

Unravelling mechanisms underlying phosphate-induced susceptibility to Bakanae disease in rice

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ABSTRACT

Rice is one of the most important crops in the world and sustains >50 % of the world's population. Rice production is, however, severely threatened by bakanae disease, caused by the fungus *Fusarium fujikuroi*. Due to low soil phosphorus bioavailability, phosphorus fertilizers are routinely used to optimize rice production, which has led to excessive P accumulation in rice fields. We show that high phosphate fertilization enhances susceptibility to bakanae. Similarly, *MIR399* overexpression increases phosphate content and enhances susceptibility to *F. fujikuroi* infection. *In vivo* imaging of the infection process using a green fluorescent protein-expressing *F. fujikuroi* isolate revealed higher fungal colonization in roots of plants grown under high-phosphate supply compared to plants under low-phosphate, which is in agreement with the observed phenotype of bakanae susceptibility in phosphate-accumulating plants. Moreover, a weaker activation of defense-related genes and reduced accumulation of ROS occurs during infection in rice plants grown under high phosphate supply. Histochemical detection of lignin and suberin showed reduced accumulation of lignin and suberin in roots of rice plants grown under high-phosphate fertilization, which was consistent with a weaker induction of lignin biosynthesis genes and suberin-related genes in those plants. Taken together, these results indicate that phosphate accumulation represses host immune responses and promotes susceptibility to bakanae. This information provides a basis to understand mechanisms underlying phosphate-induced susceptibility to pathogen infection in rice, which might be useful to reduce the use of agrochemicals, pesticides and fertilizers, in protecting rice from bakanae.

1. Introduction

Rice is a fundamental cereal crop and a staple food for over 50 % of the world's population. Nonetheless, rice production is severely threatened by pathogens causing losses of great economic significance. Bakanae disease is caused by one or more *Fusarium* species, mainly *Fusarium fujikuroi* which belongs to the *Gibberella fujikuroi* species complex (Wulff et al., 2010). Bakanae was first described in Japan in 1828 and in the recent years its incidence has increased and spread in areas of Asia, Africa, America and Europe (Italy) where this disease causes drastic yield losses (Bashyal, 2018; An et al., 2023).

Bakanae is considered a seed-born disease. Infected seeds have poor seedling emergence and surviving seedlings exhibit abnormal elongation due to the production of fungal gibberellins (GAs), the typical symptom of bakanae (Siciliano et al., 2015; Zhan et al., 2024). At later

stages, the infected plants die and the surviving plants often show sterility, thus reducing the crop yield. Notably, the *F. fujikuroi* species can produce mycotoxins which may be present in rice seeds, thus raising significant food safety concerns for both humans and animals (Munkvold, 2017). Thermal seed treatment and fungicide treatments are common management practices to arrest bakanae disease (An et al., 2023; Zhan et al., 2024). Moreover, the efficacy of plant-derived bioactive compounds (e.g., essential oil-derived compounds) as seed treatments for bakanae disease management under field conditions has been described (Mongiano et al., 2021). *F. fujikuroi* has a hemibiotrophic lifestyle, however not much information is currently available about the infection process and distribution of this fungus in tissues of the rice plant.

In response to pathogen attack, plants deploy defense strategies that involve important transcriptional reprogramming of gene expression.

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Plants have evolved two tiers of immune receptors to detect infections which are distinguished by the type of receptors that sense the presence of the pathogen. They consist of cell surface-resident pattern recognition receptors (PRRs) that sense microbial signatures, and intracellular nucleotide binding domain leucine-rich repeat (NLR) proteins that recognize pathogen effectors. Thus, recognition of conserved molecular signatures, known as Pathogen-Associated Molecular Patterns (PAMPs) by host-encoded surface PRRs activates a general defense response known as PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Couto and Zipfel, 2016; Dodds et al., 2024). PTI involves the activation of multiple signaling pathways, including the generation of reactive oxygen species (ROS), protein phosphorylation processes, and production of Pathogenesis-Related (PR) proteins (Van Loon et al., 2006; Bigeard et al., 2015). PTI protects plants against a diverse array of pathogenic microorganisms. During co-evolution, microbial pathogens have evolved mechanisms to counteract PTI by secreting virulence factors, or effectors, into the plant that are capable of suppressing the host's defense response, leading to susceptibility. In turn, intracellular receptors (NLR proteins) encoded by Resistance (*R*) genes can recognize microbial effectors to activate a robust immune response in the plant, termed effector-triggered immunity (ETI) (Cui et al., 2015). Cross-talk between PTI and ETI occurs resulting in effective defense against pathogen infection (Yuan et al., 2021).

Resistance conferred by *R* genes represents a potential strategy for managing rice diseases. However, the limited availability of bakanae disease resistant rice varieties constrains the use of *R* genes in breeding programs. Only a few bakanae disease resistant loci have been described in rice (Fiyaz et al., 2016; Volante et al., 2017; Chen et al., 2019; Lee et al., 2022). The use of biocontrol agents in addressing the challenges posed by *F. fujikuroi* spp has also been explored (Bosco et al., 2024). Elucidation of mechanisms by which rice plants defend themselves against *F. fujikuroi* is required for controlling bakanae disease.

Plants also rely on physical barriers which help to prevent infection and disease. The plant cell wall represents the first barrier that limits the entry of pathogens into the plant cell. During pathogen infection, reinforcement of this physical barrier might occur through the formation of papillae and accumulation of lignin and suberin at the infected tissue (Fröschel et al., 2021). In roots, lignin is found in the exodermis, sclerenchyma and stele layers, specifically within the secondary cell walls (Sexauer et al., 2021). The presence of lignin in xylem tissue cells is particularly vital, as it reinforces the structural integrity of the cell walls and creates a barrier against pathogen invasion (Kashyap et al., 2021). Lignin also enhances water retention within vascular bundles due to its hydrophobic characteristics. As for suberin, this polymer forms a lamellar layer between the cell wall and the plasma membrane, that also creates a hydrophobic barrier and restricts free diffusion of molecules between the root and the outer environment (Wang et al., 2021b). In roots, suberin is deposited in cell walls of the exodermis, endodermis, and vascular structures.

Increasing evidence also supports the relevance of the plant nutritional status on disease resistance (Val-Torregrosa et al., 2021; Tripathi et al., 2022; Dutta et al., 2024). In rice, high supply of nitrogen was reported to enhance susceptibility to infection by the rice blast fungus *Magnaporthe oryzae* (Ballini et al., 2013). In contrast, the application of nitrogen confers resistance to infection by *Alternaria* spp. in tomato, potato and cotton (Blachinski et al., 1996). More recently, treatment of rice plants with high phosphate (Pi) was found to increase susceptibility of *M. oryzae* infection (Campos-Soriano et al., 2020; Martín-Cardoso et al., 2024). Opposite to this, supplying *Arabidopsis* plants with high Pi enhances resistance against the fungal pathogens *Colletotrichum higginsianum* and *Plectosphaerella cucumerina* (Val-Torregrosa et al., 2022a, 2022b). A better understanding of interconnected regulations between nutrient stress and disease resistance in crop species is crucial for developing sustainable agricultural practices.

In this study we investigated the impact of Pi fertilization on bakanae disease in rice. For this, molecular, biochemical, histological and

physiological approaches were used. We show that growing rice plants under a high Pi regime, and subsequent increase in Pi content in roots, enhances susceptibility to infection by *F. fujikuroi*. A similar response occurs in rice plants accumulating Pi by overexpression of *MIR399*, a key component of the phosphate starvation response (PSR) in rice. The infection of rice roots by *F. fujikuroi* has been characterized using a *green fluorescent protein (gfp)*-expressing isolate in plants grown under contrasting Pi regimes, namely low Pi and high Pi supply. Consistent with a phenotype of bakanae susceptibility, Pi accumulation in rice roots is associated with a weaker induction of defense-related genes and reduced accumulation of ROS. Staining of root sections with fluorescent dyes and confocal laser scanning microscopy was used for the histochemical analysis of lignin and suberin accumulation in *F. fujikuroi*-infected rice roots, also in relation to Pi supply. Overall, this study identified mechanisms and processes involved in Pi-induced susceptibility to bakanae disease in rice. These results are discussed in the context of fertilizer management strategies for sustainable rice production.

