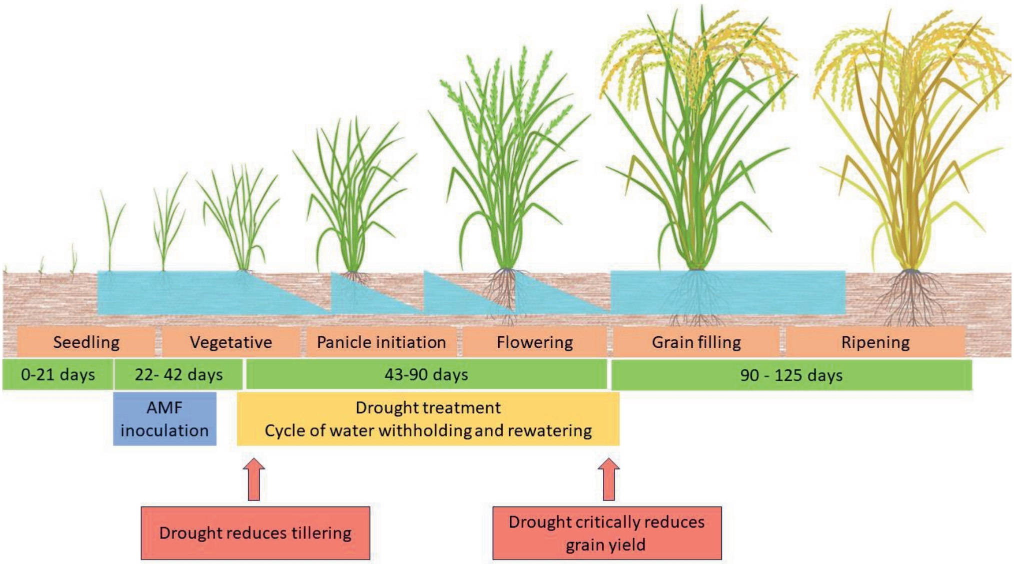


Figure 6.2: Rice growing stages, and drought impacts at different growing stages.

Rice plant adaptation mechanisms

When rice plants are subjected to drought, the plant hormone levels of ABA will increase and regulate stomatal behavior to reduce water loss via transpiration. However, high ABA can limit plant growth, and stomatal closure reduces CO_2 exchange which directly affects the photosynthetic activity. The stomatal closure of rice plants in my pot experiment (Chapter 3) started when soil water potential was reduced to below -40 kPa and was significantly reduced when soil water potential dropped below -70 kPa. After the stomatal closure, chlorophyll fluorescence decreases, and this reduces the photosynthetic activity of the plants. Prolonged or severe drought can drastically reduce plant stomatal conductance and chlorophyll fluorescence to irreversible damage.

Drought tolerant plant varieties may produce more roots to forage for water and nutrients under drought. I observed this in the first pot experiment as SR1 (the high drought-tolerant variety) produced higher root biomass than the other rice varieties. The review study of Farooq et al. (2009) points out that high-drought tolerant rice varieties have longer, thicker, and deeper roots, and thereby are able to access more water in deeper soil layers. However, in pot experiments with a fixed rooting depth, determined by the depth of the pots, varietal differences may be less relevant, and this may explain why drought tolerance behaved somewhat erratically between experiments.

Under drought, P availability decreases. As a response, plants will release strigolactone hormones in the rhizosphere to promote establishment of symbiosis with AMF. Under well-watered conditions, strigolactone exudation will be downregulated after the establishment of the mycorrhizal symbiosis. However, drought has been reported to increase strigolactone production in AMF plants (Ruiz-Lozano et al. 2016). AMF was one of the mechanisms mentioned in the program "Growing Rice Like Wheat", which was one of the programs of Wageningen University and Research to study the possibility of growing rice with less water, and drought tolerance mechanisms of rice.

Varieties and drought tolerance

I selected the rice varieties for the experiments in my thesis based on their drought tolerance and economical value. The drought tolerance of rice varieties from high to low can be ranked as SR1, KDML105, RD6, RD33, RD22 and CNT1. Drought-tolerant varieties may have root structure that allows them to forage for resources of water and nutrients under drought. Others can relate to the adaptability to drought such as drought escape and drought avoidance. Some plants can escape from drought by shortening the reproductive stage, for example, early flowering. Some plants adapt by regulating stomatal closure to prevent water loss or develop more roots to obtain the water. I did not study the root properties of the different rice varieties. If rooting structure or rooting depth play a role, the fixed pot size and depth may have precluded the effects of root properties in my pot experiments.

Under drought conditions, the reductions of root biomass were higher in CNT1, RD33 and RD 22 than SR1, RD6 and KDML105. However, the reductions of grain yield of SR1 were larger than CNT1, RD22, RD33, RD6 and KDML105 respectively. The interaction of variety x water (Chapter 2) showed that rice responds differently under different water treatments. SR1 may have escaped drought by maintaining higher root biomass under drought but had a higher reduction in grain yield. Meanwhile, KDML 105 and RD 6 had better drought tolerance by maintaining higher root biomass and grain yield. RD33, RD22 and CNT1 were not well adapted to drought as the root biomass was strongly affected by drought, and grain yield reductions were also high.

Temporal drought effects and plant recovery

The effects of drought on rice depend on drought severity and duration. In my experiments, I used type III drought according to He and Dijkstra (fig. 6.3), where drought and well-watered treatment start at the same moisture level. Then I maintained the soil moisture at around 0 kPa throughout the experiments, while letting the soil dry out in the drought treatment; and rewatering it when it reached a certain drought level (fig. 6.3). This drought pattern is most realistic in rainfed rice cultivation because drought can occur from lacking rainfall for some periods of time, after which rice can potentially recover when the rain comes again. Rice plants started to be affected by drought at 4 days after withholding water or when the soil water potential is below -40 kPa. This can be observed from the curling of the leaves. Stomatal conductance started to reduce, and continuously decreased in response to drought. The reduction became significant compared to the well-watered rice plants when the soil water potential was below -70 kPa. Stomatal conductance could not be measured after 12 days of withholding water when the soil water potential was below -90kPa. However, the rice plants recovered after receiving water again at 12 days after withholding water. The rice plants collapsed after suffering more than 14 days of drought or when soil water potential was lower than -100kPa. At this stage, it was difficult for rice plants to recover. The plant was not completely dead, but they had to start producing new shoot or tillers.

Chlorophyll fluorescence of rice plants started to reduce after 10 days of withholding water (-80 kPa), then drastically decreased after 12 days (<-90kPa). Chlorophyll fluorescence might start to reduce when plant stomata are almost completely closed.

The photosynthetic activity might critically reduce after this point. Chlorophyll fluorescence also recovered after re-watering. In practice, if rice plants do not get water for longer than 14 days, they may not be able to recover from drought effects.

Under moderate drought plants tend to close the stomata, after which the CO_2 uptake decreases but the photosynthetic capacity is initially maintained (Farooq et al. 2009). But when drought becomes more severe, the chloroplast will degrade, and the chlorophyll fluorescence will also reduce which results in decreasing photosynthetic capacity.

