

ORIGINAL ARTICLE

## Plant growth promoting rhizobacteria and *Rhizophagus irregularis*: biocontrol of rice blast in wild type and mycorrhiza-defective mutant

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### Abstract

Rice blast is one of the most destructive rice diseases known to cause considerable yield losses globally. Plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) are closely associated with rice plants and improve plant growth and health. To determine how isolated bacteria trigger rice growth, an assessment of phosphate solubilization and auxin production mechanisms was carried out *in vitro* and *in vivo*. In this study, the interactions between PGPR and *Rhizophagus irregularis* were evaluated in wild-type and *CYCLOPS* mutant plants to provide a sustainable solution against blast disease and reduce the amount of yield loss. Importantly, *Bacillus subtilis* UTSP40 and *Pseudomonas fluorescens* UTSP50 exhibited a suppressive effect on AMF colonization which shows the probable existence of a functional competition between AMF and PGPR to dominate the rhizosphere. On the other hand, *R. irregularis* decreased the biocontrol activity of *B. subtilis* UTSP40 in wild type, although this reduction was not significant in mutant plants. Results showed that the same defense-related genes were induced in the roots of wild type colonized by *B. subtilis* UTSP40 and *R. irregularis*. Therefore, plant cell programs may be shared during root colonization by these two groups of beneficial microorganisms.

**Keywords:** plant growth promoting rhizobacteria (PGPR), *Rhizophagus irregularis*, rice blast

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## Introduction

Rice (*Oryza sativa*) is a crucial crop for more than half of the world, and its supply must increase by 2050 to keep up with food demand from population growth (FAO 2009). Rice blast, caused by *Magnaporthe oryzae*, is the most destructive disease of rice causing grain losses (Filippi *et al.* 2011). Yield losses associated with blast outbreaks can reach 50% or even more in some countries including India, Japan, South Korea and Indonesia (Khush and Jena 2009). The disease can infect all parts of rice plants including leaves, leaf collars, necks, panicles, pedicels, and seeds (TeBeest *et al.* 2007). The ability of rice blast fungus to evolve new races is responsible for its partial success in favorable environments (Gnanamanikam and Mew 1992). On

the other hand, chemical control using synthetic fungicides has had undesirable effects on the environment and non-pathogenic organisms (Yoon *et al.* 2013). Furthermore, human exposure to high levels of pesticides may cause harmful side-effects (Fattahi *et al.* 2015; Damalas and Koutroubas 2016). Thus, there is a need for alternative disease management to provide successful control without negative consequences for human health and the environment (Cook *et al.* 1996).

Plants organize beneficial associations with rhizosphere microorganisms, which improve plant health and productivity. Plant growth promoting rhizobacteria (PGPR), adapted to this ecosystem, increase plant fitness and are significant in biotechnological

applications based on an integrated plant-bacteria system (Barahona *et al.* 2010). They are effectual for plant growth through various mechanisms, such as providing nutrients (e.g. inorganic phosphate) (Nouri *et al.* 2015), altering the plant hormone economy (e.g. auxin) (Zhang *et al.* 2007; Iqbal and Hasnain 2013), and increasing plant defense against pathogens (Khan and Haque 2011). Arbuscular mycorrhizal fungi (AMF) are well-known examples of symbiotic microbes which colonize more than 80% of terrestrial plants, aid in the uptake of minerals, such as phosphorus, and confer protection against biotic and abiotic stresses (Pozo and Azcon-Aguilar 2007; Smith and Read 2008; Auge *et al.* 2015). The biocontrol potential of some endophytic fungi and bacteria has been studied against rice blast disease (Li *et al.* 2011; Widiyantini *et al.* 2017; Law *et al.* 2017; Amruta *et al.* 2018).

The interactions between soil bacteria and AMF are synergistic, competitive, antagonistic or neutral (Artursson *et al.* 2005; Larimer *et al.* 2014). In the balancing of mutualistic associations between plants and beneficial microorganisms, induced systemic resistance (ISR) by PGPR and mycorrhiza-induced resistance (MIR) by AMF are actively expressed in the plants (Jung *et al.* 2012; Pieterse *et al.* 2014). Ethylene (ET) and jasmonic acid (JA) play key roles in the induction of defense-related genes (Martinez-Medina *et al.* 2011; Xie *et al.* 2011). Arbuscular mycorrhizal fungi improved rice resistance to *M. oryzae* (Campos-Soriano *et al.* 2012). *Pseudomonas fluorescens* and *Bacillus subtilis* strains were able to induce systemic resistance against rice blast (Sha *et al.* 2016).

The present study was undertaken to isolate root-associated bacteria of *O. sativa* and assess their potential as phosphate solubilizers and auxin producers. Additionally, we studied the interactions between *P. fluorescens* UTSP50, *B. subtilis* UTSP40 and *Rhizopagus irregularis* as plant beneficial microbes to control rice blast disease, and subsequently the induction of some defense-related genes against *M. oryzae* FE-13 in wild type and mycorrhiza-defective mutant. Using mycorrhiza-defective mutant plants, we explored the role of *OsCYCLOPS*, as an essential symbiotic factor, in AMF and PGPR interaction, defense-related gene expression and disease severity in rice plants.

## Materials and Methods

### Isolation and identification of the bacteria

Rice plants (*O. sativa* cv. Dylamani) were taken from fields in Mazandaran province, Tonekabon, Iran and the isolation of bacteria from root samples was carried out using soil dilution (Button *et al.* 1993). Screening

of bacteria was based on their antagonistic potential against *M. oryzae* FR-13 (data not shown), using the dual culture assay (Oldenburg *et al.* 1996). Bacterial DNA was extracted using the QIAprep Spin Miniprep kit and a microcentrifuge (QIAGEN). DNA samples were amplified using primers 27f/1492r (Lane 1991), and the cycling conditions were 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min 45 sec, with a final extension at 72°C for 7 min. Purification of the PCR samples was carried out using the QIAquick PCR purification protocol. The resultant PCR products were ligated into a pGEM-T vector and sequenced based on the 16S rRNA region with M13 Universal primers, F: 5'-d (CGCCAG GGTTCCTCCAGTCACGAC)-3' and R: 5'-d (TCA CACAGGAAACAGCTATGAC)-3', by sequencing in the Biophore building, University of Lausanne (UNIL). Databases at the National Center for Biotechnology Information (NCBI, Bethesda, MD, U.S.A.) were used to compare sequence homology to other DNA sources.