2. Materials and methods

2.1. Plant and fungal material

Rice (*Oryza sativa*) was grown at 28 °C/25 °C with a 14h/10h light/dark cycle. The rice cultivars Nipponbare, Tainung 67 (TN67), Galileo and Selenio (*japonica* cultivars) were used in this study. The transgenic rice lines overexpressing *mir399* (genotype TN67) were also assayed in this work. These *mir399* overexpressor plants were described elsewhere (Campos-Soriano et al., 2020). The rice seeds were sterilized with ethanol 70 % for 3 mins, followed by sodium hypochlorite sterilization (6 % v/v, containing 0.005 % Tween-20) for 30 mins with gentle shaking. Seeds were then washed with distilled water (5 times), pre-germinated *in vitro* for 5 days in half-strength Hoagland's nutrient solution which has a Pi concentration of 0.25 mM Pi. To assess the effect of Pi supply, the Pi concentration in the Hoagland's solution contained 0.025 mM Pi or 2.5 mM Pi (for low Pi and high Pi treatment, respectively).

The *F. fujikuroi* 297b isolate was grown on Potato Dextrose Agar (PDA, containing 30 mg/L chloramphenicol) for 12 days at 25 °C under a 16h/8h light/dark cycle. Fungal spores were collected with sterile water added to the mycelium surface, and filtered with Miracloth (Merck, Darmstadt, Germany; 25 µm pore). For infection experiments, the spore suspension was adjusted to 1×10^6 spores/mL concentration using a Neubauer counting chamber. A *F. fujikuroi* 297b isolate expressing the *Green Fluorescent Protein (GFP)* reporter gene served to monitor root colonization *in vivo* by confocal analysis. The production and characterization of the GFP-labeled fungus was previously described (Aragona et al., 2021).

2.2. Infection assays

Rice seeds are pre-germinated on petri dishes under low or high Pi supply for 5 days, (18 seeds/plate, 7.5 cm in diameter). Seedlings were inoculated with *F. fujikuroi* spores (25 mL, 10^6 spores/mL) and kept in the dark for 24 h. Control seedlings were inoculated with water. Then, rice seedlings were transplanted into inert growth substrate (mixture of 50 % peat and 50 % quartz sand) to continue growth for 10 days at 28 °C/25 °C with a 14h/10h light/dark cycle and 30–40 % relative humidity (Supplementary Fig. 1 A). Three independent experiments were conducted (at least 12 plants per condition or genotype in each experiment).

For *in vitro* assays, the *F. fujikuroi*-inoculated and mock-inoculated seedlings (24 h after inoculation) were placed on a sterile filter paper (over sterile vermiculite). High humidity was assured by covering the seedlings with transparent film. Seedlings were supplied with half-strength Hoagland's solution at the appropriate Pi concentration (0.025 mM, 0.25 mM or 2.5 mM Pi) twice per week. Three independent

experiments were conducted (at least 12 plants per condition or genotype in each experiment).

Measurements of root length were carried out by image analysis at 10 days post-inoculation (dpi) using the ImageJ/Fiji v1.54j software (<http://fiji.sc/Fiji>). Disease severity index (DSI) was also scored at 10 days after infection using a 0–100 scale as described by Amatulli et al. (2010). The rating scheme is as follows: 0, symptomless seedlings; 20, stunted seedlings with yellow and narrow leaves; 40, thin seedlings with pale-yellow and narrow leaves; 60, seedlings with pale-yellow and narrow leaves, long and thin internodes and short roots; 80, seedlings with some necrosis of the crown and severe bakanae symptoms; 100, dead seedlings. Three independent experiments were carried out (N = 15 per condition, each experiment).

2.3. Chlorophyll quantification and Pi content

Chlorophyll content was determined spectrophotometrically (Lichtenthaler and Buschmann, 2001). For this, the leaf material (30 mg) was grinded, and chlorophylls were extracted using ethanol 96 % (v/v) for 2 h at 50 °C. To remove cell debris, the samples were centrifuged for 1 min at 12,000 g. The content of photosynthetic pigments was determined by measuring the absorbance of the supernatant at 649 and 664 nm for chlorophylls, and 470 nm for carotenoids (Spectramax M3 reader, Molecular Devices, USA) and expressed in mg/g fresh weight (FW) (Lichtenthaler and Buschmann, 2001).

Measurements of Pi content were carried out as previously described (Ames, 1966; Versaw and Harrison, 2002). Briefly, rice leaves were grounded in liquid nitrogen and the leaf powder (50 mg) was treated with 1 mL of 1 % glacial acetic acid. The assay mixture contained 200 µL of 1 % glacial acetic acid, 700 µL of ammonium molybdate/ascorbic acid solution and 100 µL of the sample. The absorbance was measured at 820 nm (Spectramax M3 reader, Molecular Devices, USA).

Three independent experiments were carried out for estimation of chlorophyll content and Pi content, each experiment consisting of five biological replicates per condition (3 plants each replicate).

2.4. Microscopic analysis of rice roots inoculated with a *gfp*-expressing *F. fujikuroi* strain

Sections of rice roots were obtained using a Vibratome Series 1000 (TPI, St. Louis, MO, USA). Basal areas from rice roots (2–3 cm below the seed) were cut into segments of 1 cm. Eight segments were molded into Eppendorf tubes filled with 10 % low melting agarose. Eppendorf tubes were cooled on ice until solidification of agarose. Sections were cut at a thickness of 50 to 100 µm. For confocal analysis of roots inoculated with the *gfp*-expressing fungus, root sections were placed onto microscope slides and stained with propidium iodide (10 µg/mL). Root colonization was monitored under an epifluorescence Leica DM6 microscope (Leica, Wetzlar, Germany) using excitation/emission wavelengths of 450–490 nm and 500–550 nm for GFP and 515–561 nm and 590 nm for propidium iodide. Pictures of root sections were taken at 7–10 dpi. Three independent experiments were conducted (N = 12 per condition, each experiment).

2.5. Histochemical detection of lignin and suberin

For detection of lignin and suberin deposition, roots samples at 7–10 dpi were cut into pieces of about 1 cm in length and processed as described by Sexauer et al. (2021) with modifications. Essentially, root sections were vacuum-infiltrated with 1x PBS solution for 30 min in the dark, followed by fixation (4 % paraformaldehyde dissolved in 1x PBS, pH 6.9) at 30 °C in the dark for 1 day under constant movement (80 rpm) on an Excella E24 incubator (New Brunswick Scientific, USA). Samples were washed 3 times with 1x PBS and then vacuum infiltrated in a clearing solution (ClearSee, xylitol powder [10 % (w/v)], Na deoxycholate [15 % (w/v)] and urea [25 % (w/v)] in ddH₂O) for 30 min.

Clearing was carried out for 3 days at 30 °C in the dark under constant movement (80 rpm). Cleared roots were then sectioned on a vibratome as described earlier. Sections were placed into 6-well plates and stained for lignin with Basic Fuchsin 0.2 % (8 min) followed by a 2-step washing (one wash of ClearSee and several Milli-Q water washes until samples stopped bleeding red dye). Successively, suberin was stained with a Fluorol Yellow 088 (0.01 % solution) for 10 mins, following a wash with ethanol 50 % and three washes with MilliQ water. Samples were examined under a confocal microscope (Zeiss 980 Elyra, Jena, Germany) using excitation/emission wavelengths of 545 nm and 572 nm for basic fuchsin and 488 nm and 509 nm for Fluorol Yellow 088. Pictures were taken at 7–10 dpi. Three independent experiments were performed (N = 12 per condition, each experiment).

