Rice flooding negatively impacts root branching and arbuscular mycorrhizal colonization, but not fungal viability

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Abstract

Rice is mostly cultivated in wetlands, where arbuscular mycorrhization (AM) is reported to decrease. The mechanisms regulating such events are largely unknown. Rice uninoculated and inoculated with Rhizophagus irregularis were grown in dry and flooded conditions, allowing also for the transfer of plants from one water regime to the other. Roots were sampled at different times, from 7 to 35 d post-inoculation (dpi). The morphological and molecular parameters (root branching, aerenchyma formation, mycorrhizal colonization, AM marker gene expression) were evaluated. Root branching was more pronounced in dry conditions, and such phenotype was enhanced by the fungus. In wetlands, the colonization level was comparable till 21 dpi, when the mycorrhization then decreased, paralleled by an increase in aerenchyma. Expression of the fungal transporters was comparable under the two conditions. The root apparatus, when shifted from one water regime to the other, rapidly adapted to the new condition, revealing a marked plasticity. The reversibility of the AM rice symbiosis was also mirrored by expression changes of plant marker genes. The results demonstrate that the water regime is the driving force that regulates AM colonization under flooding conditions, by directly influencing root architecture and anatomy, but without impacting the basic AM functionality.

Introduction

As one of the most important food sources for humans, rice (Oryza sativa) plays a vital role for society and economics worldwide. Rice is cultivated in environments characterized by marked differences in soil waterlogging, changing in depth (from several metres to absence), and in duration (from the entire growing season, to transient periods or absence). During the 1990s, almost 90% of the world's rice was harvested from wetland (lowland) areas; the remaining harvest came from dryland (upland) systems (FAO 2004). Wetland rice is grown in waterlogged soils until harvest time, whereas in dryland systems, rice, like other cereals, is grown on drained soils (Grist 1986). The relationship between rice and water is complex, and flooding triggers several physiological, physicochemical and microbiological processes (De Datta 1995; Colmer 2003; Winkel et al. 2013), which may influence the plant biology and interactions.

It is known that rice plant roots form symbiotic associations with arbuscular mycorrhizal fungi (AMF). AMF belong to the phylum Glomeromycota and are considered among the oldest groups of organisms living in symbiosis with terrestrial plants (Redecker, Kodner & Graham 2000). AMF improve plants' access to phosphates, nitrogen and other mineral nutrients in exchange for photosynthates provided by the host plant. They also play important roles in triggering biotic and abiotic resistance (Pozo & Azcón-Aguilar 2007; Estrada et al. 2013), and in improving water use and plant performance, both in natural and agricultural ecosystems (Smith & Read 2008).

Understanding the response of rice to the interaction with AMF is becoming increasingly important in order to enhance plant yield and to promote sustainable agriculture. In this context, rice represents one of the model plants to understand the molecular, biochemical and physiological basis of arbuscular mycorrhiza (AM) symbiosis in non-legume plants (Paszkowski et al. 2002; Güimil et al. 2005; Sawers, Gutjahr & Paszkowski 2008; Campos-Soriano, García-Garrido & San Segundo 2010; Yang et al. 2012).

Notwithstanding the increasing amount of data published on rice–AM interactions, the mechanisms that regulate AMF colonization of rice roots in flooded fields are largely unknown. In recent decades, the occurrence of AMF colonization of rice roots under submerged conditions has been under debate. Ilag et al. (1987) stated that it is rare or absent because of the anoxic environment, while Barea (1991) concluded that AMF are obligate aerobes in nature, but are able to survive in waterlogged conditions. Solaiman & Hirata (1998) found a lower rate of AMF colonization of rice roots in wet than in dry conditions, and the rate of AMF colonization decreased during rice growth. Vallino et al. (2009) and Lumini et al. (2011) demonstrated the absence of AMF colonization in roots of rice plants grown in flooded fields after 4 and 2 months of submersion, respectively. Lumini et al. (2011) also showed that the rate of AMF rice root colonization increased over the plant life cycle only in dryland systems, and confirmed that waterlogged soils contained sufficient AMF propagules to infect plants when the soil was dried.

Recently, there has been an increasing awareness of the occurrence of AMF in various wetland ecosystems (Miller 2000; Nielsen et al. 2004; Wirsel 2004; Ipsilantis & Sylvia 2007; Wang et al. 2011), leading to the conclusion that soil conditions determine the mycorrhizal status of the host. In particular, Miller (2000) and Wang et l. (2010) found a decrease in the degree of AMF colonization with flooding along wetland gradients, while Wirsel (2004) indicated that permanent flooding could even eliminate the association. A clear explanation of these results is that AMF cannot tolerate microaerophilic conditions. Along this theme, some authors suggested that the presence of AMF in wetland ecosystems was closely related to the well-developed aerenchyma present in wetland plant roots (Miller 2000; Wang et l. 2010), which allows AMF to obtain atmospheric O2 (Purakayastha & Chhonkar 2001). However, the observations of Cornwell, Bedford & Chapin (2001) did not agree with this. They showed that the mycorrhizal status of aquatic monocot and dicot plants differed greatly, and that some monocots, which have a much higher percentage of air-filled aerenchyma in their roots than dicots (Crawford 1989), were generally not mycorrhizal. The authors suggested that this was not due to the reduced level of oxygen by itself, but rather that the specific chemistry and microenvironment of flooded soils might cause a lack of AMF colonization. Therefore, no clear explanation seems to satisfactorily describe the interactions among plant roots, water and AMF: alternative models also still need to be found for the wetland rice ecosystem, where a further level of complexity exists. It is a known fact that rice plants have three types of roots: crown roots (CRs), which emerge from the nodes on the stem and tillers; large lateral roots (LLRs), which originate from CRs and display indeterminate growth; and fine lateral roots (FLRs), which originate both from CRs and LLRs and whose growth is determinate (Gutjahr, Casieri & Paszkowski 2009; Rebouillat et al. 2009). This complex root architecture may be influenced by environmental factors (Osmont, Sibout & Hardtke 2007; Hodge et al. 2009), among which includes water management (Justin & Armstrong 1987; Parent et al. 2008; Kato & Okami 2011). Among the biotic factors, it is known that AM colonization has a deep impact on the root systems of many plants (Price, Roncadori & Hussey 1989; Yano, Yamauchi & Kono 1996; Berta, Fusconi & Hooker 2002; Paszkowski & Boller 2002; Oláh et al. 2005). However, in the case of the wetland rice, it is not clear whether the low percentage of AM colonization is sufficient to impact the rice root system, as reported in upland conditions (Gutjahr et al. 2009), or whether the AM fungus is directly affected by water management in its extraradical phase, and therefore, it cannot exert an action on the rice root architecture.