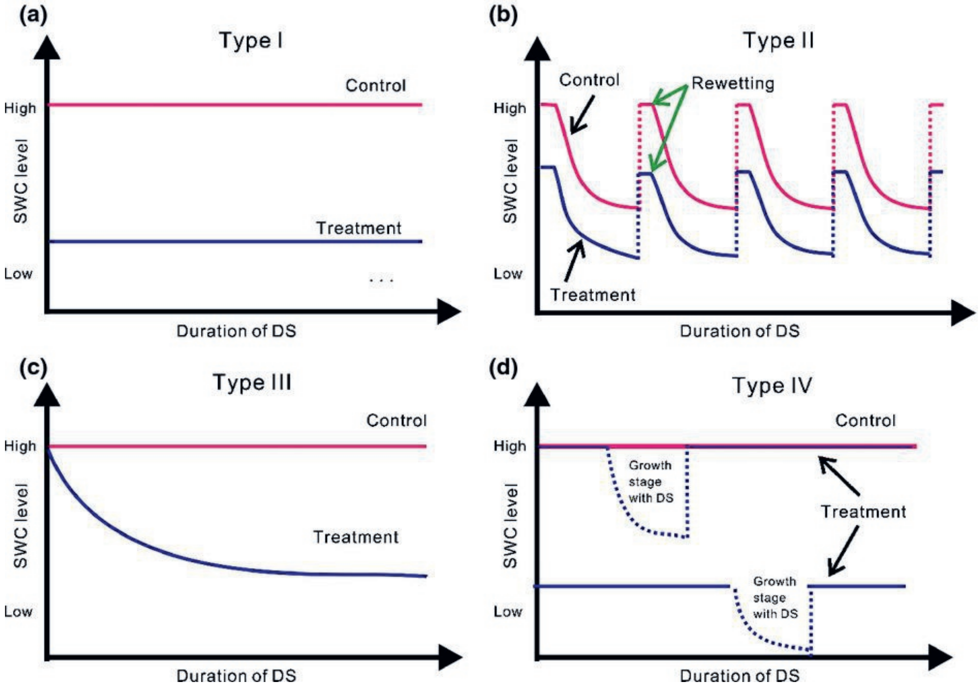


Figure 6.3: Schematic representation of the different main drought types. Type I: Drought results in a constant lower amount of soil water; Type II: Plants are exposed to different intensities of cycles of drying and rewetting; Type III: Due to less frequent rainfall events, soils dry out gradually to a lower water level; Type IV: Changes in rainfall patterns result in severe drought stress during different stages in the plant cycle (germination, onset of flowering or seed filling). N.B. Changes in frequency could also occur. From He and Dijkstra (2014). (DS = drought stress; SWC = soil water content)

AMF and drought tolerance of rice

The benefits of AMF on rice plant grown under drought

The pot experiments demonstrated the benefit of increasing AMF colonization by inoculation on rice grain yield in rice subjected to drought during their growth in field soil. The underlying mechanisms by which AMF alleviate drought stress in rice can be categorized as direct and indirect pathways. AMF may have extended the nutrients accessible to the plants via the AMF hyphae, so that the rice plants could reach sources of nutrients further away from the roots. It is still under debate whether AMF hyphae can also directly take up significant amounts of water. Ruth et al. (2011) reported that the AMF hyphae can directly and indirectly facilitate uptake of water by the plants. The possible mechanism for increasing water availability is likely to be the transportation of water via the surface of the hyphae rather than direct uptake. The size of the hyphae is too small to be able to take up and transport water to rice roots, hence the benefits from the AMF association are more related to increasing nutrient uptake that confers drought tolerance (Kuyper and Jansa 2023).

When rice plants are subjected to drought, plants will stimulate the production of the plant hormone ABA to regulate stomatal closure to reduce water loss and reduce growth hormones such as IAA. AMF colonization can regulate these plant hormones as demonstrated in Chapter 2. The pot experiment of Chapter 2 showed an increase of IAA hormone, and a trend of ABA reduction after mycorrhizal inoculation. This means AMF can maintain rice plant growth via this hormonal regulation, which can also be inferred from the increase of stomatal conductance and chlorophyll conductance of plants that have higher AMF colonization. The effects of AMF on maintaining rice stomatal conductance and chlorophyll fluorescence are also reported in the study by Porcel et al. (2015).

The benefit of having increased AMF colonization in rice plants is to maintain rice growth during a long and / or severe drought period. In my experiments, the rice plants collapsed after 14 days of drought. Higher AMF colonization can retain the plant stomatal conductance and chlorophyll fluorescence, which can maintain photosynthesis activity of the rice plant. These effects can extend the life of rice plants, expanding the time of being able to cope with drought till the next rewatering or rain event (Chapter 3). After rewatering, rice plants with high AMF colonization tended to recover faster from drought than rice plants with lower AMF colonization. This trend may infer that the recovery could be even slower in rice plant with no AMF colonization. In this case, the association with AMF may potentially facilitate rice to escape drought.

Comparing effects of high and low AMF colonization

Based on the available literature we always see a larger effect of AMF when comparing AMF plants with plants not colonized by AMF (Zhang et al. 2018). AMF are present in almost all soils and colonize plant roots of more than 90% of all plant species on this planet. Therefore, I decided to increase realism in AMF studies and to compare plant performance when grown in their actual field soil environment and compare the effects of higher (augmentation by inoculation) and lower (field soil inherent) AMF colonization. According to the meta-analysis of Lekberg and Koide (2005) increasing AMF colonization by either inoculation, shortened fallow or reduced soil disturbance increased crop yield around 23%, and increased shoot P concentration but had no significant effects on biomass at harvest.

Greenhouse and field experiments

The effects of AMF inoculation were larger in my pot experiments compared to my field experiments. This is because we could control more factors in the pots and greenhouse than in the field, even though I used non-sterilized soil from the field in the greenhouse experiments. The increase of AMF colonization in pot experiments was larger than in the field experiment. This could be due to other soil factors that may influence the colonization by AMF. In the pots rice roots have less space to grow which allows them to have more chance to contact with AMF and create more sites for colonization.

In addition, the manipulation of drought in the pot experiments is more controllable than in field experiments. In field experiments, climate factors such as wind, sunlight, temperature, fog and rainfall can affect soil moisture. These factors are the obstacles for manipulating drought in field experiments, thus the drought and AMF effects are smaller than in the pot experiments. Furthermore, most pot experiments compare non-mycorrhizal to mycorrhizal plants, therefore pot experiments likely overestimate the effects of AMF under field conditions.

Increasing AMF colonization

Using AMF inoculum in greenhouse and field experiments

I did not have native or Thai commercial inoculum when I did the experiments, so I used commercial granule inoculum from RootGrow Professional, Kent, UK. The inoculum contains five European-origin AMF species, *viz., Funneliformis mosseae, F. geosporus, Claroideoglomus claroideum, Glomus microaggregatum,* and *Rhizophagus irregularis* (Robinson Boyer et al. 2016). The spore density was about 10 spores per one gram of inoculum (Chareesri et al. 2020). A major question revolves around the potential risks of using exotic inoculum (Hart et al. 2018). For that reason, I also investigated the species composition of AMF communities in fifty rice fields around the Khon Kaen area, 5-80 km away from the experimental field where I executed a field experiment, as described in Chapter 4 (fig. 6.4). I collected soil samples, separated roots from rhizosphere soils, and extracted DNA. The material was sent to Genome Quebec for sequencing a 300-nucleotide stretch of the small subunit of the ribosomal cluster (SSU-rDNA), using the primers NS31 and AML2, in a 454-pyrosequencing. Obtained sequences of AMF were blasted against the MaarjAM database (Öpik et al. 2010) after which a phylogenetic analysis was executed. The results of the analysis are depicted in Figure 6.5.