2.6. DNA extraction and fungal biomass determination

Genomic DNA was extracted from infected roots essentially as described by Murray and Thompson (1980) but using MATAB as the extraction buffer. Quantification of fungal biomass was carried out by quantitative PCR (qPCR) using specific primers for the *F. fujikuroi* 28S DNA gene (Jeon et al., 2013). The reaction mixture (final volume of 10 µL) contained: 5 µL Sybr Green, 0.3 µL each primer (from a 10 µM stock solution), 2.4 µL ddH₂O, and 2 µL DNA (from a stock solution at 7.5ng/µL). The reaction conditions were: 1 cycle at 95 °C, 2 min followed by 45 cycles of 10 s at 95 °C (denaturation) and 60 °C for 30 s (annealing and extension). A melt curve was obtained after the amplification cycles (the temperature was increased by 0.5 °C per cycle, starting at 65 °C up to 95 °C). Primers used for qPCR are indicated in Supplementary Table 1. Quantification of fungal biomass was carried out in three independent experiments (at least 12 individual plants per condition or genotype in each experiment).

2.7. ROS staining and H₂O₂ quantification

Rice roots were stained with H₂DCFDA (2',7'-dichlorodihydrofluorescein diacetate) as described by Sánchez-Sanuy et al. (2022). For this, the rice roots were cut into 2–3 cm segments, maintained in 10 µM H₂DCFDA, vacuum infiltrated (5 mins) and kept in darkness for 10 mins. After washing the roots with distilled water (5 times), they were kept on 20 % glycerol until visualization. Roots were examined under an epifluorescence Leica DM6 microscope (Leica, Wetzlar, Germany) using excitation/emission wavelengths of 450–490 nm and 500–550 nm for GFP. Pictures were taken at 24 h post-infection (hpi). Two independent experiments were conducted for ROS staining (N = 12 per condition, each experiment).

Hydrogen peroxide (H₂O₂) analyses were performed essentially as previously described (Rossatto et al., 2017). For this, 50 mg of leaf tissue was grinded with liquid nitrogen and homogenized in trichloroacetic acid (1 %, w/v; 1 mL). The samples were centrifuged (12,000 g, 15 min, at 4 °C). The supernatant (50 µL) was mixed with 10 mM potassium phosphate, pH 7.0 (50 µL) and 1 M KI (100 µL). Absorbance was measured using Spectramax M3 reader (Molecular Devices, USA) at 390 nm. A standard curve of known H₂O₂ concentrations was constructed to determine the H₂O₂ content. Two independent experiments were conducted for quantification of H₂O₂ (4 biological replicates, each one of a pool of 5 plants).

2.8. Expression analysis by RT-qPCR

Total RNA was extracted using the Maxwell RSC Plant RNA Kit (Promega, Madison, WI, USA). First-strand cDNA was synthesized from DNase-treated total RNA (1 µg) with High Capacity cDNA reverse transcription Kit (Applied Biosystems, Waltham, MA, USA). RT-qPCR was carried out in optical 96-well plates using SYBR® green in a Light Cycler 480 (Roche, Basel, Switzerland). PCR primers were designed using the Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/>

primer-blast/). The PCR reaction contained: 5 μ L Sybr Green, 0.3 μ L each primer (from a 10 μ M stock solution), 2.4 μ L ddH₂O and 2 μ L cDNA (final concentration of cDNA, 5 ng/ μ L). The reaction conditions were: 95 °C for 2 min followed by 45 cycles of denaturalization (10 s at 95 °C), annealing and extension (30 s at 60 °C). The melt curve was obtained after the amplification cycles (95 °C for 2 ss; increasing the temperature by 0.5 °C, between 65 °C and 95 °C, 15 s each). The *ubiquitin1* gene (*OsUbi1*, Os06g0681400) was used for normalization of transcript levels. The Δ Ct method was used to calculate relative expression levels. Primers used for RT-qPCR are shown in Supplementary Table 1. Two independent experiments were conducted, each one consisting of 4 biological replicates (pool of 3 plants for each replicate).

2.9. Statistical analysis

The Student's *t*-test was used for measurements of Pi content, DSI and fungal biomass in experiments performed on Nipponbare, TN67, Galileo and Selenio plants. In experiments with miR399 OE and wild type (TN67) plants, the one-way ANOVA test was used for Pi content, DSI and fungal biomass measurements. Two-way ANOVA was used to estimate the root length, chlorophyll content, H₂O₂ content and gene expression analysis followed by Tukey's HSD test (details on statistical analyses are presented in Supplementary Table 2 and Supplementary Table 3). The thresholds for significance were as follows: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). Data was analyzed using GraphPad Prism v10.4.1 software (<https://www.graphpad.com/>)