### Rice growth conditions in phyto-chamber

Seeds of *O. sativa* cv. Nipponbar, wild type and *CYCLOPS* mutant (Gutjahr *et al.* 2008), obtained from the Department of Plant Molecular Biology (DBMV, UNIL), were sterilized in sodium hypochlorite solution for 15 min and planted in an autoclaved mixture of loam and sand (1 : 1). Plants were grown in a phyto-chamber with a 12-h day/12-h night cycle at 28°C/23°C and watered every second day with half-strength Hoagland solution.

### Inoculation of rice plants with PGPR and growth assay

Isolated bacteria were cultured in Luria-Bertani (LB) broth and incubated overnight at 28°C with shaking. Cells were collected by centrifugation (4,000 rpm for 10 min), washed and resuspended in 50 ml sterile water. One ml of the bacterial suspension containing  $10^8$  CFU · ml<sup>-1</sup> with methylcellulose 2% was applied into the soil of each pot (Suslow and Schroth 1981). Each treatment included ten replicates. Plant growth assay was carried out 4 weeks after inoculation with bacteria. The weight of shoot and root samples was measured after oven drying at 65°C.

### Assessment of phosphate solubilizers and auxin producers

To detect phosphate solubilizing bacteria, they were streaked onto Pikovskaya's agar medium (Katznelson and Bose 1959) and incubated at 28°C. The plates showing a clear zone around the colonies

within 3 days were taken as a positive test for phosphate. Quantitative measurement of phosphate solubilization was carried out using the standard method (King 1936). Production of indole acetic acid (IAA) was determined based on the standard method (Bric *et al.* 1991), and distinguished by the formation of a red halo on the paper, immediately surrounding the bacterial colonies. Auxin response assay was carried out in a phyto-chamber using *O. sativa* DR5::GUS seeds obtained from DBMV, UNIL (Bai and Demason 2008). DR5::GUS seedlings were submerged in GUS staining buffer (Jefferson 1987) for 12–18 h at 4 weeks. In this experiment, water and 10  $\mu$ M naphthalene acetic acid (NAA) were taken as negative and positive controls, respectively. Four plants were grown for each treatment. Genotyping of seven segregating seedlings was performed by PCR to identify homozygous transgenic plants (data not shown) (Kihara *et al.* 2006).

### Inoculation of rice plants with arbuscular mycorrhizal fungi (AMF)

The fungus, *R. irregularis*, commercially available inoculum (Biorhize) (Yang *et al.* 2012), was grown under aseptic conditions (Becard and Fortin 1988), and equal volumes of medium containing 500 spores/ml were added to each pot at 1.5-cm depth upon planting (Paszkowski *et al.* 2002). Ten plants were taken for each treatment.

### Evaluation of bacterial population

Ten g of each rhizospheric soil sample (6 weeks after inoculation with PGPR) was placed in an Erlenmeyer flask containing 9 ml of sterilized distilled water and shaken for 15 min. Ten-fold series dilutions were prepared, and appropriate dilutions were plated onto the LB agar medium. Cell numbers were defined as colony-forming units (CFU) after 24 h incubation at 28°C.

### Microscopic evaluation of mycorrhizal colonization

Rice roots were harvested, cleared with 10% KOH, and stained with 0.1% trypan blue according to Saito *et al.* (2007), 6 weeks after inoculation with AMF. Trypan blue-stained AM colonies were observed under a bright-field microscope (Leitz DMRB; Leica), and images were captured using a CCD camera (Penguin 600CL, Pixera). The degree of mycorrhizal colonization was determined as described by Brundrett *et al.* (1984).

### Pathogen infection and measurement of disease severity

The fungal pathogen, *M. oryzae* FR-13, was obtained from the DBMV collection (UNIL). Leaves of 6-week-old wild type and mutant plants, previously inoculated with PGPR and AMF, were sprayed with a suspension of  $5 \times 10^4$  conidia  $\cdot$  ml<sup>-1</sup> (8 ml) in 0.2% Gelatin (Deng *et al.* 2016). Infected plants were transferred to a phyto-chamber at 25°C with 80% humidity. The number of lesions per leaf was scored 7 days post inoculation (dpi) (four leaves per plant). A numerical scoring system based on disease severity (DS) was used to quantify the disease symptoms. The numerical scale reflected the percentage of the leaf area exhibiting necrosis/chlorosis: 1, 1–20%; 2, 21–40%; 3, 41–60%; 4, 61–80%; and 5, 81–100% (Park *et al.* 2009).

### RNA extraction, cDNA synthesis and real-time RT-PCR

Total RNAs were extracted from 1 g of leaf tissue (7 dpi with *M. oryzae*) using TRIzol-reagent (Invitrogen, Carlsbad, CA). Extracted RNA samples were treated with DNase I (Invitrogen) at 37°C and SuperScript-III reverse transcriptase (Invitrogen) was used to synthesize the first-strand cDNA. Relative quantification of gene expression was performed by SYBER green real-time RT-PCR. To determine which of the defense genes were transcriptionally induced upon pathogen infection, primer sequences for pathogenesis-related genes (*PR-1a*) and (*PR-10b*) were adopted from Marcel *et al.* (2010), and primer sequences for plant defensin gene (*PDF1.2*) and  $\beta$ -chitinase gene (*ChiB*) were designed for rice in this study (Supplemental Table 1). Expression values were calculated according to Güimil *et al.* (2005), and normalized to the geometric mean of amplification of three nearly constitutively expressed genes: *ACTIN* (The Institute for Genomic Research [TIGR] identifier, LOC\_Os03g50890), *CYCLOPHILIN* (TIGR identifier, LOC\_Os02g02890) and *GAPDH* (TIGR identifier, LOC\_Os08g03290) (Gutjahr *et al.* 2008). The experiment was carried out with three technical and three biological replicates.

### Statistical analysis

The program one-way ANOVA (pairwise comparisons using t-tests with pooled SD; P value adjustment method: “Bonferroni”) was used to determine the differences between treatments in the experiments. The mean differences were considered statistically significant at the 5% level. Statistical analysis was carried out with R Software (version 2.15.0). All data in the figures were presented as mean  $\pm$  SD (standard deviation).