The general goal of this work was to elucidate the primary factors that control the colonization dynamics of rice roots by AMF in different water regimes (such as flooding and dry conditions), through morphological and molecular approaches. In doing so, the relationship between rice root architecture and water treatment was first taken into account, moving from upland to wetland conditions and vice versa. As a second step, the colonization intensity of the AM fungus Rhizophagus irregularis under the same changing conditions was evaluated, and lastly, the symbiosis functionality was evaluated through the gene expression analysis of both plant and AMF marker genes. The results demonstrate that the water regime is the driving force that first influences the root architecture leading to a change in the number of LLRs. This is the parameter that has the most impact on the success of fungal colonization. The results also demonstrate that AMF can thrive under flooding conditions, keeping their nutrient transporters regularly expressed, but are impaired in their colonization capacities. This is due to the increase of the aerenchyma tissues, which are not compatible for AM development. Lastly, the functional markers of the AM symbiosis mirror such events, revealing a significant decrease in the expression of plant and fungal nutrient transporters under flooding conditions.

Materials and Methods

Biological materials and growth conditions

Seeds of O. sativa cv. Selenio (provided by the Rice Research Unit of the Agricultural Research Council, Vercelli, Italy) were germinated in pots containing sand and incubated for 7d in a growth chamber under a 14h light (23°C)/10h dark (21°C).

R.irregularis (DAOM 197198), previously identified as Glomus intraradices Schenck and Smith (Krüger et al. 2012), was produced in monoxenic cultures maintained on Agrobacterium rhizogenes-transformed chicory roots (Bécard & Fortin 1988) in two-compartment Petri plates, as described in Pérez-Tienda et al. (2011). The plates were incubated in the dark at 24°C until the fungal compartment, which contained a solid M medium without sucrose (M–C medium; Chabot, Bécard & Piché 1992), was profusely colonized by the fungus (approximately 6 weeks). The extraradical mycelium (ERM) was harvested from the fungal compartment by dissolving the solid medium with a 10mM pH 6.0 citrate buffer (1.8mM citric acid and 8.2mM sodium citrate).

Mycorrhizal roots were obtained by means of two methods: a sandwich method and a microcosm method. For the sandwich method (Supporting Information Fig.S1), 1-week-old O. sativa seedlings were first inoculated with R. irregularis ERM between two sterile nitrocellulose membranes, as described in Guether et al. (2009a). Then, the seedlings, kept between the membranes, were transferred to pots containing sterile quartz sand. Seedlings of non-mycorrhizal plants were also kept between membranes. For the microcosm experiments, 1-week-old seedlings were transferred to pots containing sterile quartz sand and inoculation with R. irregularis was performed by placing small portions of ERM on the surface of the rice root system without using nitrocellulose membrane.

Plants were grown in 9-cm-high and 11-cm-diameter pots and maintained in a growth chamber, as described above, until harvesting. For exposure to flooding conditions, plants were kept in a water-filled container, and the water level was maintained so that it was 2cm above the sand level (Supporting Information Fig.S1). Plants grown in both dry and flooding conditions were watered three times a week with tap water and every 2 weeks with a Long-Ashton solution containing a low Pi concentration (32µM Na2HPO4·12H2O; Hewitt 1966).

Plants grown using the sandwich and microcosm methods were either kept in the same water condition until harvest ('time course experiment'), or shifted from one condition to the other ('shift experiment'). For the 'time course experiment', plants were collected at different time points starting from 7 (sandwich samples) or 14 (microcosm samples) days post-inoculation (dpi) with R. irregularis, and sampled every 7d until 35dpi. For the 'shift experiment', plants were first grown in dry or in flooded conditions for 21 or 28dpi, then moved to the other water condition, grown for a further 7d and then sampled (Supporting Information Fig.S2).

Morphometric analysis

In order to describe root system architecture, the length of CRs and the number of LLRs were annotated manually for each plant. Portions of the root system from each mycorrhizal plant were collected to assess the level of mycorrhizal formation. Mycorrhizal roots were stained with cotton blue and the level of mycorrhizal formation was assessed according to Trouvelot, Kough & Gianinazzi-Pearson (1986). Over 80cm of root for three biological replicates were considered for each condition. Statistical tests were carried out through one-way analysis of variance (one-way anova) and Tukey's post hoc test, using a probability level of $P<0.05$.

To examine the presence of aerenchyma, different portions of the sampled CRs and LLRs were embedded in 8% low-melting-point agarose and sectioned with a series 1000 Microtome Sectioning System (Vibratome, St. Louis, MO, USA). Cross sections, 100µm thick, were observed under a light microscope [Primo Star Zeiss (Carl Zeiss MicroImaging, Göttingen, Germany) with a Leica DFC425 digital camera (Leica Microsystems, Wetzlar, Germany) attached].

Nucleic acid extraction and cDNA synthesis

Total genomic DNA was extracted from R. irregularis ERM and O. sativa shoots using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Plant and fungal genomic DNAs were used to test each primer pair designed for real-time PCR to exclude cross hybridization.

Total RNA was extracted from rice roots of mycorrhizal and non-mycorrhizal plant grown via the sandwich method using the Plant RNeasy Kit (Qiagen), according to the manufacturer's instructions. Samples were treated with TURBO™ DNase (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The RNA samples were routinely checked for DNA contamination by means of RT-PCR (OneStep RT-PCR, Qiagen) analysis, using OsRubQ1 (Güimil et al. 2005; Table1). For single-strand cDNA synthesis, about 700ng of total RNA was denatured at 65°C for 5min and then reverse-transcribed at 25°C for 10min, 42°C for 50min and 70°C for 15min. The final volume was 20µL and contained 10µm random primers, 0.5mm dNTPs, 4µL 5X buffer, 2µL 0.1m DTT and 1µL Super-Script II (Invitrogen, Carlsbad, CA, USA).

Table 1. List of primers used in this study

Primer ID	Primer sequences [5'-3']
GintEF α f	GCTATTTTGATCATTGCCGCC
GintEF α r	TCATTAAAACGTTCTTCCGACC
GintPTf	AACACGATGTCAACAAAGCAAC
GintPTr	AAGACCGATTCCATAAAAAGCA
GintAMT2f	AGTGCCAATGCCGCTAACATA
GintAMT2r	TGATGTACCTCCAACAATTCCA
OsPT2f	GACGAGACCGCCCAAGAAG
OsPT2r	TTTTCAGTCACTCACGTCGAGAC
OSRubQ1f	GGGTTCACAAGTCTGCCTATTTG
OSRubQ1r	ACGGGACACGACCAAGGA
OSPT11f	GAGAAGTTCCCTGCTTCAAGCA
OSPT11r	GAGAAGTTCCCTGCTTCAAGCA
OsAMT3.1f	TCCACCAAGCACGGATGGTACT
OsAMT3.1r	CATCAGACGTTCTGCGTGACGC