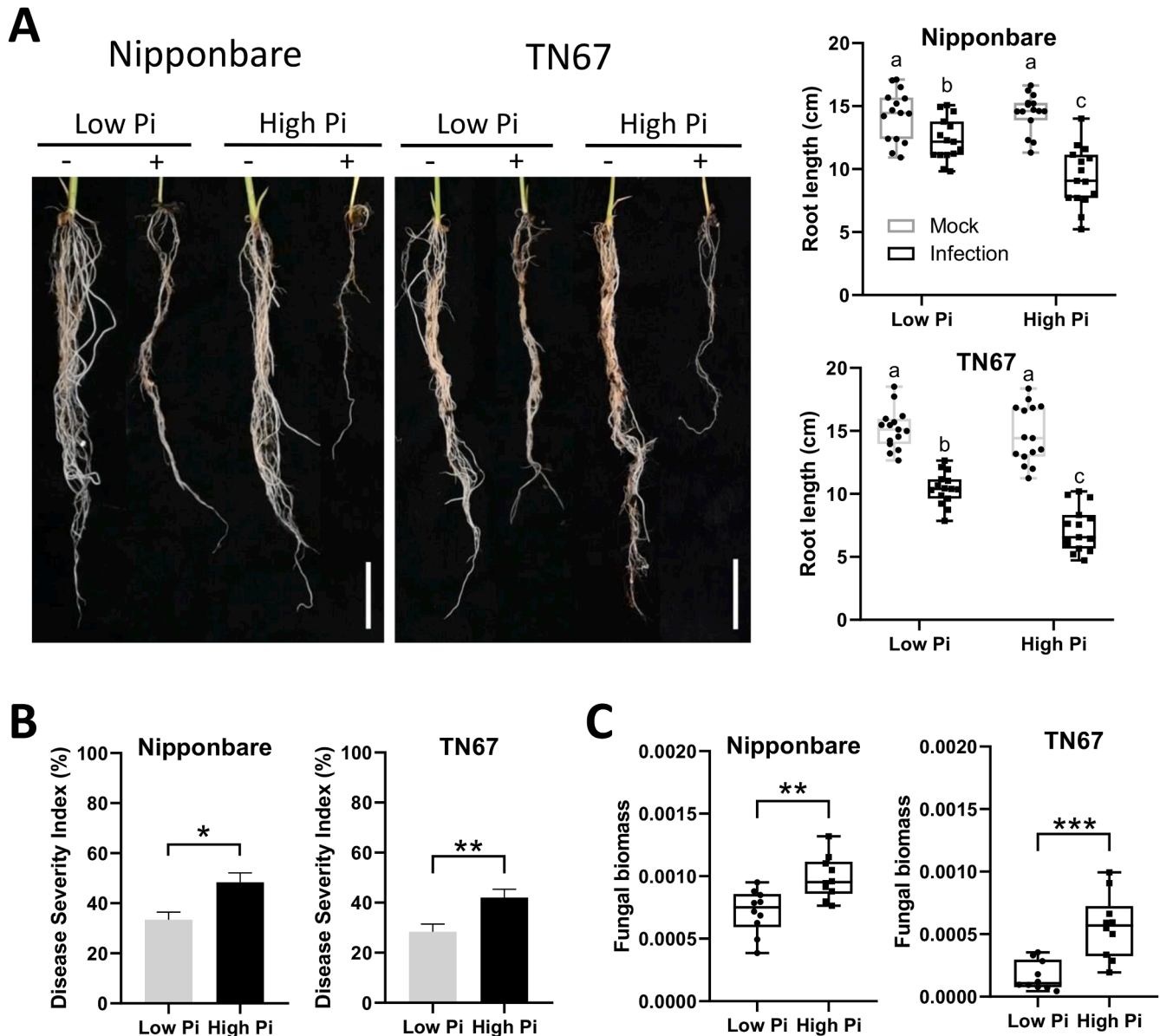


Fig. 1. Effect of Pi treatment and infection with *F. fujikuroi* in rice (cv Nipponbare and TN67). Plants were examined at 10 dpi after inoculation with fungal spores, or mock-inoculation. Three independent experiments were conducted with similar results. (A) Appearance of roots from mock-inoculated (-) and *F. fujikuroi*-inoculated (+) Nipponbare and TN67 plants. Bars = 3 cm. Right panels, root length measurements. Box plots represent median and data distribution (N = 15, each genotype and condition). Statistically significant differences were determined by two-way ANOVA followed by Tukey's HSD test (different letters indicate significant differences among conditions). (B) Disease Severity Index (DSI) in Nipponbare and TN67 plants grown under low Pi or high Pi conditions. Bars indicate mean $\pm$ SEM (N = 15) (Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$). (C) *F. fujikuroi* biomass in roots of Nipponbare and TN67 plants grown under low Pi or high Pi conditions. Box plots represent median and data distribution (N = 15, each genotype and condition). Statistically significant differences were determined by Student's *t*-test (**, $P < 0.01$; ***, $P < 0.001$).

for statistical analysis.

3. Results

3.1. High Pi fertilization enhances susceptibility to infection by *F. fujikuroi* in rice

To assess whether Pi supply has an effect on bakanae disease, plants (*japonica* rice cultivars Nipponbare and TN67) were grown under contrasting Pi regimes, namely 0.025 mM Pi and 2.5 mM Pi (from now on referred to as low Pi and high Pi, respectively). The susceptible cultivar Galileo and the resistant cultivar Selenio were used as checks for *F. fujikuroi* infections (Matić et al., 2016; Volante et al., 2017). Seeds were pre-germinated (5 days), inoculated with *F. fujikuroi* spores, or mock-inoculated, and maintained in the dark for 1 day (Supplementary Fig. 1 A). The rice seedlings were transplanted into inert substrate and allowed to continue growth for 10 days more. By this time, Nipponbare and TN67 seedlings grown in high Pi already accumulated more Pi in shoot and root tissues than seedlings grown under low Pi, thus, confirming that plants respond to Pi treatment (Supplementary Fig. 1 B, C). The *F. fujikuroi*-inoculated seedlings also showed typical symptoms of bakanae, these seedlings being taller than mock-inoculated seedlings (Supplementary Fig. 1 D).

Inoculation with *F. fujikuroi* suppressed root growth and development in Nipponbare and TN67 plants (Fig. 1A). Root growth reduction in response to pathogen inoculation was more pronounced in High-Pi plants as revealed by visual inspection of infected plants and root length measurements (Fig. 1A). Susceptibility to *F. fujikuroi* infection was determined by calculating the Disease Severity Index (DSI). In both genotypes (e.g., Nipponbare and TN67), high Pi treatment increased DSI values compared to plants in Low-Pi plants (Fig. 1B). Quantification of fungal biomass in *F. fujikuroi*-infected roots confirmed higher colonization in High-Pi plants (Fig. 1C). In the cultivars Galileo and Selenio (susceptible and resistant, respectively), treatment with high Pi also increased Pi content (Supplementary Fig. 2A) and suppressed root growth and development (Supplementary Fig. 2B). As expected, the highest DSI values were observed in the susceptible cultivar Galileo than in Selenio plants, in both cases DSI values being higher in High-Pi than in Low-Pi plants. Thus, Galileo plants had DSI values of 53.33 % and 76.19 % in low Pi and high Pi, respectively, while in Selenio plants DSI values were 23.21 % and 41.07 % in low Pi and high Pi, respectively (Supplementary Fig. 2C). Quantification of fungal biomass showed higher colonization in Galileo plants compared to Selenio plants, fungal colonization being higher in High-Pi plants than in Low-Pi plants (Supplementary Fig. 2D). Finally, inoculation with *F. fujikuroi* caused a reduction in chlorophyll content in leaves of all the rice genotypes assayed in this study (Nipponbare, TN67, Galileo and Selenio (Supplementary Fig. 3), regardless of their level of susceptibility.

From these results, it is concluded that treatment of rice plants with high Pi increases susceptibility to infection by *F. fujikuroi*, supporting that Pi has an effect on the outcome of the rice/*F. fujikuroi* interaction.

3.2. Pi accumulation by miR399 overexpression increases susceptibility to infection by *F. fujikuroi* in rice

As a complementary approach to Pi treatment, we investigated whether *MIR399* overexpression influences bakanae disease. Previous studies in *Arabidopsis* demonstrated that Pi deprivation increases miR399 accumulation (Chiou et al., 2006). The upregulation of miR399 suppresses *PHOSPHATE2* (*PHO2*) expression, this gene encoding a ubiquitin-conjugating enzyme involved in proteasome degradation of Pi transporters (Chiou et al., 2006). This, in turn, results in an increase in Pi uptake. Equally, *MIR399* overexpression leads to Pi accumulation in rice tissues (Campos-Soriano et al., 2020). Knowing this, in this work, miR399 overexpressor lines (hereinafter miR399 OE lines) were assayed in infections experiments with *F. fujikuroi*. The characterization of

transgenic lines overexpressing *MIR399* (TN67 genotype) was previously described (Campos-Soriano et al., 2020). Measurement of Pi content demonstrated Pi accumulation in miR399 OE plants prior *F. fujikuroi* infection (Supplementary Fig. 4A).

Next, miR399 OE plants (3 independently generated transgenic lines) were challenged with *F. fujikuroi* and their level of resistance/susceptibility to bakanae was determined. *F. fujikuroi* infection caused a reduction in root growth and development in miR399 OE lines, this reduction being more important in miR399 OE plants than in wild-type plants (Fig. 2A). Importantly, miR399 OE plants showed higher DSI values (Fig. 2B) and higher fungal colonization in their roots than wild-type plants (Fig. 2C). Chlorophyll loss in response to pathogen infection was also observed in miR399 OE plants, this reduction being more important in miR399 OE plants than in wild-type (TN67) plants (17.82 % and 46.06 % reduction in chlorophyll content in wild-type and miR399 OE lines, respectively) (Supplementary Fig. 4 B).

Collectively, these results indicated that miR399 overexpression, and subsequent Pi accumulation, negatively affects bakanae disease. These results are in agreement with those obtained in wild-type rice plants upon treatment with high Pi supply, thus, reinforcing the notion that Pi accumulation in rice tissues increases susceptibility to infection by *F. fujikuroi* in rice.

3.3. Expression of phosphate transporter genes in rice roots during infection by *F. fujikuroi*

Plant roots acquire P from the soil in the form of inorganic P, or Pi, through the activity of Pi transporters (PTs, also known as PHTs). PTs are classified into 5 major subfamilies based on their sequences and subcellular localizations (PHT1 to PHT5) (Gu et al., 2016; Wang et al., 2017). Members of the PHT1 family take up Pi from the soil using a Pi/H⁺ symport mechanism.