**Table 1.** Primers used for quantification of shoot RNA and gene expression by real-time RT-PCR

ID Putative function	Gene	ID Gene	Reference	Primer 1 (forward)	Primer 2 (reverse)
Pathogenesis related	<i>PR1a</i>	LOC_Os07g03710	(Marcel <i>et al.</i> 2010)	GCTACGTGTTTATGCATGTATGG	TCGGATTATTCTCACCAGCA
Pathogenesis related	<i>PR-10b</i>	LOC_Os12g36830	(Marcel <i>et al.</i> 2010)	TCTCCGTATTGCTGCTTCCT	CACTCTCACAAAATCAAACACCA
Defensin	<i>PDF1.2</i>	LOC_Os02g12060	in this study	ATTCAAGGGGTTGTGCTTG	ATGCAGCGTCGAGTCAAGT
$\beta$ -chitinase	<i>ChiB</i>	LOC_Os05g33130	in this study	GTACGGCGTGATCACCAAC	TGAACGGCCTCTGGTTGTAG

## Results

### Growth assay for rice plants inoculated with PGPR

The experiment was done with two identified bacteria using BLASTn and the region sequence of 16S rRNA which revealed 98% homology to *P. fluorescens* and *B. subtilis* (accession numbers HQ288938 and JF313215, respectively). Sequences were deposited in Genbank with accession numbers KM974652 for *P. fluorescens* UTSP50 and KM974651 for *B. subtilis* UTSP40. The bacteria were selected from 100 isolates based on their antagonistic potential against *M. oryzae* and, also their abilities to produce auxin and solubilize phosphate.

The phenotype of *O. sativa* cv. Nipponbar was shown in Figure 1A, B. *Pseudomonas fluorescens* UTSP50 showed the ability to promote rice growth. By contrast, *B. subtilis* UTSP40 significantly decreased shoot growth, but this reduction was not significant with respect to the root samples (Fig. 2A, B). In this experiment, the re-isolated bacteria were identical to

the original strains based on the sequencing results and databases at NCBI.

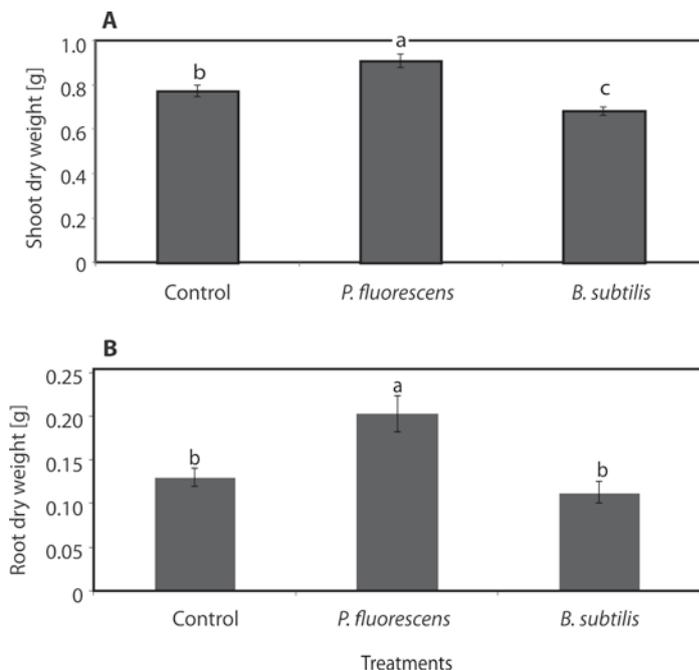
### Phosphate-solubilizing and auxin-producing PGPR

In this study, *P. fluorescens* UTSP50 by inducing a clear zone on Pikovskaya's agar medium was considered as a phosphate-solubilizing isolate (Fig. 3A). Dissolved phosphate was measured around 35 to 156 mg · l<sup>-1</sup> during the 10 days of growth in Pikovskaya's liquid medium (Fig. 4A), and a significant reduction was observed in the medium pH (Fig. 4B).

The bacteria tested *in vitro* showed no ability to produce auxin (Fig. 5). GUS staining was detected in the roots of DR5::GUS transgenic plants treated with the bacteria (Fig. 6), however, it was much stronger in root samples inoculated with *P. fluorescens* UTSP50. DR5::GUS is the synthetic auxin response reporter construct, to localize regions of auxin responsiveness (Bai and Demason 2008). NAA- (the synthetic auxin) and water-treated plants showed the most and



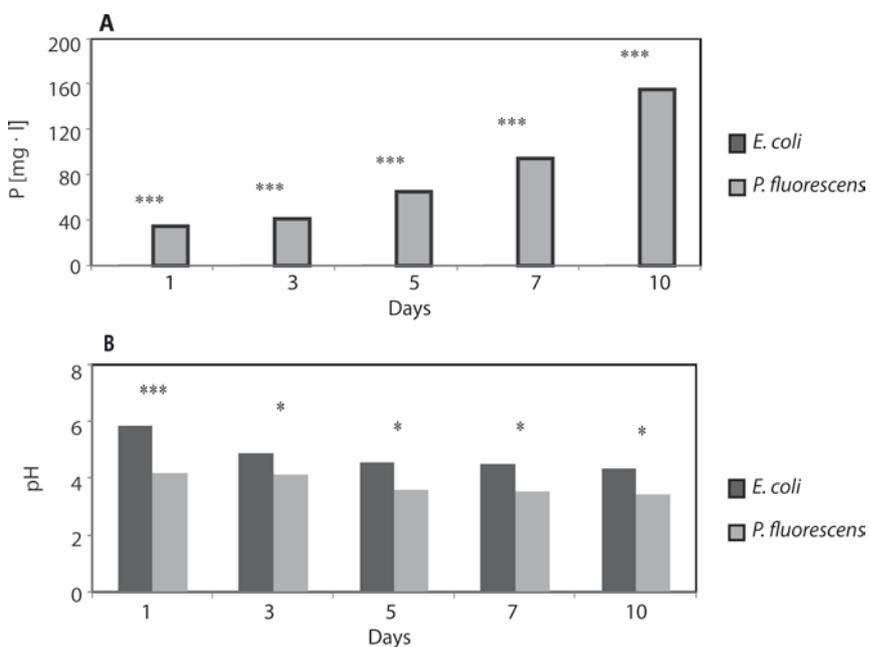
**Fig. 1.** Phenotype of *Oryza sativa* cv. Nipponbar, 4 weeks after inoculation with PGPR: A – control/*Pseudomonas fluorescens* UTSP50, B – control/*Bacillus subtilis* UTSP40