Real-time quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed using an iCycler apparatus (Bio-Rad, Hercules, CA, USA). Each PCR reaction was carried out in a total volume of 20µL containing 1µL diluted cDNA (about 20ng), 10µL 2X SYBR Green Reaction Mix and 3µL of each primer (3µm). The following PCR programme was used: 95°C for 90s, 50 cycles of 95°C for 15s, 60°C for 30s. A melting curve (80 steps with a heating rate of 0.5°C per 10s and a continuous fluorescence measurement) was recorded at the end of each run to exclude the generation of non-specific PCR products (Ririe, Rasmussen & Wittwer 1997). All reactions were performed on at least three biological and three technical replicates. Baseline range and Ct values were automatically calculated using iCycler software. Transcript levels were normalized to the Ct value of GintEF1α (Gonzàlez-Guerrero et al. 2010) for the fungal genes GintPT (Fiorilli, Lanfranco & Bonfante 2013) and GintAMT2 (Pérez-Tienda et al. 2011), and OsRubQ1 (Güimil et al. 2005) for the plant genes OsPT2, OsPT11 (Güimil et al. 2005) and OsAMT3.1 (Table1). Only Ct values leading to a Ct mean with a standard deviation below 0.5 were considered (Pérez-Tienda et al. 2011). Statistical tests were carried out through one-way anova and Tukey's post hoc test, using a probability level of $P<0.05$. All statistical elaborations were performed using PAST statistical package (version 2.16; Hammer, Harper & Ryan 2001).

Phosphate quantification in mycorrhizal roots

Rice shoots and roots of mycorrhizal and non-mycorrhizal plant grown via the sandwich method were powdered in a mortar under liquid nitrogen. Aliquots of about 3mg of dry material were suspended in 1mL of twice-distilled water containing 20mg of insoluble PVPP to remove phenolic compounds. After shaking for 1h at 4°C, samples were boiled for 15min, and centrifuged for 5min and then for 10min, at 16.000g and 4°C. The clear supernatant was used for Pi analysis by anion-exchange chromatography. In all samples, anions were separated on a IonPac column (AS9-SC, 250×4mm; Dionex, Sunnyvale, CA, USA) eluted with a mixture of 1.8mM Na2CO3 and 1.7mM NaHCO3 at a flow rate of 1.1mLmin−1. Pi was detected by a conductivity detector module (CDM, Dionex). Ten biological replicates were considered for each biological condition. Statistical tests were carried out through one-way anova and Tukey's post hoc test, using a probability level of $P<0.05$.

Results

Dry versus flooding conditions impact the root branching

In a pilot experiment, the root systems of rice plants grown in dry condition were compared with the root systems of plants grown in flooding condition. Morphological differences were already apparent at the macroscopic level, where roots grown in dry condition exhibited a more pronounced branching (Supporting Information Fig.S3). As the most clear differences seemed to be caused by a different number of LLRs along the length of the CR, the ratio between these two parameters was named as the branching index (BI=nLLR/cmCR), which describes the degree of the root apparatus branching (Gutjahr et al. 2009).

FLR dynamics were not investigated because in agreement with Gutjahr et al. (2009), AMF colonization was absent also in the FLRs of the Selenio cultivar.

Root branching was first examined in a time course experiment utilizing two sets of mycorrhizal (+Myc) and non-mycorrhizal (-Myc) rice plants grown in flooding and dry conditions, using both the sandwich and the microcosm methods.

Data obtained are summarized in Table2 and shown in Fig.1 (sandwich method) and Supporting Information Fig.S4 (microcosm method). The BI of the rice root apparatus grown in dry conditions was higher than the BI of roots grown in flooded conditions. In view of the sandwich method (Fig.1), the BI of –Myc plants grown under flooded conditions was quite stable and ranged between 0.1 and 0.4, while the BI of plants grown in dry conditions ranged between 0.4 and 1.4, peaking at 21dpi. As an average, the BI of plants grown in dry conditions is about three times the BI of plants grown in flooded conditions (Table2). Similar results were obtained from the microcosm method, as the average BI for plants grown in dry conditions was two times higher than the BI of plants grown in flooded conditions (Supporting Information Fig.S4, Table2).

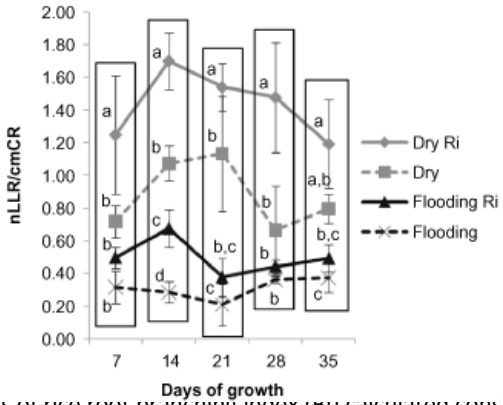

Figure 1. Time course analysis of rice root branching index (BI) calculated considering the ratio between the number of large lateral root (LLR) and cm of crown root (CR) in: mycorrhizal plants grown in dry (dry Ri) and in flooding (flooding Ri) conditions and non-mycorrhizal plants grown in dry (dry) and flooding (flooding) conditions. Plants were sampled after 7, 14, 21, 28 and 35d of growth using the sandwich method. Error bars represent the SD of three replicates. One-way analysis of variance (anova) and Tukey's post hoc test were performed considering data coming from a single time point (rectangular box): different letters indicate a significant difference ($P<0.05$).

Table 2. Branching index (BI) values of mycorrhizal plants grown in dry (dry Ri) and in flooding (flooding Ri) conditions, and non-mycorrhizal plants grown in dry (dry) and flooding (flooding) conditions at 7, 14, 21, 28 and 35dpi, using both the sandwich (s) and microcosm (m) methods. The two growth methods gave comparable results. The upper part shows the BI values for each condition; the lower part shows the ratio between BI values of all different conditions. Mean values were calculated among values of the different time points.

Days of growth	Branching index (nLLR/cmCR)							
	Dry Ri		Dry		Flooding Ri		Flooding	
	s	m	s	m	s	m	s	m
7	1.3		0.7		0.5		0.3	
14	1.7	1.3	1.1	0.9	0.7	0.5	0.3	0.4
21	1.5	1.2	1.1	0.9	0.4	0.7	0.2	0.4
28	1.5	1.0	0.7	0.9	0.4	0.5	0.4	0.5
35	1.2	1.1	0.8	0.8	0.5	0.6	0.4	0.5
Mean value	1.4	1.1	0.9	0.9	0.5	0.6	0.3	0.4

Days of growth	Ratio of branching index									
	Dry Ri/dry		Flooding Ri/flooding		Dry/flooding		Dry Ri/flooding Ri		Dry Ri/flooding	
	s	m	s	m	s	m	s	m	s	m
7	1.7		1.6		2.3		2.5		3.9	
14	1.6	1.4	2.3	1.3	3.7	2.4	2.5	2.6	5.9	3.4
21	1.4	1.3	1.8	1.8	5.4	2.4	4.1	1.7	7.3	3.1
28	2.2	1.1	1.2	1.1	1.8	1.8	3.4	1.8	4.1	2.0
35	1.5	1.4	1.3	1.1	2.1	1.5	2.4	1.8	3.2	2.1
Mean value	1.7	1.3	1.7	1.3	3.1	2.0	3.0	2.0	4.9	2.6

In the presence of the fungus (+Myc plants), a similar phenomenon was observed (Fig.1, Supporting Information Fig.S4). The BI of plants grown in dry conditions ranged from 2.4 to 4.1 fold (for the sandwich method) and from 1.7- to 2.6-fold (for the microcosm method) higher than the BI of plants grown under flooding conditions. However, irrespective of the environmental conditions, the presence of the fungus led the BI values to nearly double when the sandwich method was utilized (Table2): in fact, under flooded conditions, the BI value was between 0.3 and 0.7, while under dry conditions, it ranged between 0.9 and 1.9. Similar results were obtained from the microcosm method, where the presence of the fungus increased the BI values almost one and a half times (Supporting Information Fig.S4, Table2).