The phylogenetic tree of the AMF shows that close relatives of *F. mosseae*, *F. geosporus*, *C. claroideum* and *R. irregularis* occur in rice fields around Khon Kaen. I was unable to obtain a SSU sequence of *G. microaggregatum* from MaarjAM or GenBank. However, a study by Sýkorová et al. (2016) had a commercial inoculum of *G. microaggregatum* sequenced; it turned out to be a species of *Diversispora*, a genus also well represented in my samples. My data therefore show relatively small differences in taxonomic composition between the AMF in the original field soil and the commercial AMF inoculum I used in my experiments. It is therefore unlikely that the beneficial effects of inoculation are due to particularly high performance of these exotic species. The relatively small increases in AMF colonization across the experiments described in my empirical chapters could indicate inoculum limitation in soils, due to the general management of those fields that include regular flooding. I did not assess the AMF species composition (native and introduced) in the pot experiments, so I cannot evaluate to what extent there was competition between both sources of inoculum for root space. The large similarity in species composition with indigenous and commercial inoculum argues, in my view, also against major hypothetical risks of invasive AMF species.

I observed an increase of AMF colonization under the drought treatment in the pot experiment (Chapter 2) and field experiment (Chapter 4). However, the colonization in the well-watered treatment was higher than in the drought treatment in another pot experiment (Chapter 3). In the varietal-mixture pot experiment (Chapter 5), there was no significant difference in AMF colonization between the well-watered and drought treatment. Variation of AMF colonization under different watering regimes has been reported in different studies. A study by Das et al. (2022) reported that the AMF colonization in rice roots grown at – 15 and 30kPa soil water potential was significantly lower than in soil at 0kPa. However, their reported colonization levels (around 50%) were much higher than what I observed. They also reported increased AMF colonization with increasing phosphorus application, a pattern rarely seen with AMF. In other crops, moderate drought increases AMF colonization in pasture legumes (Jongen et al. 2022), and in wheat (Rehman et al. 2022).

I expected AMF colonization under drought to be higher than in the well-watered treatment, because plants most likely establish the symbiosis with AMF under stressed conditions with more severe nutrient-limitation, especially P-limitation, more than in more favorable conditions. However, if the water deficit becomes too severe, the AMF colonization can be reduced as well. This can be due to the decay of the plant roots that dried out and died when suffering severe drought. In addition, plants may not have enough carbon available for AMF, if drought reduces photosynthesis, and there may be an unfavorable cost:benefit ratio of the AMF symbiosis under severe drought conditions.

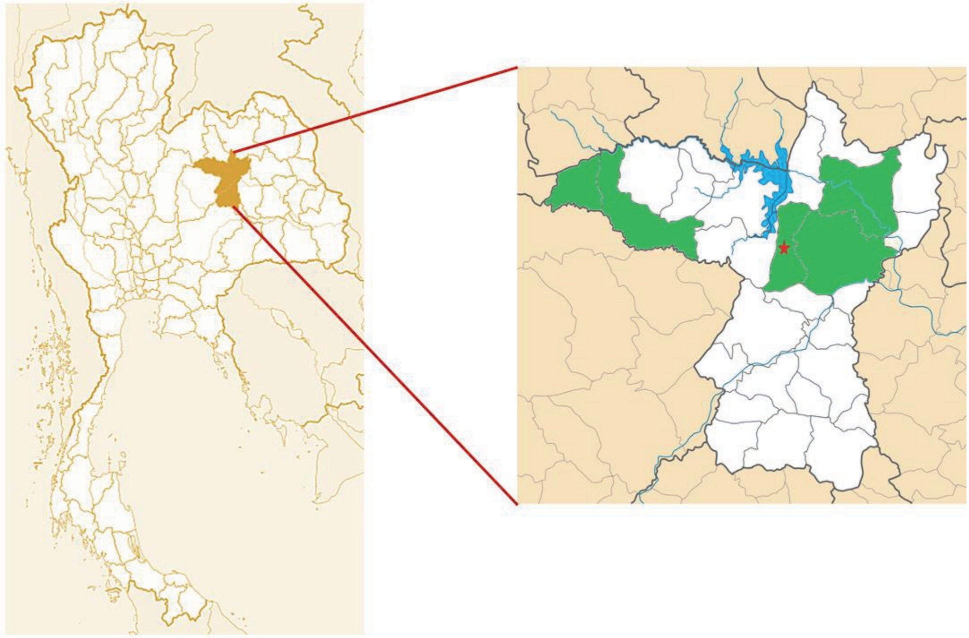
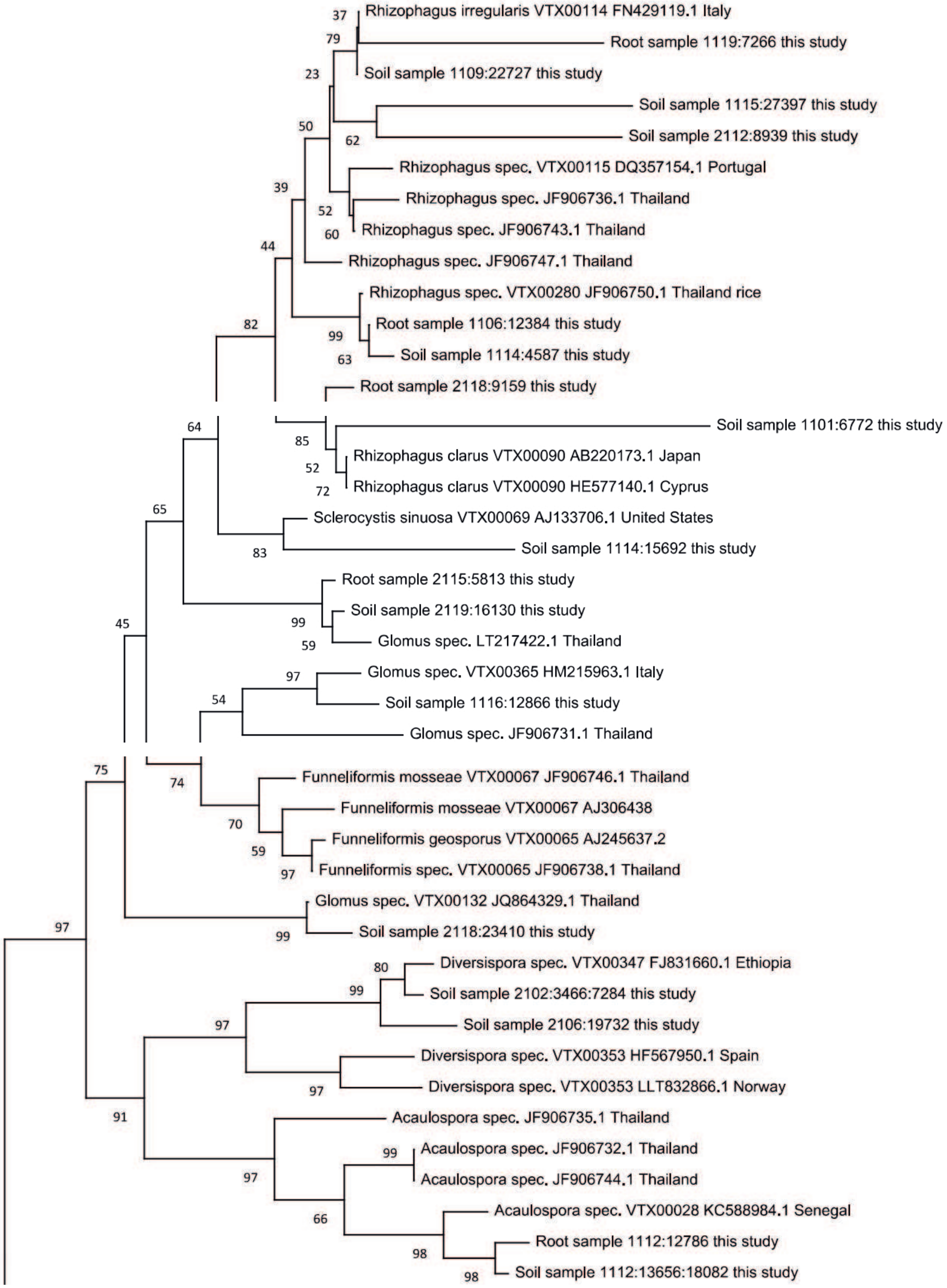


Figure 6.4: The map of the area in Thailand where I collected paddy soil and rice root samples for AMF species identification. The green areas on the map are the sampling areas. The red star is the location of the field experiments and the soil I used for the pot experiments.