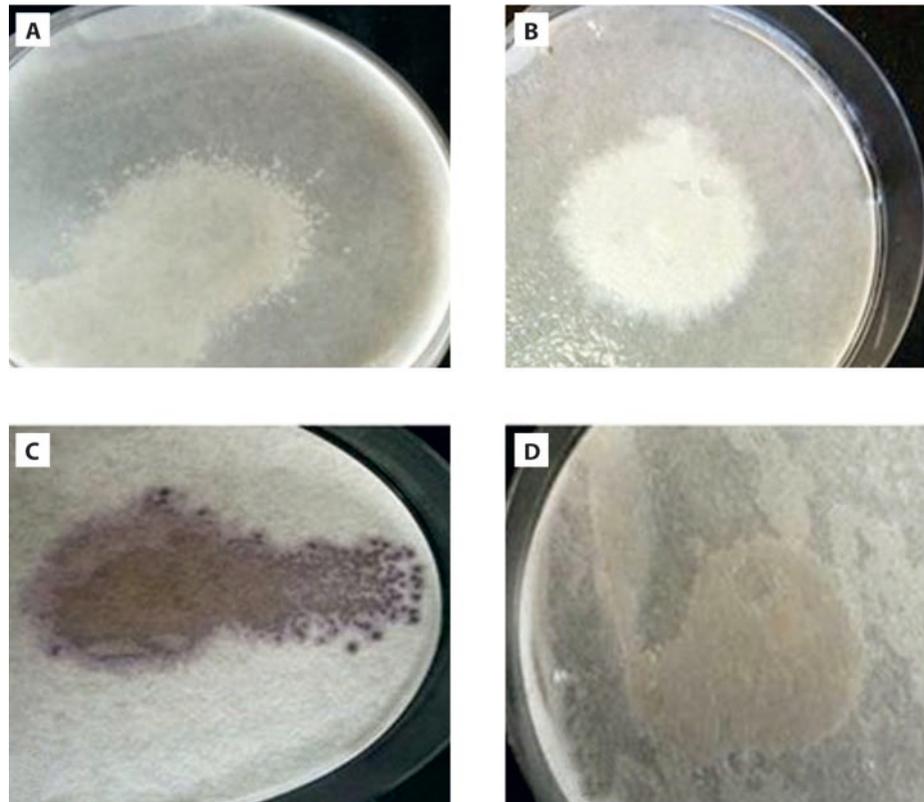
**Fig. 2.** Effects of *Pseudomonas fluorescens* UTSP50 and *Bacillus subtilis* UTSP40 on rice (*O. sativa* cv. Nipponbar) growth: A – shoot dry weight, B – root dry weight. Bars represent LSD ( $p < 0.05$ ) for comparisons between treatments with four replicates



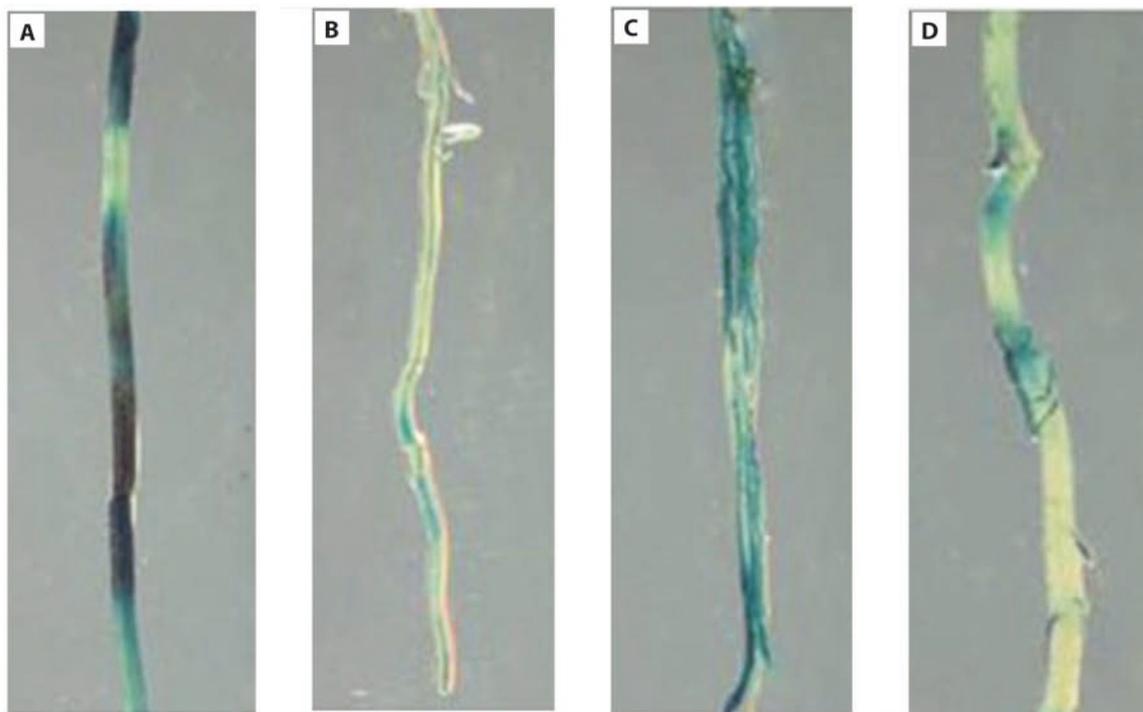
**Fig. 3.** Phosphate solubilization assay on Pikovskaya's agar medium: A – *Pseudomonas fluorescens* UTSP50, B – *Bacillus subtilis* UTSP40, C – *Escherichia coli* as a negative control in this experiment. A clear zone was induced by *P. fluorescens* UTSP50



**Fig. 4.** Phosphate-solubilizing ability of *Pseudomonas fluorescens* UTSP50: A – Tricalcium phosphate solubilizing ability of *P. fluorescens* UTSP50, B – pH value of Pikovskaya's liquid medium, over a 10 day period of inoculation by *Escherichia coli* and *P. fluorescens* UTSP50. The data represent the average of three replicates. \*\*\* $p < 0.0005$  and \* $p < 0.05$



**Fig. 5.** Indole-3-acetic acid (IAA) production assay *in vitro*: A – *Pseudomonas fluorescens* UTSP50, B – *Bacillus subtilis* UTSP40, C – *P. mosselii* UTSP6 (positive control), D – *Escherichia coli* (negative control). *In vitro*, only *P. mosselii* UTSP6 as positive control was able to produce IAA and a red halo was observed surrounding the bacterial colonies. *Pseudomonas moselii* UTSP6 (accession number KM974649) and *E. coli* were used as the positive and negative controls, respectively



**Fig. 6.** GUS histochemical staining of transgenic rice plants, *Oryza sativa* cv. Nipponbar, containing *DR5::GUS* fusion construct expression, 4 weeks after inoculation (4-week-old rice): A – NAA (positive control), B – water (negative control), C – *Pseudomonas fluorescens* UTSP50, D – *Bacillus subtilis* UTSP40. The blue color observed in roots showed auxin production in treatments

the least auxin production as positive and negative controls, respectively.