In this work, we investigated the expression of members of the PHT1 family of rice Pi transporters in response to infection by *F. fujikuroi*. The rice PHT1 family consists of 13 members (*OsPT1* to *OsPT13*) (Wang et al., 2021a). Their expression was examined in roots of *F. fujikuroi*-inoculated and mock-inoculated roots of plants that have been grown under low Pi or high Pi supply. Under non-infection conditions, the expression level of PHT1 family members varied, *OsPT1* showing the highest level of expression followed by *OsPT2* (Fig. 3). When comparing expression levels of Pi transporter genes in response to Pi treatment (Low Pi vs High Pi, mock conditions), *OsPT10* was found to be up-regulated in plants treated with high Pi (Fig. 3). No significant alterations were observed in the expression of other Pi transporter genes between Pi conditions (e.g., *OsPT1*, *OsPT2*, *OsPT3*, *OsPT4*, *OsPT5*, *OsPT6*, *OsPT7*, *OsPT8* and *OsPT9*) (Fig. 3, mock).

Not only Pi treatment, but also *F. fujikuroi* infection was found to modulate the expression of Pi transporter genes, a response that is dependent on the Pi condition in which the rice plants are grown (Fig. 3, infection). *OsPT1* and *OsPT4* expression was up-regulated by pathogen infection in both Pi conditions, but these genes reached lower expression levels in High-Pi plants compared to Low-Pi plants. On the other hand, *OsPT2*, *OsPT6* and *OsPT10* were down-regulated by pathogen infection in both Pi conditions (Fig. 3). As for *OsPT5*, its expression was induced by pathogen infection in Low-Pi plants but not in High-Pi plants (Fig. 3). Finally, *OsPT11*, *OsPT12* and *OsPT13* expression was barely detectable in mock-inoculated or *F. fujikuroi*-inoculated roots.

Taken together, these results indicated a differential regulation in the expression of PHT1 family members of Pi transporters whose expression is regulated not only by Pi treatment, but also by infection with the root pathogen *F. fujikuroi*.

3.4. Treatment with high Pi fosters invasive growth of *F. fujikuroi* in rice roots

To further establish a relationship between Pi content and *F. fujikuroi*

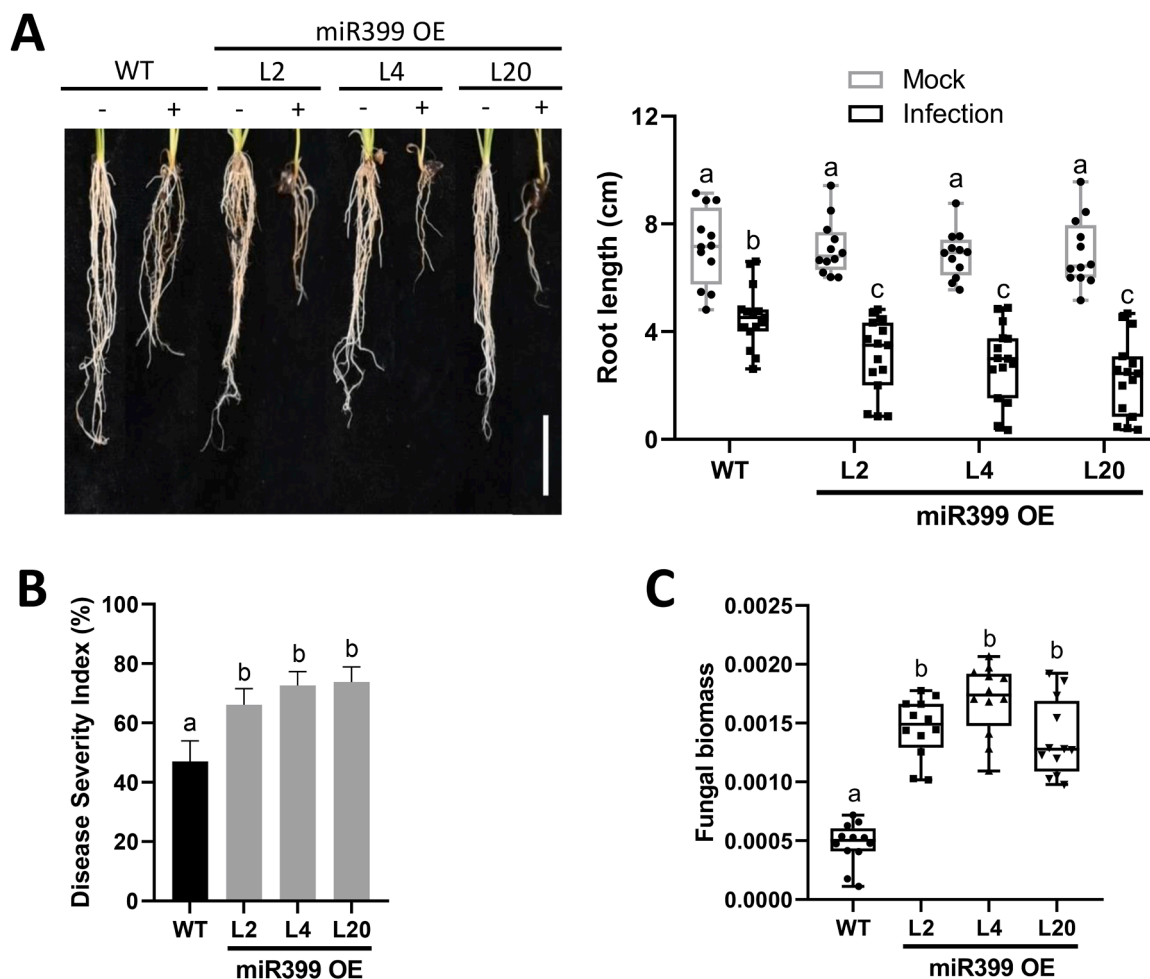


Fig. 2. Infection by *F. fujikuroi* in rice plants overexpressing *MIR399*. Wild-type (WT, cv TN67) and *miR399* OE lines (L2, L4 and L20) were grown under normal Pi supply (0.25 mM Pi) and inoculated with *F. fujikuroi* spores (10^6 spores/mL), or mock-inoculated. Three independent experiments were conducted with similar results. (A) Appearance of roots from mock-inoculated (-) and *F. fujikuroi*-inoculated plants. Bars = 3 cm. Right panels, root length measurements. Boxplots represent median and data distribution (N = 15). Different letters indicate significant differences among genotypes (two-way ANOVA followed by Tukey's HSD test). (B) Disease Severity Index (DSI) (left panel) in roots of wild-type and *miR399* OE lines at 10dpi. Bars indicate mean $\pm$ SEM (N = 15). Different letters indicate significant differences among genotypes (one-way ANOVA). (C) *F. fujikuroi* biomass in roots of wild-type and *miR399* OE lines at 10 dpi. Boxplots represents median and data distribution (N = 15). Statistically significant differences are indicated by different letters (one-way ANOVA).

infection, colonization of rice roots was monitored *in vivo* using a *F. fujikuroi* strain constitutively expressing the *green fluorescent protein* (*gfp*) reporter gene. The obtention and pathogenicity of the *gfp*-expressing *F. fujikuroi* isolate was previously described (Aragona et al., 2021). Confocal microscopy allowed visualization of fungal invasion in roots of plants grown under contrasting Pi regimes at different times after inoculation with the *gfp*-expressing *F. fujikuroi* isolate (Chen et al., 2020).

Early during infection (e.g., 24 hpi), hyphae growing along the root surface were visualized (Fig. 4A). By this time of infection, penetration events into the root cells were barely seen, regardless of the Pi condition (Fig. 4A, 24 h). At 48 hpi, the fungus has already penetrated the epidermal root cells. By this time, invasion of epidermal cells was more frequently observed in roots of High-Pi plants than in roots of Low-Pi plants (Fig. 4A, 48 h). At 72 hpi, the fungus invaded contiguous cells in High-Pi plants but not in Low-Pi plants (Fig. 4A, 72 h).