37
Rhizophagus irregularis VTX00114 FN429119.1 Italy
79
Root sample 1119:7266 this study
23
Soil sample 1109:22727 this study
Soil sample 1115:27397 this study
50
62
Soil sample 2112:8939 this study
Rhizophagus spec. VTX00115 DQ357154.1 Portugal
39
Rhizophagus spec. JF906736.1 Thailand
52
60
Rhizophagus spec. JF906743.1 Thailand
44
Rhizophagus spec. JF906747.1 Thailand
Rhizophagus spec. VTX00280 JF906750.1 Thailand rice
82
Root sample 1106:12384 this study
99
Soil sample 1114:4587 this study
63
Root sample 2118:9159 this study
64
Soil sample 1101:6772 this study
85
Rhizophagus clarus VTX00090 AB220173.1 Japan
52
72
Rhizophagus clarus VTX00090 HE577140.1 Cyprus
65
Sclerocystis sinuosa VTX00069 AJ133706.1 United States
Soil sample 1114:15692 this study
83
Root sample 2115:5813 this study
Soil sample 2119:16130 this study
45
99
Glomus spec. LT217422.1 Thailand
59
97
Glomus spec. VTX00365 HM215963.1 Italy
54
Soil sample 1116:12866 this study
Glomus spec. JF906731.1 Thailand
75
Funneliformis mosseae VTX00067 JF906746.1 Thailand
74
Funneliformis mosseae VTX00067 AJ306438
70
Funneliformis geosporus VTX00065 AJ245637.2
59
97
Funneliformis spec. VTX00065 JF906738.1 Thailand
Glomus spec. VTX00132 JQ864329.1 Thailand
97
99
Soil sample 2118:23410 this study
80
Diversispora spec. VTX00347 FJ831660.1 Ethiopia
99
Soil sample 2102:3466:7284 this study
97
Soil sample 2106:19732 this study
Diversispora spec. VTX00353 HF567950.1 Spain
97
Diversispora spec. VTX00353 LLT832866.1 Norway
91
Acaulospora spec. JF906735.1 Thailand
99
Acaulospora spec. JF906732.1 Thailand
97
Acaulospora spec. JF906744.1 Thailand
66
Acaulospora spec. VTX00028 KC588984.1 Senegal
Root sample 1112:12786 this study
98
98
Soil sample 1112:13656:18082 this study

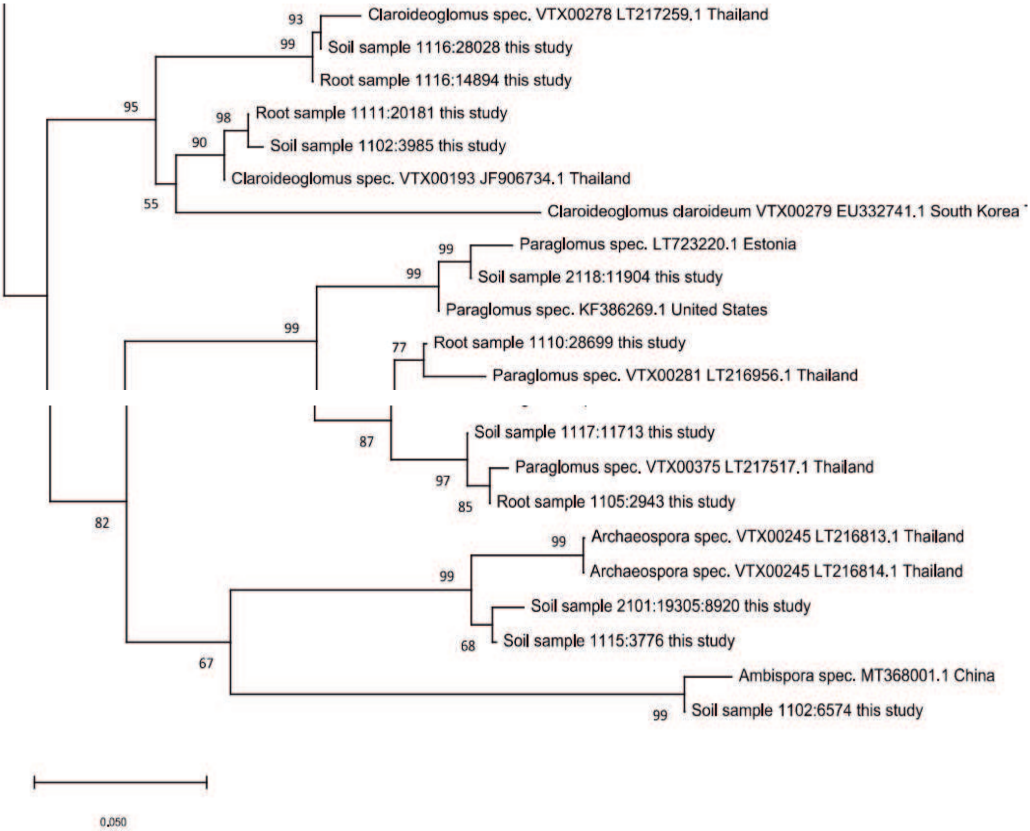


Figure 6.5: The phylogenetic tree of AMF species found in the rice fields in Khon Kaen, Thailand. Phylogenetic tree (Neighbor Joining, Bootstrap n = 10,0000) based on SSU rDNA sequences obtained after 454-pyrosequencing. Sequences from this study involve 50 samples in rice fields, separated in root samples and rhizosphere (soil) samples. Sequences from Thailand starting with JF9067 are from rice fields studied by Watanarojanaporn et al. (2013), sequences from Thailand starting with LT21 are from rubber plantations and cassava fields studied by Herrmann et al. (2016). VTX refer to Virtual Taxa in the MaarjAM database by Öpik et al. (2010).

Increasing AMF colonization via farm management such as varietal mixture or intercropping

For farmers inoculation of AMF inoculum in field crops may not be the most efficient practice in terms of cost and management. Although I have successfully increased AMF colonization in the field experiment by using AMF inoculum, the increase in colonization was not as high as in my pot experiments. Alternative options to increase mycorrhizal colonization, and hence mitigate the effects of drought on rice performance, should therefore be considered. One other option to increase AMF in rice fields is farm management, for instance intercropping. Intercropping is the cultivation method where a mixture of different plant species or different varieties of the same species are grown together. Intercropping of complementary crops can benefit in increasing aboveground and belowground plant biomass, as well as biodiversity. Previous studies have shown that intercropping can increase AMF colonization. The higher interspecific genetic diversity through intercropping could provide more niches for AMF, resulting in higher AMF species richness and root colonization and subsequently plant benefits. However, it is less likely that this mechanism is important when different varieties of the same species are grown together. Nevertheless, in an experiment by Wang et al. (2020), growing two maize varieties increased the colonization of AMF and extraradical hyphal length in maize roots. The authors also noted that there was a positive correlation between the increase in fungal performance, as assessed through intraradical colonization or extraradical hyphal length, and maize performance, suggesting that the intercropping benefits were driven by the AMF symbiosis. Yield benefits of varietal mixing was also absent when the plants were non-mycorrhizal. In my pot experiment (Chapter 5), growing KDML105 and SR1 rice together increased AMF colonization. In my earlier pot experiment (Chapter 2) I found that SR1 had higher AMF colonization than KDML105, so growing these two varieties together may have complementarity effects on increasing AMF colonization. This increase was positively correlated with an mixture-driven increase in grain yield, confirming that mixtures of different varieties does not only result in yield increases due to rice blast suppression (Zhu et al. 2000), but also in yield increases due to enhanced AMF performance.