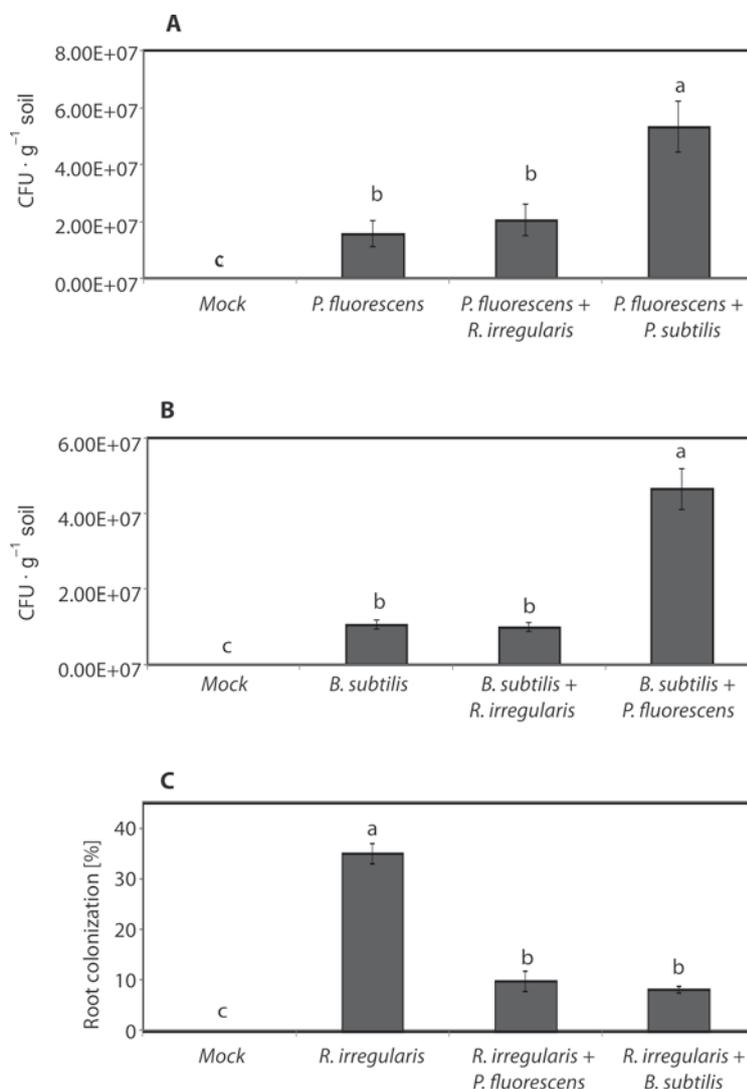
### Bacterial populations and arbuscular mycorrhizal fungi (AMF) colonization

The interactions between *P. fluorescens* UTSP50, *B. subtilis* UTSP40, and *R. irregularis* were evaluated in a phyto-chamber. A synergistic interaction was detected between *P. fluorescens* UTSP50 and *B. subtilis* UTSP40 (Fig. 7A, B). In contrast, the bacteria exhibited an inhibitory effect on the percentage of root colonization by *R. irregularis*, which was reduced to less than 10% (Fig. 7C). The presence of *R. irregularis* in the rhizosphere had no effect on the populations of *P. fluorescens* UTSP50 and *B. subtilis* UTSP40 (Fig. 7A, B). All typical symbiotic structures (extraradical hyphae, spores and arbuscules) are shown in Figure 8 (data not shown).

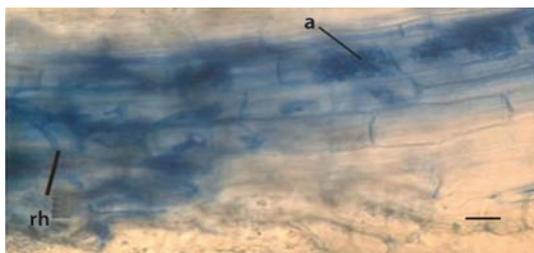
### PGPR and *Rhizophagus irregularis* combination against *Magnaporthe oryzae* FR-13 in wild type and *CYCLOPS* mutant plants

The symptoms of disease were detectable in the infected leaves 7 days after infection with the pathogen (Fig. 9). Disease severity was scored more than four in the wild type plants infected with the pathogen. Although all the treatments showed significant differences in comparison with the control, *B. subtilis* UTSP40 was the most effective isolate against the pathogen and DS was less than 1.5. Importantly, the presence of *R. irregularis* significantly reduced the biocontrol potential of *B. subtilis* UTSP40 against *M. oryzae* FR13. However, *R. irregularis* did not affect the biocontrol ability of *P. fluorescens* (Fig. 10).

Unlike the wild-type plants, *R. irregularis* colonization was aborted at the epidermis in *CYCLOPS* mutant