For a more detailed analysis of root colonization, we examined transversal sections of *F. fujikuroi*-infected roots at a later stage of the infection process (e.g., 10 dpi). Confocal examination *F. fujikuroi*-infected roots from the Nipponbare and TN67 cultivars is presented in Fig. 4B. Infection of roots from *miR399* OE plants is presented in Fig. 4C. In all the rice genotypes, the fungus penetrated the root cortex and

invaded cortical cells. However, root cortical cells in Nipponbare and TN67 plants grown under high Pi supply were more highly colonized by the fungus than roots of Low-Pi plants (Fig. 4B). We also noticed that Nipponbare roots were more susceptible to colonization by *F. fujikuroi* than TN67 roots (Fig. 4B). The intensity of colonization in *miR399* overexpressor plants accumulating Pi was also higher than in wild-type plants (Fig. 4C). Quantification of fungal biomass in *F. fujikuroi*-infected roots confirmed higher colonization in roots of plants accumulating Pi (e.g., plants treated with high Pi and *miR399* OE plants) (Fig. 4D).

Regarding the control rice cultivars, as expected, fungal invasion was clearly observed in the susceptible cultivar Galileo, but not in the resistant cultivar Selenio (Supplementary Fig. 5). In the susceptible cultivar Galileo, fungal invasion was more abundant in plants grown under high Pi supply compared to plants in low Pi, as revealed by confocal microscopy analysis of roots inoculated with the *gfp*-expressing fungal isolate and quantification of fungal biomass (Supplementary Fig. 5A). The fungus was barely detected in roots of the resistant cultivar Selenio, not even in plants grown under high Pi supply (Supplementary Fig. 5B).

Together, these results support that overaccumulation of Pi either by growing the plants under a high Pi regime or by overexpression, enhances susceptibility to colonization by *F. fujikuroi* in rice roots.

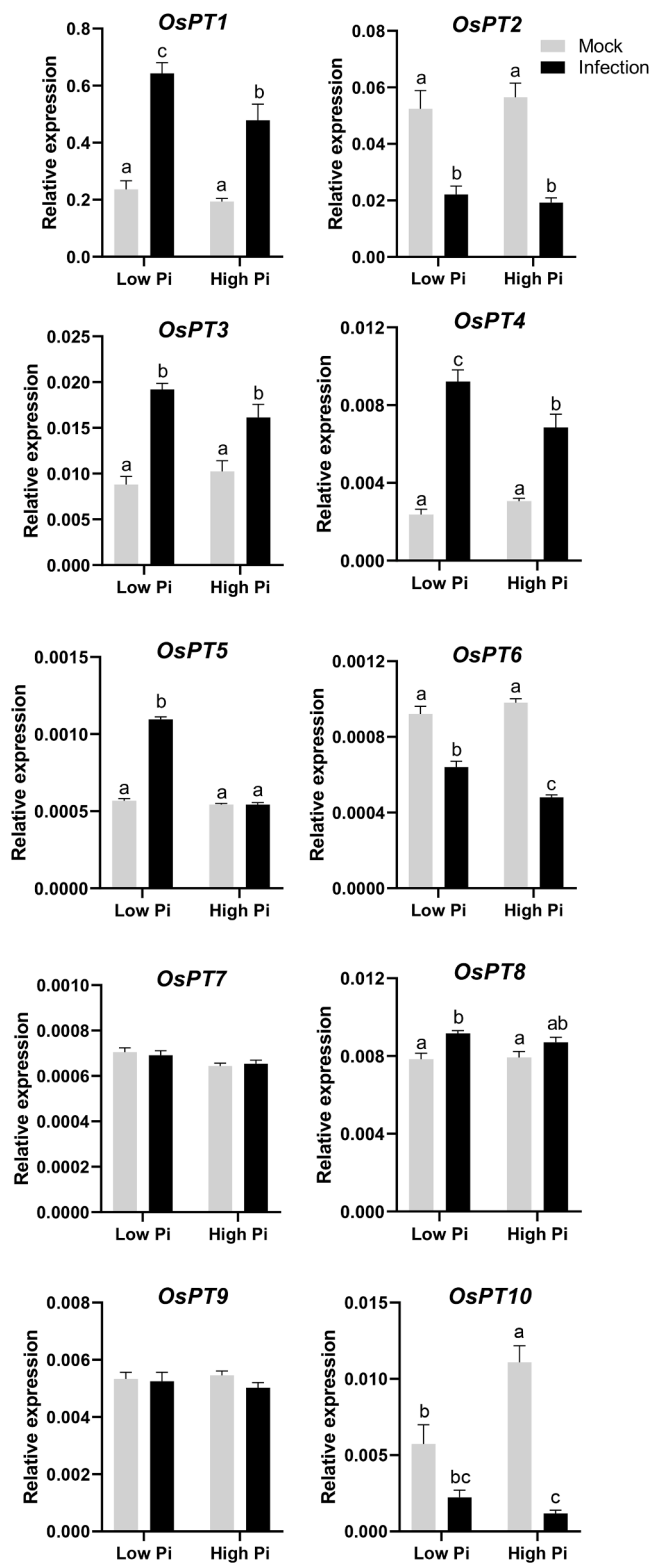


Fig. 3. Expression of members of the *PHT1* family of Pi transporter genes in roots of rice (cv Nipponbare) plants grown under low Pi or high Pi, and inoculated with *F. fujikuroi* spores, or mock-inoculated. Expression of RT-qPCR was carried out using the *OsUbi1* rice gene as the internal control. *OsPT11*, *OsPT12* and *OsPT13* expression was undetectable in the same plant material. Bars represent mean $\pm$ SEM of 4 biological replicates, each one from a pool of three different plants. Different letters indicate significant differences among conditions (two-way ANOVA followed by Tukey's HSD test).