Since it is well known that AMF stimulate root branching (Oláh et al. 2005; Maillet et al. 2011), it can be hypothesized that a synergic effect occurs between the fungal presence and the positive impact of the aerated soil. In fact, BI values of roots grown under dry conditions in the presence of the fungus were about five times (for the sandwich method) and three times (for the microcosm method) higher than the BI values of roots grown under flooded conditions and without the fungus (Table 2).

Dry and flooding conditions change the degree but not the phenotype of AM colonization

The degree of fungal colonization was determined on roots of plants grown in the presence of R. irregularis after cotton blue staining. Roots were first observed under a stereomicroscope (Fig. 2a,b,c), and intraradical and extraradical fungal mycelia were clearly visible. Colonization was detected in CRs and in LLRs, but was absent in FLRs. While in the LLRs, the fungus extensively occupied the root (Fig. 2b), in CR, fungal structures were unevenly located (Fig. 2c), and this pattern was more evident in plants grown in flooded conditions. Each observed plant exhibited the typical AM structures, such as hyphopodia, intracellular hyphae, arbuscules and vesicles (Fig. 2d,e,f,g). In addition, the morphology of the arbuscules in dry versus flooding conditions was comparable (Fig. 3a,b).

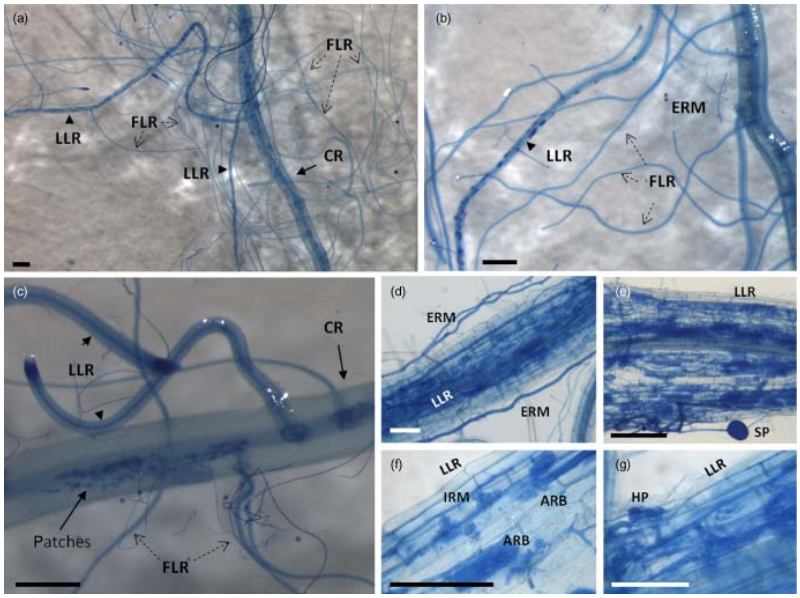

Figure 2. Root system of rice plants grown using the sandwich method in dryland conditions at 28dpi with R. irregularis. The roots are cotton blue stained. (a) The three types of roots are shown: crown roots (CR, arrows); large lateral roots (LLR, arrowheads); fine lateral roots (FLR, dashed arrows). (b) A general view of the LLRs shows a widespread colonization, while no intraradical fungal structures are detectable inside the FLR, notwithstanding the web of extraradical mycelium (ERM). (c) A detail of the CR roots shows how fungal structures have a patchy location. (d–g) Details of LLR colonization and extraradical and intraradical fungal structures: ERM; spore (SP); hyphopodium (HP); intraradical mycelium (IRM) and arbuscules (ARB). Scale bars: 1mm (a–c); 100µm (d–g).

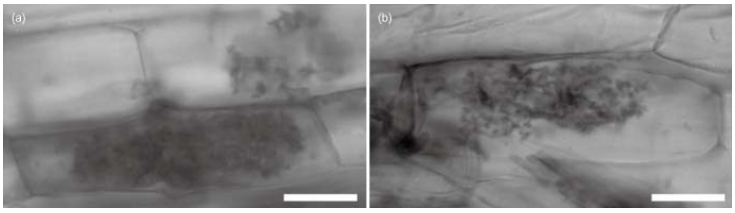

Figure 3. Morphology of R. irregularis arbuscules in rice roots growing in dry (a) and flooding (b) conditions is comparable. Scale bars: 40µm.

A quantitative analysis of the colonization revealed that the intensity of mycorrhization (M%) and the arbuscule abundance (A%) for the sandwich method increased from 7 to 21dpi under both dry and flooding conditions. However, starting from 21dpi there was a significant increase in the M% and A% under dry conditions, and a dramatic decrease under the flooded conditions. Comparing the degree of colonization in dry versus flooding conditions, the greatest difference in M% and A% was detected at 35dpi where the value was six times lower because of flooding (Fig. 4). Similarly, in the microcosm method, the M% and A% increased significantly only after 21dpi under dry conditions (Supporting Information Fig. S5).

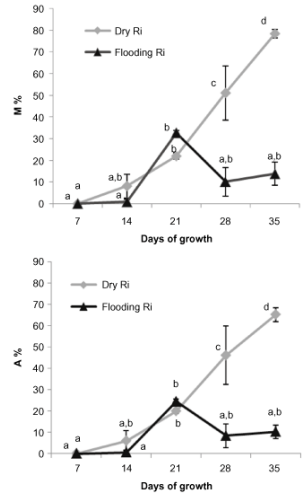

Figure 4. Time course experiments illustrating the intensity of the mycorrhizal colonization (M%) expressed as the percentage of the root length colonized by R. irregularis and arbuscule abundance (A%) of the same samples in the root system of mycorrhizal rice plants grown using the sandwich method, under dry and flooding conditions at 7, 14, 21, 28 and 35dpi. One hundred root fragments of 1cm lengths were analysed for each sample. Data are mean values of three replicates. Error bars indicate SD. Different letters indicate a significant difference ($P<0.05$).

When the BI and the root colonization values were compared, a temporal delay between the two parameters became apparent. The effect of water regime on root branching was almost immediate (7d), while the effect on mycorrhization was significant after 21d of growth.