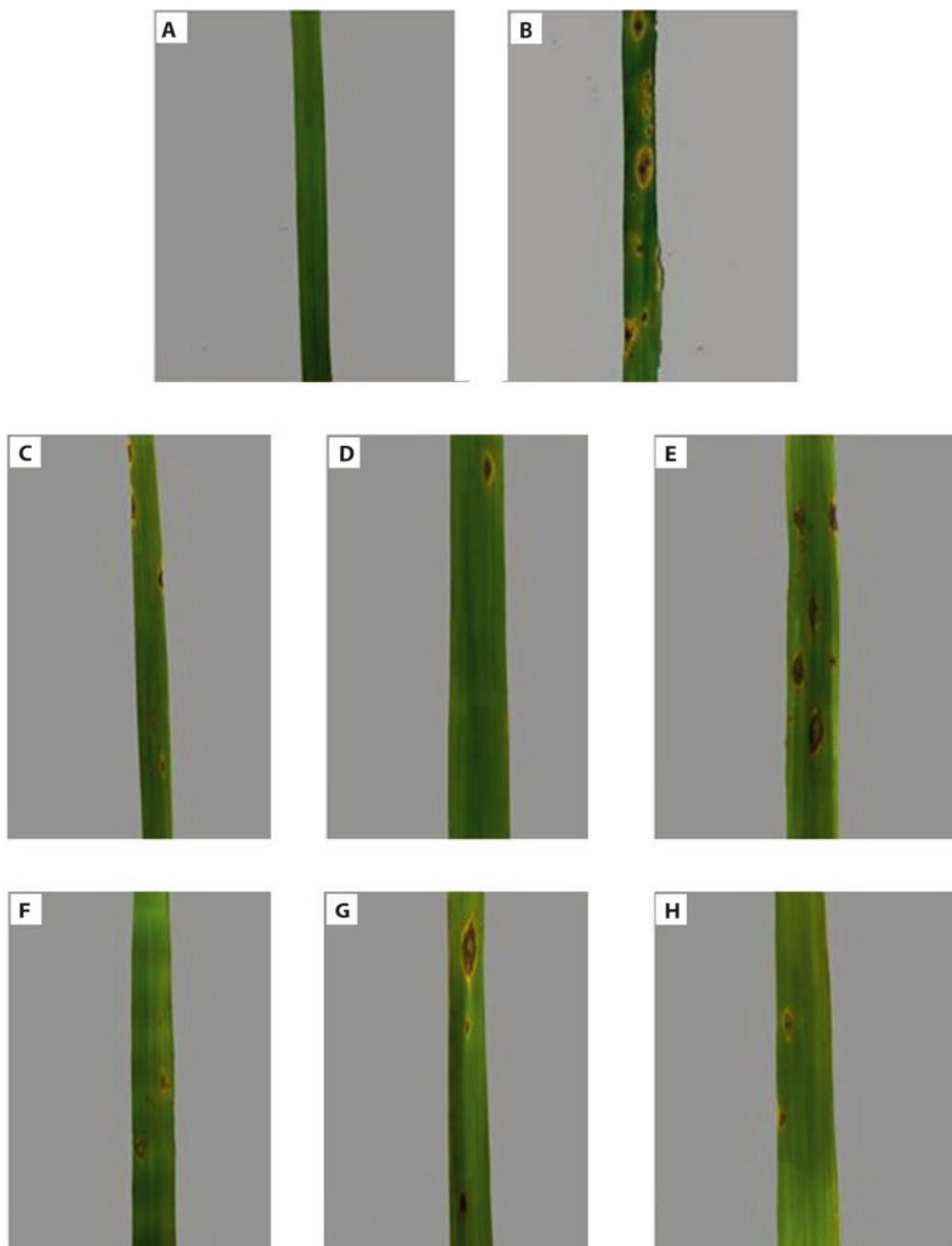


**Fig. 7.** Populations of PGPR and root colonization by *Rhizophagus irregularis*: A – population of *Pseudomonas fluorescens* UTSP50 in rice rhizosphere in combination with *Bacillus subtilis* UTSP40 and *R. irregularis*, B – population of *B. subtilis* UTSP40 in rice rhizosphere in combination with *P. fluorescens* UTSP50 and *R. irregularis*, C – the percentage of rice root system colonized by *R. irregularis* in combination with *P. fluorescens* UTSP50 and *B. subtilis* UTSP40, determined by a modified grid-line intersect method, 6 weeks after inoculation. Bars represent LSD (least significant differences,  $p < 0.05$ ) for comparisons between treatments with three replicates

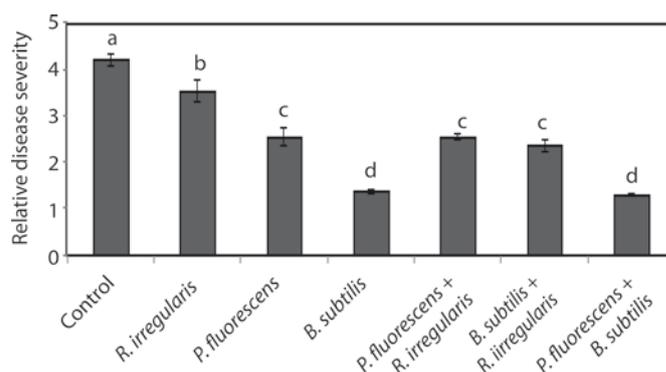


**Fig. 8.** AMF symbiosis phenotype: Light micrographs of trypan blue-stained roots were shown by Leica TCS SP2 AOBS confocal microscopy. The fungus, *Rhizophagus irregularis*, structures were indicated by a: arbuscule and rh: running hyphae. Scale bars: 20  $\mu$ m

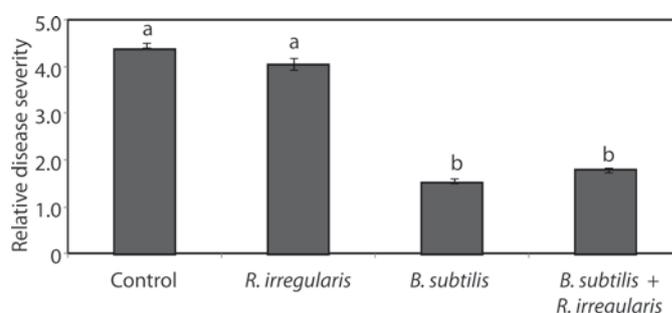
without arbuscule formation within cortical root cells (data not shown). The *OsCYCLOPS* gene is classified into a common symbiosis pathway (CSP) in rice that controls mycorrhizal root endosymbiosis and has been certified as an essential factor for AM symbiosis in rice (Gutjahr *et al.* 2008; Yano *et al.* 2008). There was no significant difference between the control and *CYCLOPS* mutant plants treated with *R. irregularis*. *B. subtilis* UTSP40 exhibited considerable deterrence against the pathogen in mutant plants. Using mutant plants, *R. irregularis* did not show any effect on the bio-control ability of *B. subtilis* UTSP40 (Fig. 11).