Other agronomic practices to increase AMF colonization

Alternate wetting and drying

Submerged rice fields are not a favorable condition for AMF, although there are some AMF species that are found in submerged rice roots. Several studies have compared AMF species composition under different agronomic practices, but linking this differential species composition to actual rice performance has been challenging. Under submerged conditions for longer than two months the presence of AMF in rice fields can be significantly reduced (Vallino et al. 2009, Lumini et al. 2011). Alternate wetting and drying (AWD) cycles are a cultivation method that farmers use to manage the field by having some period that they let the field dry to reduce water use and increase soil oxygen. This drying period with increased oxygen availability in the soil may enhance AMF viability and increase AMF colonization of rice roots. Under AWD the drying period does not cause water deficiency for rice and is therefore important as a water-saving mechanism. It is not clear how AWD will impact AMF functioning, a topic that should be further investigated. Whereas the drought scheme that I applied in my experiments is somewhat like the practice of AWD, the severity of water shortage in my experiments was much higher than under AWD; and results of my experiments cannot therefore be easily linked to that practice.

The system of rice intensification (SRI) is a specific rice-growing practice that not only involves water management, like AWD, but also the replacement of mineral fertilizer by compost and a different management of seedlings (transplanting seedlings at a younger stage, singly, and with wider spacings). Effects of SRI on the functioning of the AMF symbiosis have hardly been investigated apart from a study by Watanarojanaporn et al. (2013) on species composition of rice fields under conventional management and SRI. Their study indicated higher species richness and higher root colonization by AMF under SRI than under conventional management.

Fertilizer management

Intensive farming with heavy use of mineral fertilizer often reduces the abundance and activity of AMF in the soil (Verbruggen and Kiers 2010). Whereas the application of mineral fertilizer will increase plant performance like the effects of AMF, indirect effects of AMF, as for instance through conferring enhanced drought tolerance, might not be mimicked by mineral fertilizer. Also, the amount of fertilizer savings should not be neglected. A study by Zhang et al. (2015) showed that grain yield of mycorrhizal rice that received around 20-40% of the local fertilizer-P application was like that of non-mycorrhizal yield that received a full fertilizer-P dose. Reducing mineral fertilizer in rice cultivation may increase AMF presence and colonization. Verbruggen et al. (2013) has proposed three success factors for increasing AMF colonization consisting of (i) species compatibility with native species, (ii) field carrying capacity (the field condition may not favorable and weaken the capacity of inoculum), and (iii) priority effects (the presence of eventual native species that may be very competitive).

Applying inoculum prior to planting

It is still impractical to apply AMF to field crops, because it requires a huge amount of inoculum and additional labor and thus requires financial investment. The possible way to use less inoculum can be through applying AMF in the nursery prior to transplanting in the field. This will save the amount of inoculum and cost for the farmers. Solaiman and Hirata (1997) reported that inoculation of rice in the nursery stage under both dry and wet conditions increased root colonization and plant performance. After transplanting the young rice plants in the field these plants that were nursery-inoculated did not produce more shoot biomass, but the grain yield was significantly increased (+15%).

Limitations of experimental setup

Drought manipulation in field experiments

In field experiments, it might be difficult to manipulate drought cycles consistently according to the experimental method. The soil might dry out faster or slower depending on the weather, and rainfall.

Land levelling is one of the most important aspects to take care off for field experiments concerning water treatments. In my experiment I did not have laser land levelling equipment, as a result there were still some slopes in the field plots. This unevenness caused difficulty for water management and for imposing equal treatment levels of flooded or drought conditions, respectively.

Other uncontrollable factors in field experiment

In the field experiment, non-sterile soil can contain millions of soil organisms. There are other factors that might influence the AMF, for instance earthworms, antagonistic microbes, and other plant endophytes that can compete with AMF for root colonization. Earthworms or other animals in the soil may have influences in soil disturbance and the colonization of AMF. Zaller et al. (2011) reported that earthworms could alleviate AMF colonization via their burrows which facilitated plant roots and AMF symbiosis. The variation of these organisms in the field might not be the same throughout the field, and this might cause some different effects on AMF and rice plant growth.

Further study

Effects of other root-inhabiting fungi

AMF are not the only root-inhabiting fungi that have positive impacts on plant performance. Recently there has been increased interest in the role of other root-inhabiting endophytic fungi (Andreo Jimenez et al. 2019). Molecular assessment of sequences from my 50 field plots (fig. 6.4) yielded sequences of these root-endophytic fungi. However, the phylogenetic resolution of SSU-rDNA was inadequate to identify these fungi on species or genus level. For that pyrosequencing or Illumina barcoding based on ITS sequences would have been required.

Endophytic fungi live inside plant tissue, but do not form a symbiosis that extends into the soil like AMF. However, co-inoculation of an endophyte and AMF can reduce AMF colonization in rice roots, for instance, AMF and *Trichoderma harzianum* can compete for root space for colonization (Green et al. 1999).

Intercropping rice with legume

I found benefits of mixing two rice varieties on AMF colonization and rice grain yield. However, in practice mixtures of two rice varieties may not be practical in the field for rice quality reasons, because of the difference in harvesting date, and market quality demand. Leguminous plants are commonly grown after rice harvest for green manure, and they can be sown at the same time as rice. Several studies have assessed the performance of rice-legume intercrops, and the role of the AMF symbiosis in intercropping. Li et al. (2009) observed that intercropping rice with mung bean increased mycorrhizal colonization, especially in rice. Because of enhanced mycorrhization, P uptake in rice also increased. There were no effects of intercropping on shoot or root biomass of rice; unfortunately, no data on changes in yield were reported. When legume plants decompose in the field, they will increase soil N, and this legacy effect would also have beneficial effects on rice grain yield under conditions of N-limitation.

AMF colonization at different growth stages of rice

In my experiments I quantified AMF root colonization at harvest, that is at the end of the growing period. Assessment at that time may overlook functional consequences of increased AMF colonization at earlier growth stages, such as onset of flowering or grain filling. My observations that mycorrhizal effects were larger on grain yield than on aboveground biomass suggest that enhanced mycorrhizal activity at earlier growth stages is critical. A study by Zhang et al. (2015) confirmed a major mycorrhizal role on resource allocation. Mycorrhizal plants allocated more carbon, nitrogen, and phosphorus to grains than non-mycorrhizal plants, suggesting that early uptake of nutrients might be paramount to achieve high yields. It would therefore be important to sample the roots and quantify AMF colonization at different growing stages. The effects of sampling time should both be investigated for well-watered plants, and for plants subjected to drought, where timing of the applied drought treatment in relation to both plant and AMF phenology is important. In my experiments I induced drought 14 days after transplanting, as I assumed that by then the AMF had colonized the rice roots. If my assumption was right, AMF may have started contributing to drought tolerance of rice since the vegetative stage. Then the benefits will become larger when the colonization increases at the flowering and grain filling stage. However, it is still unclear to me whether the AMF benefits I observed are resulting from the increase of colonization at specific rice growing stages.