When the two colonized root types were considered separately, the value of the two parameters was always higher in LLRs than in CRs (Fig.5a,b). After 28d of growth in flooding conditions, however, the difference was no longer significant.

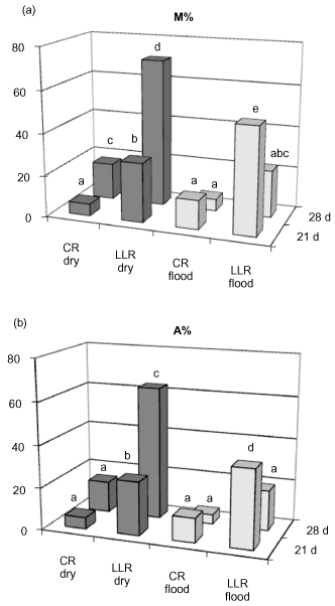

Figure 5. (a) Intensity of the mycorrhizal colonization (M%) expressed as the percentage of the root length colonized by R. irregularis and (b) arbuscule abundance (A%) of the same samples in crown root (CR) and large lateral root (LLR) of rice plants grown, using the sandwich method, under dry and flooding conditions at 21 and 28dpi. One hundred

root fragments of 1cm lengths were analysed for each sample. Data are the mean values of three replicates. Different letters indicate a significant difference ($P<0.05$).

To better understand why the AMF were losing their colonization capacities under flooding conditions, even if they could thrive at least for the first 21d, roots were further inspected under a light microscope to see whether there was a correlation between their anatomy and AM colonization. The observations revealed a negative correlation between the aerenchyma presence in the LLRs and the AM colonization percentage. In fact, in early stages of growth, aerenchyma was present only in CR in comparable amount in plants grown under dry and flooded conditions, while, afterwards, aerenchyma almost completely occupied also LLRs roots grown under flooding conditions (Fig. 6). By contrast, the presence of the aerenchyma in the CRs was relatively unchanged. We conclude that the progressive disappearance of the fungus under flooded conditions is a consequence not only of a modification in the root architecture (decrease of LLRs, prevalence of CRs), but also of an anatomical change (increase of aerenchyma), which leads to the disappearance of the cortical cells, which are required for coils, intercellular hyphae and arbuscules.

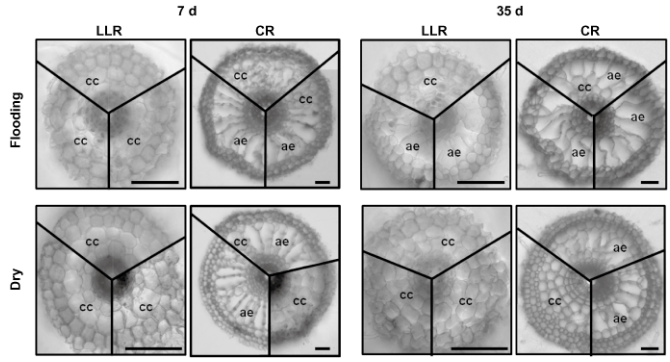

Figure 6. Cross sections of large lateral roots (LLR) and crown roots (CR) grown in dry and flooding condition for 7 and 35d. For each image, three different roots are shown. Cortical cells (cc) and aerenchyma (ae) are indicated. Scale bar is 100µm.

Root branching and AM colonization: a shift from dry to flooding conditions and vice versa

Having demonstrated that the effect of flooding on root branching (decreasing of BI values) was rapid, while its effect on mycorrhization was significant only after 21dpi (decrease in the mycorrhization percentage), we wondered whether rice plants retain these adaptation capacities also when transferred from flooding to dry conditions, and vice versa. To evaluate whether changing environmental conditions have an effect on root architecture and AM colonization, similar experiments to those previously described were performed on mycorrhizal plants, which were shifted from one water condition to the other at two time points (21 and 28dpi), and grown for a further 7d in the new regime.

After 21dpi, the transition to the new condition significantly changed the BI values. The experimental condition of 21dpi dry/7d flooding led to a significant reduction in the BI value compared with plants grown for 28dpi under dry conditions. Simultaneously, plants grown for 21dpi flooding/7d dry showed an increase in the BI value when compared with plants grown for 28dpi under flooding conditions (Fig.7 shows the sandwich method; Supporting Information Fig.6 shows the microcosm method).

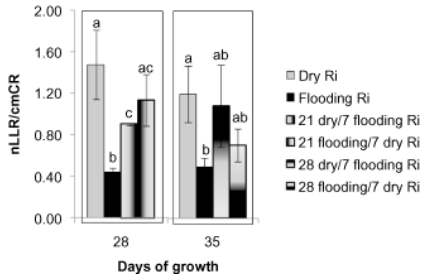

Figure 7. Dynamics of rice root branching in dry versus flooding and during a shift experiment: dry/flooding and flooding/dry. The rice root branching index was calculated considering the ratio between the number of large lateral root(LLR) cm−1 crown root (CR) in mycorrhizal plants grown, using the sandwich method, in dry (dry Ri), flooding (flooding Ri) and in changing environment (dry/flooding Ri; flooding/dry Ri) at 28 and 35 dpi. Error bars represent the SD of three replicates. One-way analysis of variance (anova) and Tukey's post hoc test were performed considering data coming from a single time point (rectangular box): different letters indicate a significant difference ($P<0.05$).

When the experiment was extended and plants were retained in the first water condition for 28dpi, followed by 7d in the second condition, only a slight and not significant variation of the BI values was observed (Fig. 7 shows the sandwich method, Supporting Information Fig. S6 shows the microcosm method).

The transition from aerobic to anaerobic conditions and vice versa also had an effect on the degree of mycorrhization. In particular, under the experimental conditions of 21dpi/7d, flooding exerted a significant inhibitory effect on the root colonization rate. By contrast, the transfer from flooding to dry conditions led to a less evident enhancement of the mycorrhization level (Fig. 8a,b shows the sandwich method; Supporting Information Fig. S7a,b shows the microcosm method). Under the experimental conditions of 28dpi/7d, this phenomenon appeared even more evident and statistically significant in both water transfers considered (Fig. 8a,b shows the sandwich method; Supporting Information Fig. S7a,b shows the microcosm method).