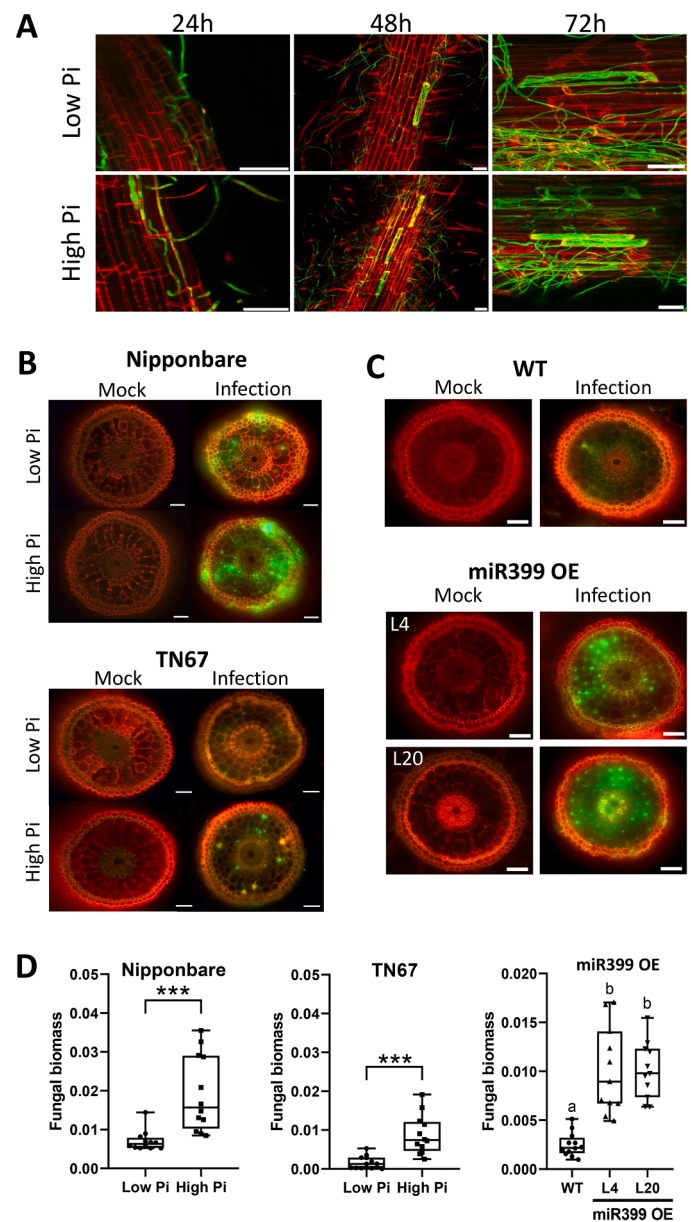


Fig. 4. Confocal laser scanning microscopy of roots from rice cultivars (Nipponbare, TN67 and miR399 OE lines) that have been inoculated with the GFP-expressing *F. fujikuroi* isolate. Three independent experiments were conducted with similar results. Seedlings were grown *in vitro* under low Pi or high Pi supply (Nipponbare, TN67 plants). MiR399 OE plants (and corresponding wildtype TN67 plants) were grown under normal Pi supply. The rice plants were then inoculated with *F. fujikuroi* spores (10^6 spores/mL), or mock-inoculated. Root cells were stained with propidium iodide (in red). (A) Longitudinal views of the primary root from Nipponbare plants. Images were taken at 24, 48 and 72 hpi with *F. fujikuroi* spores. Similar results were obtained in TN67 plants. Bars = 25 μ m. (B) Transverse sections of primary roots of Nipponbare and TN67 plants (upper panel and lower panel, respectively). Images were taken at 10 days post-inoculation (dpi). Bars = 50 μ m. (C) Transverse section of primary roots of wild-type (TN67) and miR399 OE lines grown under normal Pi supply. Images were taken at 10 days post-inoculation (dpi). Bars = 50 μ m. (D) Fungal biomass in roots of Nipponbare, TN67 and miR399 OE lines at 10 dpi with *F. fujikuroi* spores. Boxplots represent median and data distribution (N = 12). Statistically significant differences were determined by Student's *t*-test (***, *P* < 0.001) and one-way ANOVA (different letters indicate significant differences among genotypes).

3.5. Pi accumulation compromises the induction of defense responses in *F. fujikuroi*-infected rice roots

Knowing that Pi accumulation increases susceptibility to bakanae disease, we investigated the impact of Pi treatment on defense responses

in rice roots during infection by *F. fujikuroi*. Initially, we examined the expression of PR genes *OsPR1a* and *OsPBZ1*. These genes are considered markers for the induction of the rice defense response to pathogen infection. *OsPR1a* and *OsPBZ1* expression was examined at different times after inoculation with *F. fujikuroi* spores (24 h and 48 h post-

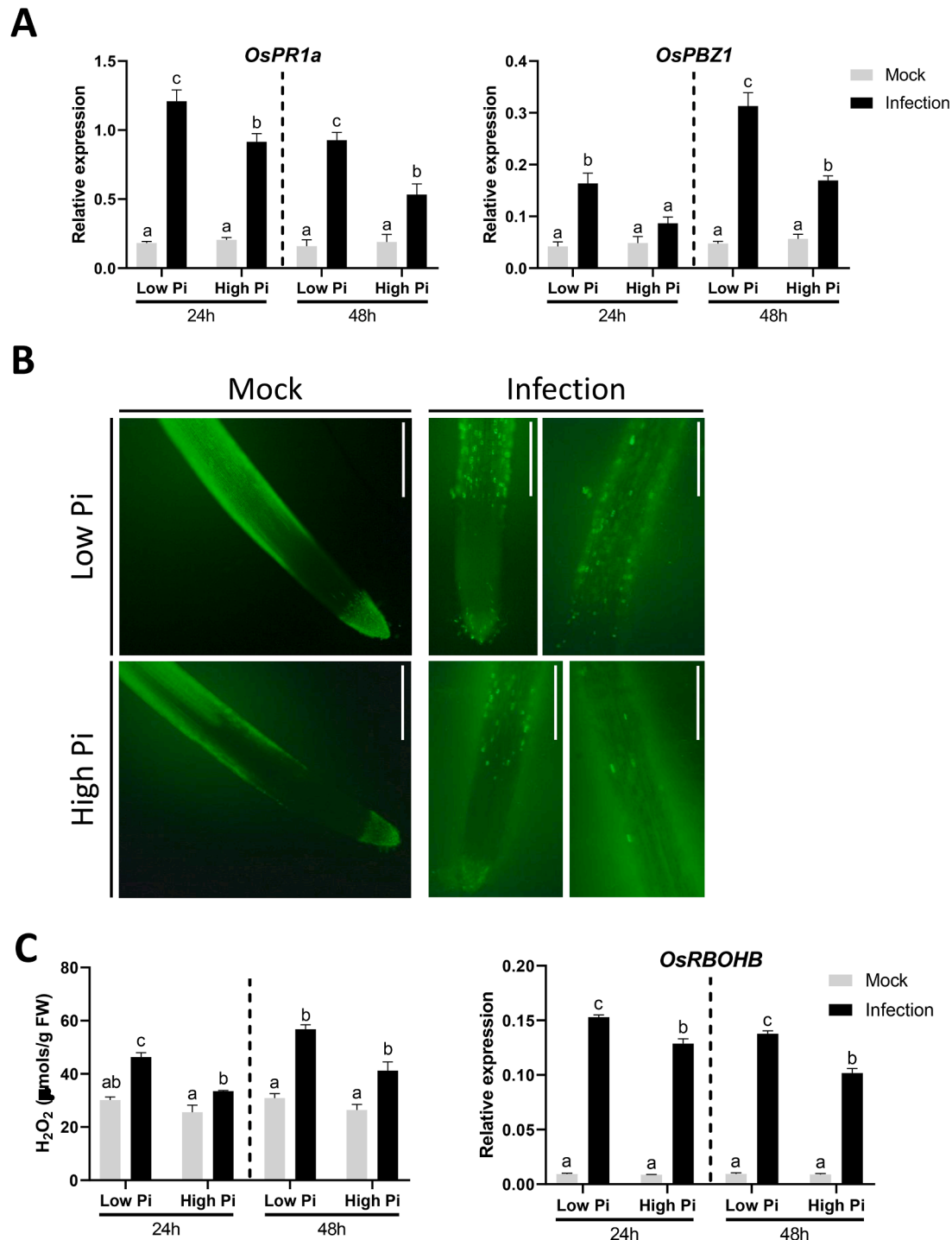


Fig. 5. Defense response against *F. fujikuroi* in roots of rice plants that have been grown under low Pi or high Pi conditions. Rice plants (cv Nipponbare) were inoculated with fungal spores (10^6 spores/mL), or mock-inoculated. Statistically significant differences in A and C were determined by two-way ANOVA followed by Tukey's HSD test. (A) Expression of the *Pathogenesis-Related* genes, *OsPR1a* and *OsPBZ1*. RT-qPCR was carried out at 24 h and 48 h after infection using the *OsUbi1* rice gene as the internal control. Bars represent mean $\pm$ SEM of 4 biological replicates, each one from a pool from three different plants. (B) Microscopic observation of ROS accumulation in the root tip region of rice plants grown under low Pi or high Pi supply and inoculated with *F. fujikuroi* spores, or mock-inoculated (24 hpi). ROS (H_2O_2) was detected using the fluorescent probe H_2DCFDA under a fluorescence microscope. Representative images are shown. Bars = 50 μ m. (C) H_2O_2 quantification (left panel) and *OsRBOHB* expression (right panel) in roots at 24- and 48-hours post-inoculation. RT-qPCR was carried out using the *OsUbi1* rice gene as the internal control. Bars represent $\pm$ SEM of 4 biological replicates, each one from a pool of five different plants.

inoculation). Under non-infection conditions, no differences were observed in the expression of these genes across Pi treatments (Fig. 5A, mock). As expected, pathogen infection induced *OsPR1a* and *OsPBZ1* expression (Fig. 5A, infection). Interestingly, *OsPR1a* and *OsPBZ1* were induced to a lesser extent in roots of High-Pi plants than in roots of Low-Pi plants during *F. fujikuroi* infection (Fig. 5A). A weaker induction of defense gene expression during infection is consistent with results presented above on disease susceptibility to *F. fujikuroi* infection in High-Pi plants (see Fig. 1B, C) and root colonization by the *gfp*-expressing fungal strain (see Fig. 4).