Appendix

The appendix is added to clarify the figures in Chapter 2. Chapter 2 has been published in Mycorrhiza (2020), so I do not want to change it in the chapter. I found the letters indicating significant differences between treatments on top of the bars not straightforward in figure 2.2, 2.3 and 2.4. This might be unclear. In this appendix the figures are revised to a simpler form.

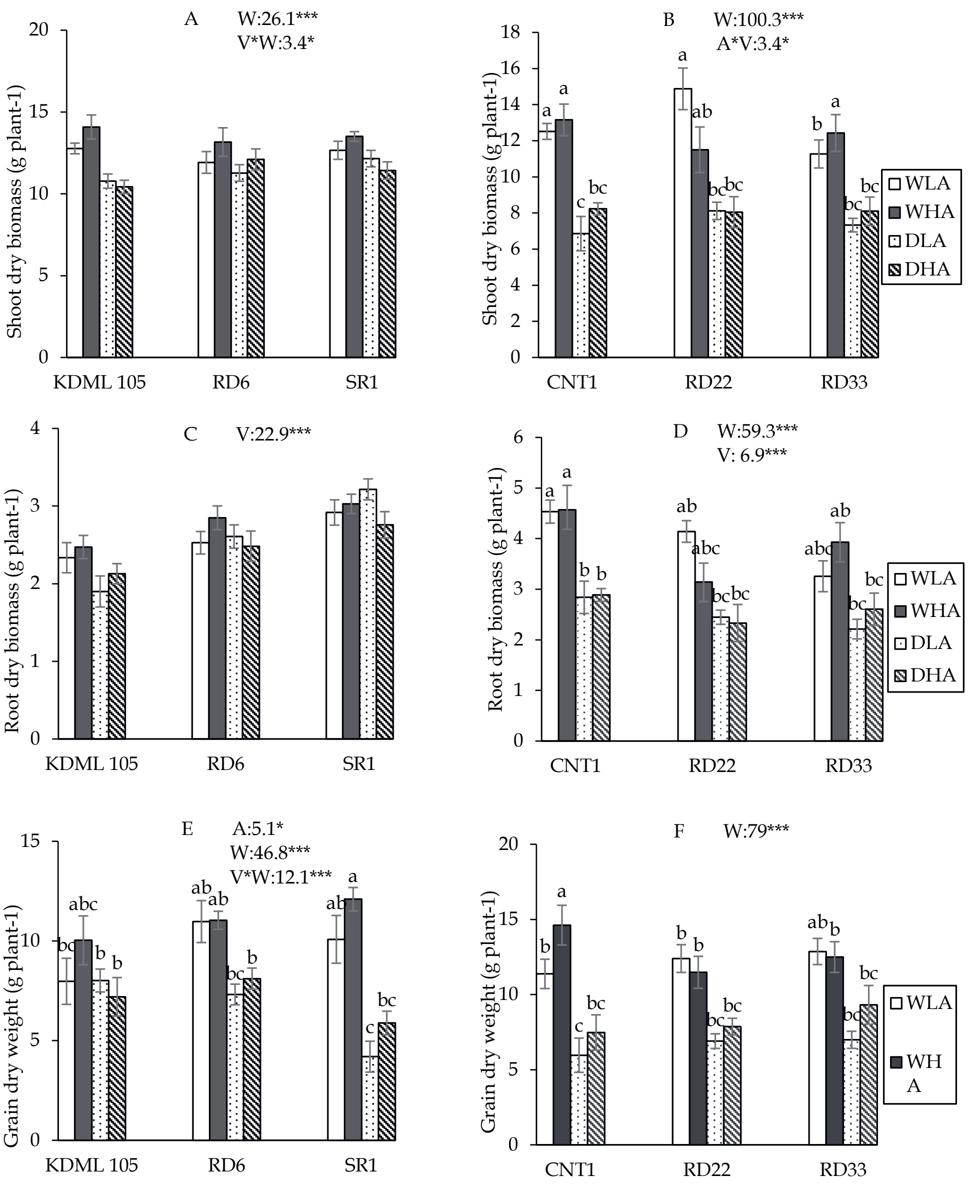


Figure 2.2: The shoot and root biomass and grain yield of six rice varieties in relation to drought and AMF inoculation. A, C, E= Experiment 1 and B, D, F = Experiment 2. WLA = Well-watered and low AMF, WHA = Well-watered and high AMF, DLA = Drought and low AMF and DHA = Drought and high AMF. The error bar is related to SE. Bars topped by the same letter are not significantly different by Tukey HSD ($P < 0.05$). The significant effects of mycorrhiza (A), Water (W) and variety (V) and their interactions are presented as the F value with test of significance (*$P< 0.05$; ** $P < 0.01$; *** $P < 0.001$) of

three-way ANOVA. Non-significant factors are not shown (see Supplementary Table 3 and Table 4 for more information).

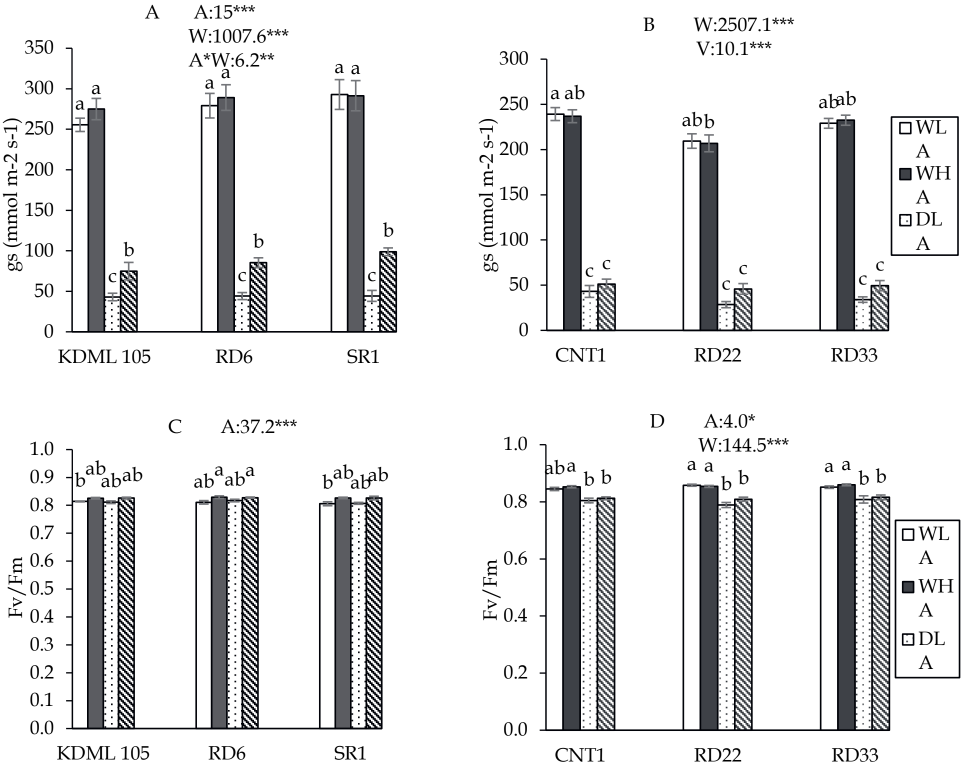


Figure 2.3: The stomatal conductance (g_s) and the maximum quantum efficiency of PS II photochemistry (F_v/F_m). A, C= Experiment 1 and B, D = Experiment 2. WLA = Well-watered and low AMF, WHA = Well-watered and high AMF, DLA = Drought and low AMF and DHA = Drought and high AMF. The error bar is related to SE. Bars topped by the same letter are not significantly different by Tukey HSD ($P < 0.05$). The significant effects of mycorrhiza (A), Water (W) and variety (V) and their interactions are presented as the F value with test of significance ($^*P< 0.05$; ** $P < 0.01$; *** $P < 0.001$) of three-way ANOVA. Non-significant factors are not shown (see Supplementary Table 3 and Table 4 for more information).