**Fig. 9.** Disease symptoms, caused by *Magnaporthe oryzae* FR-13, in *Oryza sativa* cv. Nipponbar leaves at 7 dpi: A – control without infection, B – control infected by *M. oryzae* FR-13, C – *Pseudomonas fluorescens* UTSP50, D – *Bacillus subtilis* UTSP40, E – *Rhizophagus irregularis*, F – *R. irregularis* + *P. fluorescens* UTSP50, G – *R. irregularis* + *B. subtilis* UTSP40, H – *P. fluorescens* UTSP50 + *B. subtilis* UTSP40



**Fig. 10.** Relative disease severity in *Oryza sativa* cv. Nipponbar infected with *Magnaporthe oryzae* FR-13: Disease severity was scored at 7 dpi using a numerical scoring system as described in "Materials and Methods". Bars represent LSD ( $p < 0.05$ ) for comparisons between treatments with four replicates



**Fig. 11.** Disease severity (DS) in rice (*Oryza sativa* cv. Nipponbar) *CYCLOPS* mutant after 7 dpi by *Magnaporthe oryzae* FR-13: Disease severity was scored at 7 dpi using a numerical scoring system as described in "Materials and Methods". Bars represent LSD ( $p < 0.05$ ) for comparisons between treatments with four replicates

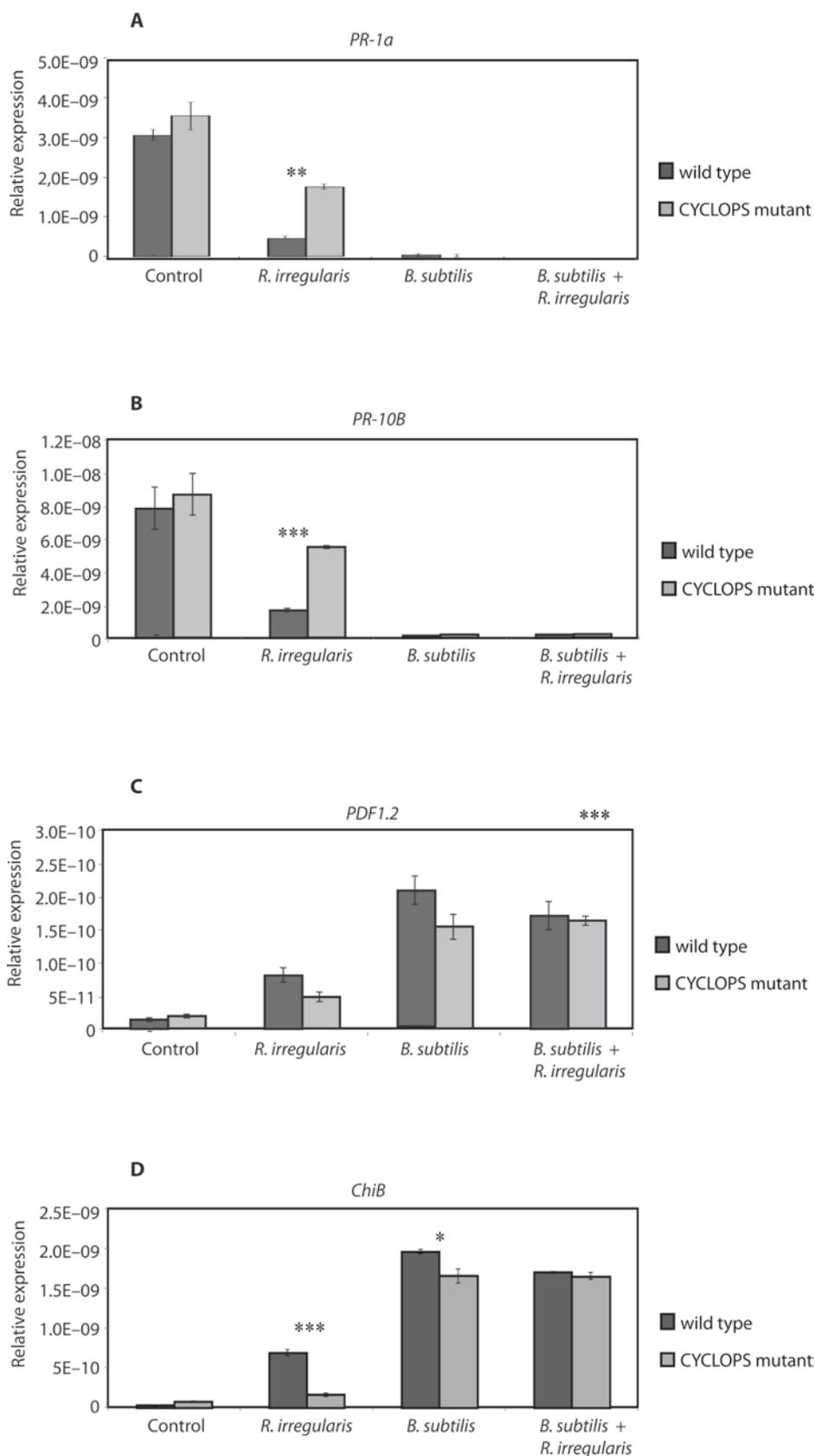
### Expression of some defense-related genes against *Magnaporthe oryzae* FR-13 in wild type and *CYCLOPS* mutant plants

The expression of four defense-related genes was assessed in wild type and *CYCLOPS* mutants. The transcript levels of *PR-1a* and *PR-10b* genes were low in both wild type and mutant plants treated with *B. subtilis* UTSP40, alone and in combination with *R. irregularis*. In contrast, the expression of *PR-1a* and *PR-10b* genes increased in mutant plants treated with *R. irregularis*, however, there was no significant difference in comparison with wild type (Fig. 12A, B). Plants treated with *B. subtilis* UTSP40, alone or in combination with *R. irregularis*, showed higher transcript accumulation of *PDF1.2* and *ChiB* genes in both wild type and mutants. The expression levels of *PDF1.2* and *ChiB* genes were enhanced in wild type plants treated with *R. irregularis* (Fig. 12C, D), considering that they were affected in mutant plants.