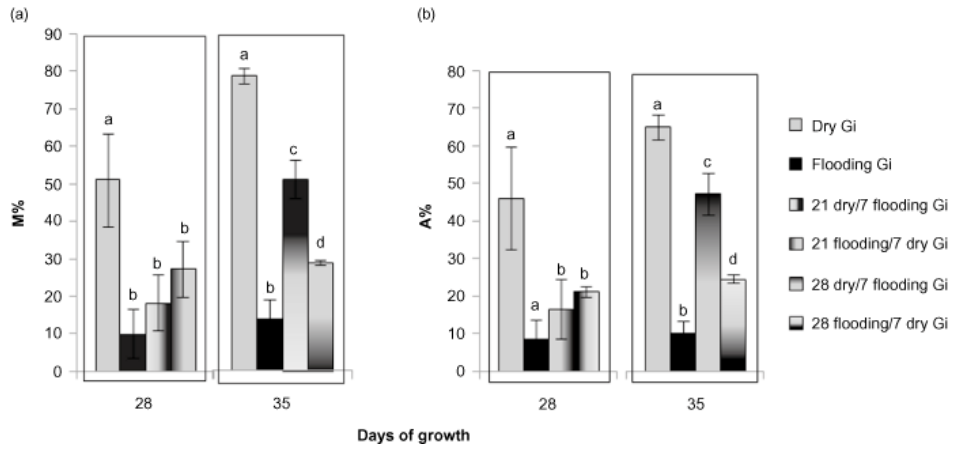

Figure 8. Dynamics of rice root colonization in dry versus flooding and during a shift experiment (dry/flooding; flooding/dry) at different time points. Two parameters are considered (a) the intensity of the mycorrhizal colonization (M%) expressed as the percentage of the root length colonized by R. irregularis and (b) arbuscule abundance (A%) of the same samples in the root system of mycorrhizal rice plants grown using the sandwich method, in dry (dry Ri),

flooding (flooding Ri) and in changing environment (dry/flooding Ri; flooding/dry Ri) at 28 and 35dpi. One hundred root fragments of 1cm lengths were analysed for each sample. Data are mean values of three replicates. Error bars indicate SD. One-way analysis of variance (anova) and Tukey's post hoc test were performed considering data coming from a single time point (rectangular box): different letters indicate a significant difference ($P<0.05$).

Taken as a whole, this new set of experiments showed that when shifted from one water condition to the other, the root apparatus rapidly adapts to the new situation, and that the anatomical changes are mirrored by changes in AM colonization.

Since the data obtained from the two systems (sandwich and microcosm) were comparable, the sandwich method was selected for further experimentations.

Expression analysis of AM functional-marker genes

In order to evaluate the functionality of the symbiosis in dry and flooding conditions, the expression of plant and fungal phosphate and ammonium transporters (AMTs) were tested in mycorrhizal roots of plant grown via the sandwich method at the time points where a good degree of colonization was reported (21, 28, 35dpi). For the host plant, we tested a constitutively expressed phosphate transporter (PT) implicated in direct Pi uptake and induced in roots in response to low Pi conditions, OsPT2 (Yang et al. 2012), an AM-induced PT gene, OsPT11, whose expression was detected specifically in arbusculated cells and whose coded protein has been localized on the periarbuscular membrane (Paszkowski et al. 2002; Gutjahr et al. 2008; Kobae & Hata 2010) and an AMT, which is considered to be the putative homolog of LjAMt2;2 (AM-induced Lotus japonicus gene, Guether et al. 2009b) known as OsAMT3.1 (Supporting Information Fig. S8; Guether, personal communication). In addition, on the fungal side, we tested the high-affinity PT gene, GintPT (Maldonado-Mendoza, Dewbre & Harrison 2001), and the high-affinity NH+4 gene GintAMT2, which is involved in nutrient transport and is considered to be a marker of fungal metabolism (Pérez-Tienda et al. 2011). We observed that the transcription profiles of OsPT11 and OsAMT3.1, which are considered AM-induced genes, were in line with the degree of mycorrhization. In fact, the expression levels of OsPT11 and OsAMT3.1 were comparable in dry and flooded conditions at 21dpi, where the degree of colonization was similar. The expression levels of OsPT11 and OsAMT3.1 were significantly higher in dry than in flooded conditions at 35dpi, mirroring the higher colonization degree (Fig. 9b,c). By contrast, the Pi transporter of the direct uptake pathway, OsPT2 was expressed independently from the colonization level (Fig. 9a). Interestingly, both the fungal transporter genes (GintPT and GintAMT2) showed comparable expression profiles in both conditions, indicating that the AM fungus, when established, was active and functional (Fig. 9d,e) irrespective of the water regime.

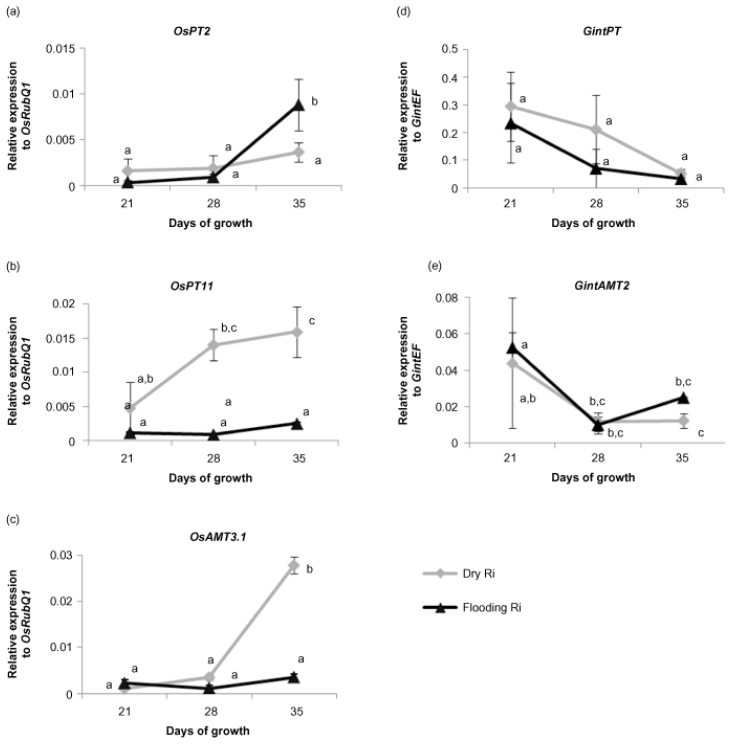

Figure 9. Time course of the expression of rice and R. irregularis genes evaluated by quantitative real time. The selected genes are considered functional markers for the arbuscular mycorrhization (AM) symbiosis. The expression of (a) OsPT2, (b) OsPT11, (c) OsAMT3.1 and R. irregularis (d) GintPT, (e) GintAMT2 has been evaluated in mycorrhizal rice roots grown using the sandwich method, in dry and flooding conditions at 21, 28 and 35dpi. Error bars indicate the SD of three replicates. Different letters indicate a significant difference ($P<0.05$).