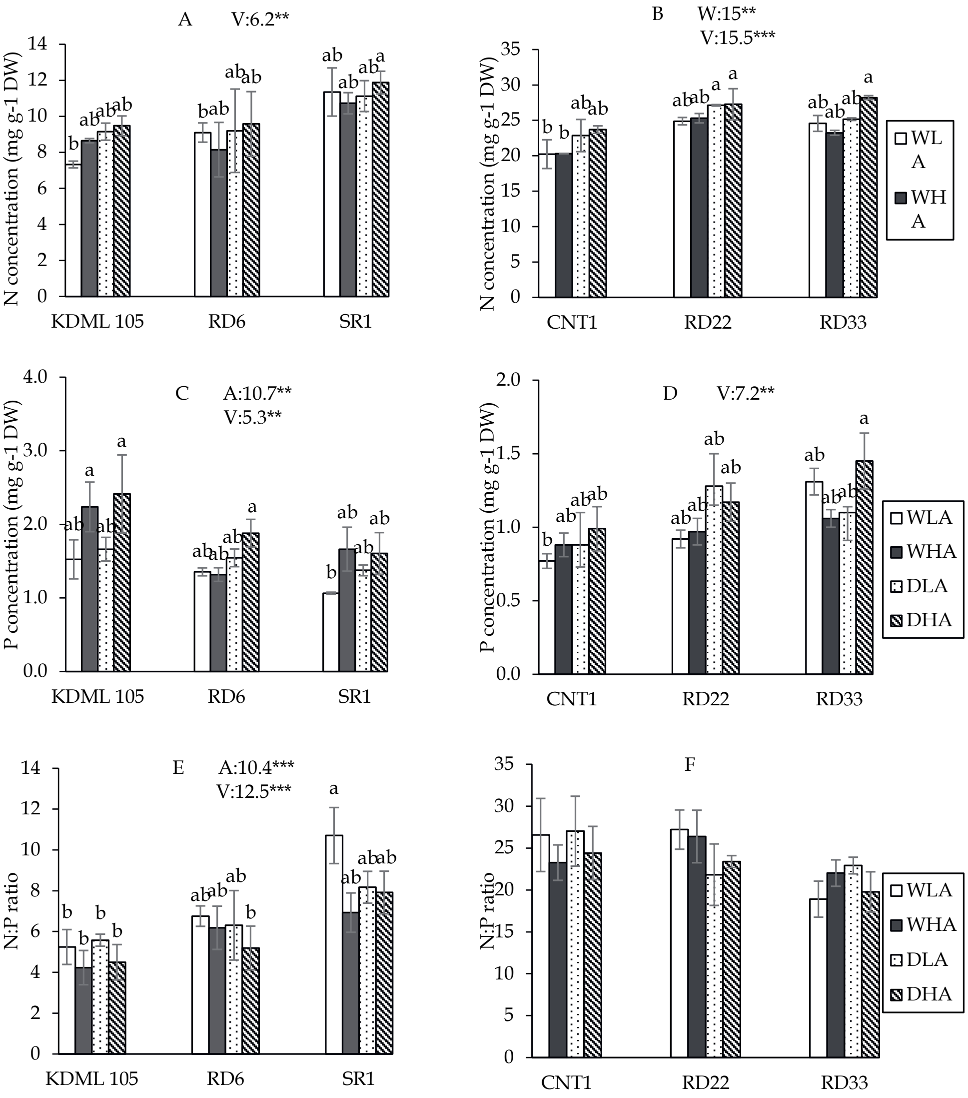


Figure 2.4: N, P concentrations in rice leave and N:P ratio. A, C, E = Experiment 1 and B, D, F = Experiment 2. WLA = Well-watered and low AMF, WHA = Well-watered and high AMF, DLA = Drought and low AMF and DHA = Drought and high AMF. The error bar is related to SE. Bars topped by the same letter are not significantly different by Tukey HSD (*P* < 0.05). The significant effects of mycorrhiza (A), Water (W) and variety (V) and their interactions are presented as the F value with test of significance (**P*< 0.05; ** *P* < 0.01; *** *P* < 0.001) of three-way ANOVA. Non-significant factors are not shown (see Supplementary Table 3 and Table 4 for more information).

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Summary

Rice (*Oryza sativa*) is one of the staple foods consumed by more than half of the world population. Most rice production comes from rainfed rice cultivation, where its productivity is mainly dependent on rainfall. Increased variation of rainfall due to climate change leads to more frequent and more severe drought in many regions affecting crop production including rice. Naturally, rice can adapt to drought conditions by producing more roots, reducing water loss via stomatal closure, regulating plant growth hormones, and early maturation. Many studies have focused on plant breeding and farm management to improve or benefit more from these drought adaptation mechanisms. Furthermore, the association of rice roots with soil microorganisms such as arbuscular mycorrhizal fungi (AMF) have a potential in improving drought tolerance of rice. AMF benefits for plant performance are well known such as increased nutrient uptake especially phosphorus (P). Studies have also reported the benefits of AMF for improving plant drought tolerance. However, the role of AMF and its mechanisms in alleviating rice drought tolerance have not been investigated. Thus, this dissertation focuses on investigating roles and mechanisms of AMF symbiosis on improving rice growth under drought conditions. Furthermore, this study extends the knowledge of increasing AMF colonization in rice via varietal mixing to increase the potential for alleviating drought tolerance for rice plants. More background, knowledge gaps, and research questions are described in Chapter 1.

In Chapter 2, the role of AMF on alleviating drought tolerance of six rice varieties (KDML105, RD6, SR1, CNT1, RD22 and RD33) were tested in pot experiments in two rice cropping seasons. I used unsterilized soil in the experiments to compare the benefits of low (natural) and high (additional inoculation) AMF colonization. This method is more realistic to actual farming practice in which the farmers may apply AMF inoculum in rice fields, compared with experiments where mycorrhizal and non-mycorrhizal plants are

compared under controlled conditions. AMF inoculation significantly increased AMF colonization in rice roots between low and high AMF colonization treatments. Drought at -40 kPa and -80 kPa soil water potential decreased the growth of rice plants and grain yield. The reduction of rice grain yield was lower in rice plants in high AMF colonization treatments. These AMF benefits are related to the roles of AMF in regulating plant growth hormones such as abscisic acid (ABA) and indole acetic acid (IAA), P uptake, leaf stomatal conductance, and chlorophyll fluorescence or photosystem II (PSII) of rice plants. AMF tended to reduce ABA and increase IAA in rice leaves. Rice plants with higher AMF colonization had higher leaf P concentration, stomatal conductance and PSII. These pathways contribute to sustaining rice productivity under drought conditions.