## Discussion

In this study, *P. fluorescens* UTSP50 considerably increased rice growth. Fluorescent pseudomonads

enhanced plant growth using different mechanisms, e.g. improvement of soil nutrient status and production of plant growth hormones (Reetha *et al.* 2014; Oteino *et al.* 2015). Since root exudates can alter microbial consortia in the rhizosphere, it was hypothesized that plant genotypic variants, based on their unique exudate profile, play a critical role for specific rhizobacteria (Micallef *et al.* 2009), and consequently deliver them to the right place at the right time. Phosphate solubilizing microorganisms (PSMs) have been employed in the agriculture industry and are considered to be important microbes due to their potential of soil amelioration. It has been shown that PSMs reduced P fertilizer application by 50% (Oteino *et al.* 2015). In this study, *P. fluorescens* UTSP50 was able to solubilize phosphate *in vitro*, and pH decreased in the basic medium, confirming observations by Jha *et al.* (2009) and Marra *et al.* (2012). The initial pH of the culture medium influenced the production of organic acids by *Acinetobacter* and *Paenibacillus* but had no effect on calcium phosphate solubilization (Marra *et al.* 2015). Additionally, an optimum level of auxin is required for several aspects of plant growth (Sreevidya *et al.* 2010). PGPR affected root architecture by altering root auxin economy (Iqbal and Hasnain 2013). Here, both *P. fluorescens* UTSP50 and *B. subtilis* UTSP40



**Fig. 12.** Accumulation of mRNAs from four defense-related genes in infected wild-type rice and *CYCLOPS* mutant, 7 days after infection with *Magnaporthe oryzae* FR13: Transcript accumulation of *PR-1a*, *PR-10b*, *PDF1.2* and *ChiB*. Means  $\pm$  SE represented by three technical and three biological replicates, \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ . Expression levels are shown relative to the constitutively expressed rice *CYCLOPHILIN* gene

were able to produce auxin and/or alter auxin maxima, in a phyto-chamber.

An inhibitory interaction was spotted between PGPR and *R. irregularis*, and the bacteria inoculations

reduced AMF rice root colonization, confirming the observation of Larimer *et al.* (2014), showing the probable existence of 'functional competition' between beneficial microorganisms. However, many studies have shown a synergistic interaction between AMF and PGPR (Bharadwaj and Alstrom 2012; Hashem *et al.* 2016). Based on the results obtained by Ordonez *et al.* (2016), AMF and PSMs, the major groups of beneficial microorganisms, interacted with each other in the rhizosphere, and such interactions could potentially lead to synergistic effects depending on the ability of bacteria to solubilize phosphate in the rhizosphere. Several factors, such as soil type, plant species, bacterial and fungal species, and even other microbial communities in the rhizosphere, may affect the interactions between PGPR and AMF. In the rhizosphere, where there are root exudates, more specialized microbial communities can be established (reviewed by Braga *et al.* 2016), since plants play a critical role in microbial interactions and microorganisms interact differently in response to root exudates. Artursson *et al.* (2005) suggested that gram-positive bacteria may more commonly associate with AMF than gram-negative ones. A level of specificity may exist between PGPR and AMF, however, the possibility needs to be more rigorously confirmed.

The fungus *M. oryzae* is a hemibiotroph fungus, which initially grows intracellularly without causing host cell death, and later promotes necrosis (Park *et al.* 2009). Therefore, a complex network of signaling events could be involved in resistance against this pathogen. The effects of AMF on shoot diseases largely relied on the lifestyle, challenging the strategy of the attacker (reviewed by Pozo and Azcon-Aguilar 2007). The same defense-related genes were induced by *B. subtilis* UTSP40 and *R. irregularis* in the plants infected by the pathogen, supporting the hypothesis that some plant cell programs may be shared during root colonization by different beneficial microorganisms, following a similar signaling pathway against the plant pathogens (Pieterse *et al.* 2014). The results of the *CYCLOPS* mutant experiment were clearly different.

*PR-1a* and *PR-10b*, the pathogenesis-related (*PR*) genes responsible for systemic acquired resistance (SAR) against pathogens (Zhang *et al.* 2010), were markedly down-regulated in wild type plants treated with *B. subtilis* UTSP40 and *R. irregularis*, unlike infected control plants. Salicylic acid (SA) signaling negatively affects root colonization by PGPR and AMF (Doornbos *et al.* 2011). The expression of defense-related genes was prominent during the early stages of the interaction between plants and AMF, and subsequently declined by developing symbiosis (Kape *et al.* 1992). Increase in the expression of SA-dependent genes in *CYCLOPS* mutant plants colonized by

*R. irregularis* confirms the importance of recognition of symbiotic factors in plant-AMF interaction, confirming Gao *et al.* (2004) results. The *CYCLOPS*-mutant impaired AMF interaction and blocked the penetration of the root surface or invasion of the root cortex by the fungus (Gutjahr *et al.* 2008). Arbuscule is the main structure in the mutualistic arbuscular mycorrhizal symbiosis, which is not formed in mutant plants (Gutjahr *et al.* 2008; Bona *et al.* 2016).

The expression levels of *PDF1.2* and *ChiB*, involved in the JA/ET-dependent defense pathway (Abe *et al.* 2008), were significantly increased in both wild type and mutant plants treated with *B. subtilis* UTSP40, and only in wild type treated by *R. irregularis*, confirming the observations of Ryu *et al.* (2004) in *Arabidopsis*, Campos-Soriano *et al.* (2012) in rice, Song *et al.* (2015) in tomato. During root colonization by beneficial microbes, the plant immune system leads to a primed state and allows for more efficient activation of defense-related genes against pathogens (Pieterse *et al.* 2014). *Pseudomonas* EA105 not only inhibited appressorium formation and growth of *M. oryzae* *in vitro*, but also triggered ISR in rice plants through the mechanism dependent on JA and ET signaling, and finally, there were fewer blast lesions (Spence *et al.* 2014). However, Gutjahr *et al.* (2015) showed that JA is not essential for AM colonization of rice, and high levels of JA in the roots suppress AM development, probably through the induction of defense. Importantly, a conserved response to fungal colonization was revealed in rice, since a set of genes was similarly expressed in both symbiotic and pathogenic interactions (Güimil *et al.* 2005), which may lead to trade-offs between symbiosis and disease resistance (Jacott *et al.* 2017). Furthermore, gene expression is affected by the AMF species and stage of fungal colonization (Gao *et al.* 2004). Khaosaad *et al.* (2007) suggested that a high degree of AM root colonization is essential for mycorrhizal induced systemic resistance. Interestingly, the expression of *PDF1.2* and *ChiB* genes was significantly reduced in mutant plants treated not only with *R. irregularis* but also with *B. subtilis* UTSP40, although further research is needed to confirm the importance of the *CYCLOPS* gene in plant colonization by PGPR.

In conclusion, selection of an accurate combination of PGPR and AMF is significant in their performance in the rhizosphere and impressively affects plant health and productivity. Rice is a monocot model crop that can be used to study the molecular mechanisms of fungal symbiosis (reviewed by Nakagawa and Imaizumi-Anraku 2015). In future studies, identification of novel and appropriate combinations of PGPR and AMF can provide good comparative clues for the investigation of mechanisms that facilitate microbial development and impact management strategies for the optimization of supportable agriculture systems.

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