Gene expression analysis was also carried out on plants grown via the sandwich method shifted from dry to flooding conditions and vice versa. Expression analysis revealed that the AM-induced plant genes (OsPT11 and OsAMT3.1) exhibited an even stronger link between AM colonization and lateral root availability. In particular, 7d of exposure to flooding conditions were sufficient to dramatically decrease the accumulation of the OsPT11 and OsAMT3.1 transcripts (32.8-fold and 5-fold, respectively) compared with the values detected at 28dpi in dry conditions (Fig. 10b,c). On the other hand, the opposite phenomenon occurred at 21dpi flooding/7d dry where the OsPT11 and OsAMT3.1 expression levels were restored (Fig. 10b,c). In agreement with the previous transcription results, the OsPT2 gene maintained the inversely proportional trend with respect to AM-induced gene: rising when exposed to flooding and decreasing when shifted to dry conditions (Fig. 10a). The same trend was also reported in the second experimental condition (28dpi/7d; Fig. 10a). With respect to the fungal genes, we observed that in the case of the treatment involving 28dpi/7d, the transfer to dry conditions induced the opposite transcription pattern. GintPT was strongly up-regulated during the transfer from dry to flooding conditions, while GintAMT2 was significantly induced during the transfer from flooding to dry conditions (Fig. 10d,e).

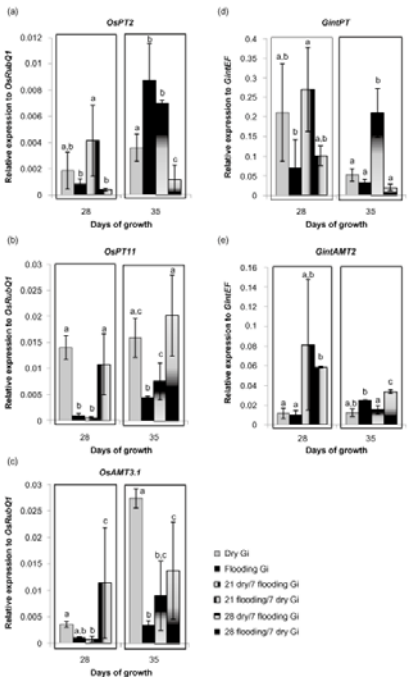

Figure 10. Quantitative real-time expression analysis of plant and fungal AM marker genes from rice root colonized with R. irregularis in dry versus flooding and during a shift experiment (dry/flooding; flooding/dry) at different time points. Analysis of the expression of O. sativa (a) OsPT2, (b) OsPT11, (c) OsAMT3.1 and R. irregularis (d) GintPT, (e) GintAMT2 in mycorrhizal rice roots grown, using the sandwich method, in dry (dry Ri) and flooding (flooding Ri) conditions and in changing environment (dry/flooding Ri; flooding/dry Ri) at 28, and 35dpi. Error bars indicate the SD of three replicates. One-way analysis of variance (anova) and Tukey's post hoc test were performed considering data coming from a single time point (rectangular box): different letters indicate a significant difference ($P<0.05$).

Phosphate quantification

In order to investigate the effect of R. irregularis colonization on phosphate (Pi) acquisition in the different water regimes, the mineral content of shoots and roots of plants grown for 28d in the presence (Myc) or the absence (C) of R. irregularis in flooding and dry conditions was measured by anion-exchange chromatography. With respect to dry conditions, Pi uptake was significantly higher in Myc plants (Fig. 11). By contrast, in flooding conditions, these differences were not detectable (Fig. 11). In addition, it is worth noting that the amount of Pi detected in leaves was much higher (7.6-fold) than in roots (Fig. 11) in plant exposed to flooding conditions.

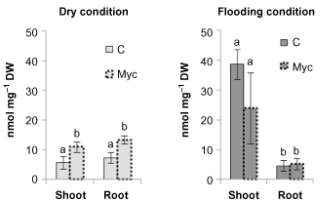

Figure 11. Phosphate content in shoot and root of mycorrhizal (Myc) and non-mycorrhizal (C) O. sativa plants, grown using the sandwich method in dry and flooding conditions at 28dpi with R. irregularis. Error bars represent the SD of 10 replicates. Different letters indicate a significant difference ($P<0.05$).

Discussion

Rice is derived from a semi-aquatic ancestor, and thus has unique characteristics in its root anatomy and physiology, making it suitable for wetland conditions (Fukai & Inthapan 1988). However, at the same time, it can adapt to and grow in soil that is water deficient (Gowda et al. 2011). In flooded soil, rice plants develop an extensive aerenchyma and have a compact root system. Besides influencing plant root architecture, water also influences several physicochemical processes and the microbiological components of the soil (De Datta 1995) creating a peculiar microenvironment.

This work focused on the role of water regime in shaping roots in the context of root–AMF interactions in rice plants. The results show that the water regime is the parameter that drives rice–AM interactions. The BI values of the rice root systems under the four conditions (upland versus wetland, presence versus absence of the fungus), and taking into account the shift from one condition to the other, conclusively demonstrate that flooding influences the root architecture leading to a decrease of the LLRs and to a proliferation of aerenchyma tissues. The results also show that the AMF retains the physiological possibility to thrive under flooding conditions, as demonstrated by the expression of their functional markers, but it is impaired in the colonization process because its preferential niches (LLRs) are progressively decreasing.

Branching in rice is positively affected by aerobic conditions and by the presence of the AMF

The increased number of LLRs during the course of exposure to dryland conditions supports the results obtained by Mitchell, Owusu & Fukai (2012), in which 41-day-old aerobically grown rice plants had significantly greater root branching when compared with plants exposed to flooding conditions. It is worth mentioning that the role of oxygen was indicated as a primary factor influencing the function of the root system (Dean 1933 and references therein). In particular, Dean (1933) demonstrated that the root systems of different aquatic plants were more branched when exposed to aerated soils. More recently, it was proposed that the reduction in water supply is possibly offset by increased root branching to face the potential exploitation of the soil volume (Mitchell et al. 2012), and to increase the surface for mineral nutrient absorption.

Branching was also increased by the presence of R. irregularis in the soil, in both dry and wet conditions. Interestingly, the increase was independent of the degree of mycorrhization: it was evident even when the colonization was absent or low and it remained constant over time. Therefore, it seems that the effect of AMF on root branching was a consequence of the presence of diffusible signals from the fungus, not of the establishment of the symbiosis. Data obtained in this study are in agreement with Gutjahr et al. (2009), who showed that R. irregularis induced the formation of LLRs in upland rice (cv. Nipponbare) and that this effect was similar in wild-type and SYM mutants. SYM mutants are defective in components of the common SYM pathway and block AMF at the outer cell layers inhibiting cortex colonization (Gutjahr et al. 2008; Parniske 2008): the induced formation of LLRs also in these mutants suggests that the presence of extraradical fungal hyphae is sufficient to induce changes in the root system architecture.