In Chapter 3, my aim was to gain more insight into the roles of AMF in maintaining rice leaf stomatal conductance and PSII under drought conditions. The pot experiments in Chapter 2 showed the increase of stomatal conductance and PSII in rice leaf when AMF colonization was higher, but the temporal pattern of these processes was not well understood. The pot experiment was set up to investigate the temporal effects of drought and AMF colonization on stomatal conductance and PSII. Over 14 days of drought, leaf stomatal conductance started to decline after four days subjected to drought, and PSII started to decline after eight days of drought. The reductions of rice leaf stomatal conductance and PSII were delayed by the increase of AMF colonization. This resulted in higher shoot biomass and tendency to have a higher grain yield in rice plants with higher AMF colonization. This delay and lower reduction of stomatal conductance and PSII could also increase the chance for rice plants to recover from drought.

In Chapter 4, the discovered benefits of AMF in alleviating negative consequences of drought in pot experiments, described in Chapter 2 and Chapter 3, were tested in field conditions. I hypothesized that the benefits of increasing AMF colonization in the pot experiments will occur as well in the field. Like pot experiments, drought significantly reduced rice biomass and grain yield, and AMF colonization increased with addition of AMF inoculum. However, the increase of AMF colonization did not provide a significant effect on rice performance under drought conditions. We could not firmly conclude that AMF has an effect on improving drought tolerance of rice, because other field factors may have limited the beneficial AMF effects.

Increasing AMF colonization in rice fields by applying AMF inoculum may not be an efficient practice for farmers, because it requires large amounts of inoculum and high investment cost for farmers. Farm management such as varietal mixing can be an alternative to increase field colonization. In Chapter 5, I investigated the benefits of varietal mixing on increasing AMF colonization and thereby to increase drought tolerance of rice. The pot experiments were conducted by growing a mixture of two rice varieties (KDML105 and SR1), compared with monocultures of those two rice varieties. The complementarity of mixtures of two rice varieties enhanced AMF colonization and promoted rice grain yield. The effect of varietal mixing was larger in well-watered conditions compared to drought conditions. The increase of AMF colonization was positively correlated with relative yield total (RYT) of rice grain weight, but not for shoot and root biomass. The results of this experiment indicate that varietal mixing could be a potential means to increase AMF colonization in rice fields and thereby enhance drought tolerance of rice plants.

In Chapter 6, the four empirical chapters were discussed according to the main findings and relevant literature. The benefits of AMF in alleviating drought tolerance of rice were pointed out as shown by possible explanations related to AMF and drought tolerance mechanisms. In conclusion, increasing AMF colonization in rice roots can potentially enhance rice performance under drought conditions, by regulating plant hormones, increasing P uptake, maintaining stomatal conductance and PSII. However, the effects of AMF are less in field experiments than in pot experiments. Increasing AMF inoculum potential can be done by applying AMF inoculum, and, more efficiently and more cost-effective, by mixing varieties. Further research should be carried out in relation to other root-inhibiting fungi that may influence AMF benefits and play roles in drought tolerance of plants. Furthermore, the study of AMF colonization and effects at different rice growing stages may provide more insight about the crucial timing of AMF benefits. Lastly, increasing AMF colonization by intercropping rice with other crop such as legumes in rice field can be another challenge that needs to be investigated.

List of publications

1. **Chareesri A**, De Deyn GB, Sergeeva L, Polthanee A, Kuyper TW (2020) Increased arbuscular mycorrhizal fungal colonization reduces yield loss of rice (Oryza sativa L.) under drought. Mycorrhiza 30(2): 315-328.
https://doi.org/10.1007/s00572-020-00953-z

2. Ros M B, Hiemstra T, van Groenigen J W, **Chareesri A**, Koopmans G F
(2017) Exploring the pathways of earthworm-induced phosphorus availability.
Geoderma, 303, 99-109.
https://doi.org/10.1016/j.geoderma.2017.05.012

3. Rasmussen P U, **Chareesri A**, Neilson R, Bennett A E, Tack A J (2019) The
impact of dispersal, plant genotype and nematodes on arbuscular mycorrhizal fungal colonization. Soil Biology and Biochemistry, 132, 28-35.
https://doi.org/10.1016/j.soilbio.2019.01.018

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review/project proposal (9 ECTS)

- Arbuscular mycorrhizal fungi and drought tolerance of rice

Post-graduate courses (4.2 ECTS)

- Introduction to R for statistical user; PE&RC (2017)
- Mixed linear model; PE&RC (2017)
- Soil ecology; PE&RC (2019)
- World soil and their assessment; ISRIC (2019)

Laboratory training and working visits (1.8 ECTS)

- International training on in vitro culture of AMF; Universite Catholique de Louvain, Belgium (2019)

Invited review of journal manuscripts (2 ECTS)

- Plant biology: AM fungi reduce susceptibility of rice to root knot nematodes and striga (2016)
- Pedosphere: improvement of growth and yield of maize plant *Zea mays L.* fertilized with rock phosphate and triple superphosphate under water stress by co-inoculation with *Funneliformis mosseae* and *Pseudomonus fluorescens* (2017)

Competence, skills and career-oriented activities (4.3 ECTS)

- Data management; WGS (2015)
- Reviewing a scientific paper; WGS (2017)

- Scientific writing; WGS (2018)
- Scientific publishing; WGS (2018)
- Ethics in plant and environmental sciences; WGS (2023)
- Apply for Adobe Illustrator scientific artwork & infographics; WGS (2023)

PE&RC Annual meetings, seminars and the PE&RC retreat (1.8 ECTS)

- PE&RC First year weekend (2015)
- PE&RC Day (2015)
- PE&RC Last year weekend (2018)

Discussion groups/local seminars or scientific meetings (4.5 ECTS)

- Plant-soil interaction; Wageningen University (2016-2017)
- Agri-systems group meeting; Khon Kaen University (2016-2017)
- SOQ Discussion group; Wageningen University (2017-2019)

International symposia, workshops and conferences (5 ECTS)

- Global soil biodiversity conference; oral presentation; China (2017)
- International conference of Mycorrhizae; poster presentation; Mexico (2019)

About the author

Anupol Chareesri was born on 8th October 1985 in Amnatchroen Province, Northeastern Thailand. He was born in a rice farmer family, and surrounded by rice farmer neighbors. During his childhood, he learned about growing rice and helped his parents in rice farming since he was eight years old. Anupol always wanted to help rice farmers to have a better life and improve their wellbeing.

In 2004, he was the only student from the district that got the government scholarship under One District One Scholarship program. This scholarship offered him an opportunity to study aboard. He chose to study in a Bachelor of Agri-Systems Management at Van Hall Larenstein University of Applied Science in Deventer (later moved to Wageningen), the Netherlands. After graduation, Anupol went back to work with the organic rice farmer group in Yasothorn Province, which is neighboring to his hometown. After two years of work, he liked to gain more knowledge and experience. He applied and got the scholarship for a Master and PhD study in soil biology and biodiversity from the Agriculture Research and Development Agency (ARDA), Thailand.

In 2012 Anupol was back in Wageningen to study in the MSc Environmental Sciences, specializing in soil biology. He did a Master thesis about phosphorus availability in earthworm casts. Then, he had a chance to work with arbuscular mycorrhizal fungi during his internship at the James Hutton Institute, Dundee, Scotland. He got really interested in arbuscular mycorrhizal fungi. After his MSc graduation, he contacted Prof. Dr Thomas W. Kuyper to discuss about the possible research for his PhD. In 2015, he started his PhD under the supervision of Prof. Dr Thomas W. Kuyper, Prof. Dr Gerlinde B. De Deyn, and Prof. Dr Anan Polthanee. After a long journey of his study, Anupol has gained more knowledge about soil biology, arbuscular mycorrhizal fungi and rice with which he can go back to Thailand and contribute his knowledge and experience to help rice farmers in Thailand.

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