AM fungi thrive in flooding conditions, but changes in root anatomy cause a progressive decrease of the symbiosis

This study also definitively concluded that exposure to dryland conditions increases and exposure to wetland conditions depresses the degree of AMF colonization. While this situation has already been described by other authors (Miller 2000; Wirsel 2004; Wang et al. 2010), the experimental designs utilized in this study allowed for changes in the root systems and changes in AM colonization to be monitored. Direct relationships between the three factors (water regime, aerenchyma increase and AM decrease) were observed, indicating that the change in AM colonization is a consequence of a different number of LLRs and a different amount of aerenchyma in the two water conditions and seems to be independent of the fungal physiology (see below). R. irregularis preferentially colonizes LLRs (Gutjahr et al. 2009; this study), whose number increases under dryland conditions. Aerenchyma cannot offer a physical substrate for AM colonization because, due to the lysigenous–schizogenous nature of rice aerenchyma (Rebouillat et al. 2009), only remnants of parenchyma cell walls are observed (Fig. 6). The morphological observations in this study are in agreement with those by Butterbach-Bahl, Papen & Renneberg (2000) and Rebouillat et al. (2009), who showed the progression of aerenchyma from 7–13-day-old rice roots (abundant intercellular spaces) to 39–40-day-old rice roots (schizogenous aerenchymes occupying 70–90% of the cortex).

In conclusion, at the end of this developmental process, both CRs and LLRs do not possess a layer of active cortical cells, and as the FLRs lack a complex cortex, they cannot support AMF colonization. A crucial part is probably played by ethylene, which has been identified as the hormonal signal that mediates aerenchyma formation in wheat, maize, rice and Arabidopsis (Justin & Armstrong 1991; Watkin, Thomson & Greenway 1998; Gunawardena et al. 2001; Mühlenbock et al. 2007; Shiono et al. 2008). Interestingly, ethylene was shown to be a factor, which leads to an impairment in mycorrhizal development (Herrera-Medina et al. 2007; Martín-Rodriguez et al. 2010).

Taken as a whole, the data shows that AMF can grow and colonize rice roots even in flooded soil, and that flooding itself is not responsible for the lack of the symbiosis. However, continuous flooding may exert multiple effects on root morphology and physiology, leading to a progressive decrease of the tissues available for colonization, including both diminishing LLR formation and widening the area occupied by the aerenchyma. These root changes, which become more pronounced over time, may explain why in conventional flooded fields, AMF colonization in rice roots diminishes with the age of the plant (Solaiman & Hirata 1995, 1998), and was not detected after 4 (Vallino et al. 2009) and 2 months (Lumini et al. 2011) of submersion.

Mycorrhizal rice functionality in upland versus wetland conditions

Rice responds to the presence of AMF by activating various genes involved in nutrient transfer, and these are considered beneficial markers for functionally active symbiosis (Paszkowski et al. 2002; Güimil et al. 2005; Yang et al. 2012). To understand whether changes in AM colonization (which was dependent on the water regime and root branching) were mirrored by changes in the transcriptional behaviour of marker genes, the expression of genes involved in phosphate and ammonium transport in rice were examined. As expected, OsPT2 expression was inversely related to the degree of colonization, while AM-induced genes (OsPT11 and OsAMT3.1) were up-regulated in dry conditions, where the degree of mycorrhization was increased.

On the other hand, the expression profiles of the two fungal marker genes, GintPT and GintAMT2, mainly involved in Pi transport and in NH4+ retrieving, respectively, were very surprising. The profiles of GintPT and GintAMT2 were similar in dry and flooded conditions, showing that the activity of R. irregularis was not influenced by the water regime. This result was well in line with the novel observations in this study, which showed that the morphology of the infection structures was not impacted by flooding in and of itself. The results showed that the fungus develops regular arbuscules, and its uptake activities are independent of the flooding conditions.

The activity of R. irregularis was also confirmed by the measurement of the Pi content of mycorrhizal and non-mycorrhizal plants grown in both water regimes. Inside the roots, an increased amount of Pi was observed in Myc plants grown in dry conditions when compared with plants grown in wet conditions, while no significant difference was observed in control plants, and this result mirrored the expression pattern of OsPT11. Interestingly, a strong Pi accumulation was detected in leaves collected from both Myc and control plants exposed to flooded conditions. This fact was probably due to the characteristics of the Selenio cultivar, which, as all Italian cultivars, is adapted for wetland conditions. In fact, in these cultivars, flooding improves the grain formation process compared with dry conditions (Solaiman & Hirata 1995), inducing plants to allocate more Pi to the aerial portions of the plant. These data suggest that the establishment of AM symbiosis led to a significant mineral nutrient benefit only in dry conditions, while in flooding conditions, the effect could be mitigated by the low AM colonization level, and/or masked by the presence of the preferential water regime.

How rice roots and AMF adapt to the environmental conditions

In this study, the experimental set-up simulating the changing from upland to wetland conditions showed that root architecture and root–AMF interaction are characterized by a marked plasticity, and that there is a time window to such events. In fact, moving plants from dry to wet conditions resulted in an increase in the number of LLRs and root colonization, while moving from wet to dry conditions led to a decrease in both the number of LLRs and root colonization. Therefore, in this case, AMF colonization seemed to be linked to LLR availability. Interestingly, the response was less evident when plants were retained in the first condition for a longer period of time, and this would suggest that root branching plasticity is high in a certain time frame, and, once exceeded, it needs a longer period in the new growth condition for reprogramming.

The plasticity and reversibility of the rice–AM symbiosis was even more evident when the plant marker genes were examined. The expression levels of OsPT11 and OsAMT3.1 were reversed by both water transfer conditions.

The effect of the shift on GintPT and GintAMT2 expression was not so linear. Initially, no changes were expected as these genes seemed not to be influenced either by water regime or by the level of colonization. This was partially true only for GintPT, whose expression changed significantly when plants were exposed to a regime of 28 dry/7 flooding. However, GintAMT2 expression increased when plants were moved from flooding to dry conditions. This behaviour was probably the result of other soil properties affected by the water regimes, for example, mineral speciation. It is worth noting that flooding increases the solubility of Pi and NH4+, and therefore, their availability to the plant (Roy & De Datta 1985; De Datta 1995). Current experimental evidence indicates that GintAMT2 is not only a marker of NH4+ transport but is also a marker of fungal metabolism (Pérez-Tienda et al. 2011). In this context, while the NH4+ transfer

from the fungus towards the plant is favoured under flooding conditions, such a transfer could be impaired under dry conditions. Dry conditions might induce NH_4^+ starvation in the fungus, leading to an up-regulation of GintAMT2. However, these aspects require further investigation.

In conclusion, our findings conclusively show that AMF can grow and colonize rice in flooded soil, while maintaining their signalling properties (effect on root branching) and functional capacities (gene expression, P uptake). However, the degree of colonization progressively decreases, since over time, deep morphological changes occur in the root apparatus. Furthermore, the results obtained from water transfer experiments revealed that both the root apparatus and AMF adapt rapidly to the new environment, triggering both morphological and transcriptomic changes. Shifting experiments showed that the rice symbiosis is a flexible and reversible process, which can be improved given a short period of exposure to dry conditions. Taken as a whole, this work highlights novel traits for AMF exploitation in rice cultivation. The turnover between lowland and upland conditions might offer a new and innovative strategy to reduce water use while also preserving the traditional paddy fields, which are protected habitats for plants and animals in Europe through the Natura 2000 network (Council Directive 92/43/EEC) because they play an ecological role as artificial wetlands.

Acknowledgments

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