ROS molecules, in particular H_2O_2 , play a key role in defense reactions against pathogens, acting as signaling molecules for the induction of *PR* gene expression (Torres et al., 2006). H_2O_2 is also important in root growth and development with a function in the regulation of cell wall modification (Kärkönen and Kuchitsu, 2015; Tsukagoshi, 2016). ROS (H_2O_2 and O_2) have been proposed to determine the transition between cell proliferation and elongation in *Arabidopsis* roots (Zhou et al., 2020; Eljebbawi et al., 2021). Less is known about the association between ROS and root development or between ROS and defense reactions to infection by root pathogens in rice.

ROS accumulation was examined in roots of Low-Pi and High-Pi plants, in the presence or absence of pathogen infection. For this, we performed histochemical staining of rice roots with the fluorescent probe H_2DCFDA (2', 7'-dichlorofluorescein diacetate), a stain that mainly detects H_2O_2 accumulation (Fichman et al., 2019). Except in the transition zone, a generalized distribution of ROS occurred at the meristematic and elongation zone of the rice roots in Low-Pi and High-Pi plants in the absence of pathogen infection (Fig. 5B, mock). Upon pathogen infection, however, ROS preferentially accumulated at discrete sites in roots of both Low-Pi and High-Pi plants, the GFP-labeled sites being more frequently observed in roots of Low-Pi plants (Fig. 5B, infection). Regarding total ROS accumulation in roots, no significant differences occurred in ROS accumulation between Low-Pi and High-Pi plants under non-infection conditions (same regions as those examined by confocal microscopy). Although H_2O_2 accumulation increased in response to pathogen infection in both Pi conditions, its level was reduced in High-Pi plants compared to Low-Pi plants (24 and 48 hpi) (Fig. 5C, left panel).

It is well known that RBOH (Respiratory Burst Oxidative Homolog) enzymes in the plasma membrane are the primary source of ROS production in the apoplast during pathogen infection. Among rice *RBOH* genes, *OsRBOHB* has been shown to respond to pathogen infection in rice (Martín-Cardoso et al., 2024). Consistent with results obtained by histochemical detection and H_2O_2 quantification, a weaker induction of *OsRBOHB* expression occurs in roots of High-Pi plants compared to Low-Pi plants (Fig. 5C, right panel).

Collectively, this study indicated that Pi accumulation on rice roots compromises the induction of defense-related mechanisms, including ROS production and *PR* expression. A reduced defense capacity of roots from rice plants treated with high Pi would be consistent with increased susceptibility to infection by *F. fujikuroi* in these plants.

3.6. Pi-mediated regulation of lignin and suberin deposition in rice roots

Traditionally, the plant cell wall has been considered as a passive barrier against pathogen infection. Currently, this view has evolved and the plant cell wall is now considered a dynamic structure with a function in resistance to pathogens (Miedes et al., 2014). Furthermore, alterations of cell wall components can lead to the activation of immune signaling responses (Wolf, 2022). Several types of cell-type specific barriers contribute to resistance to pathogen infection in roots, including deposition of lignin and suberin. For instance, reinforcement of the xylem vessel secondary cell wall with lignin hinders entry of the pathogen to the vasculature, hence pathogen movement throughout the plant via the vascular system. Although many reports described the relevance of reinforcement of plant cell walls in arresting pathogen

infection, there are also examples in which “weakening” of the cell wall (e.g., by knocking out essential genes involved in the biosynthesis of the cell wall) results in enhanced resistance towards specific pathogens (Gallego-Giraldo et al., 2011; Miedes et al., 2014).

We investigated whether Pi supply has an effect on lignin and suberin accumulation in rice roots, in the presence or absence of pathogen infection. Lignin was visualized with Basic Fuchsin, whereas suberin was visualized with Fluorol Yellow 088 (Sexauer et al., 2021). Results obtained on the histochemical analysis of lignin and suberin accumulation are presented in Fig. 6 (lignin, red color; suberin, yellow color). In mock-inoculated plants, lignin was detected in the exodermis, sclerenchyma and endodermis, with no important differences in lignin staining between Low-Pi and High-Pi plants (Fig. 6, mock). As for suberin, the root exodermis in Low Pi-plants showed a slightly higher and discontinuous suberin staining relative to High-Pi plants, in both cases in the absence of infection (Fig. 6, mock). In the endodermis, suberin staining occurred with similar intensity in one or another Pi condition (Fig. 6, mock). Upon pathogen infection, both lignin and suberin accumulation increased regardless of the Pi condition. However, suberin staining in exodermis and vascular tissues was higher in roots of Low-Pi plants compared to roots of High-Pi plants (Fig. 6, infection).

The impact of Pi treatment and infection on the expression of lignin biosynthesis genes or genes involved in the suberin pathway was investigated. A schematic representation of the lignin biosynthesis pathway indicating genes whose expression was examined in this study is presented in Fig. 7A. Compared to High-Pi plants, most of the genes involved in lignin biosynthesis exhibited a higher expression in Low-Pi plants, in the absence of pathogen infection in both cases (Fig. 7B). This was the case of *OsPAL*, *Os4CL*, *OsCOMT*, *OsCCoAOMT*, *OsF5H*, *OsCCR*, *OsCAD*, and *OsPRX* genes (Fig. 7B). Although *F. fujikuroi* infection induced the expression of lignin biosynthesis genes in both Pi regimes, all the genes here examined reached higher levels of expression in roots of Low-Pi plants than in High-Pi plants (Fig. 7B). Other lignin-related genes, such as those involved in lignin biosynthesis, or genes related to formation of the Casparian strip (e.g., *OsCASP1* and *OsLOT1*) showed similar expression patterns, most of these genes showing higher expression in roots of Low-Pi plants in the absence of pathogen infection, and stronger induction during infection (Supplementary Fig. 6 A, B).

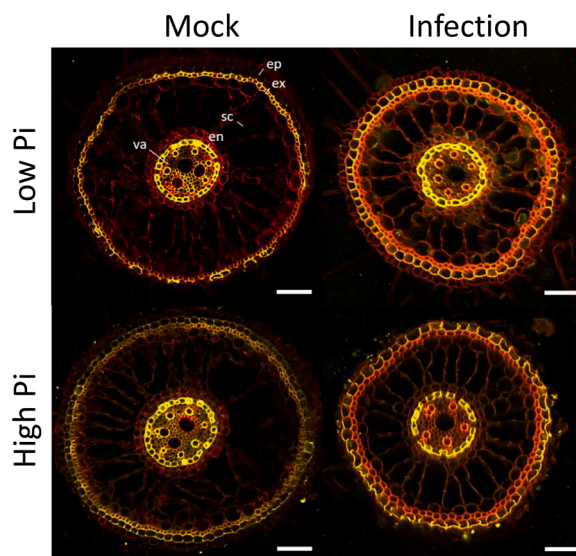


Fig. 6. Histochemical analysis of lignin and suberin accumulation in roots of rice (cv Nipponbare) plants grown under low Pi or high Pi conditions, and inoculated with *F. fujikuroi* spores, or mock-inoculated. Staining was carried out at 10 dpi. The red color corresponds to lignin and the yellow color to suberin. Scale = 50 μ m. Ep; epidermis; ex, exodermis; en, endodermis; sc, sclerenchyma; va, vascular tissues. Bars = 50 μ m.

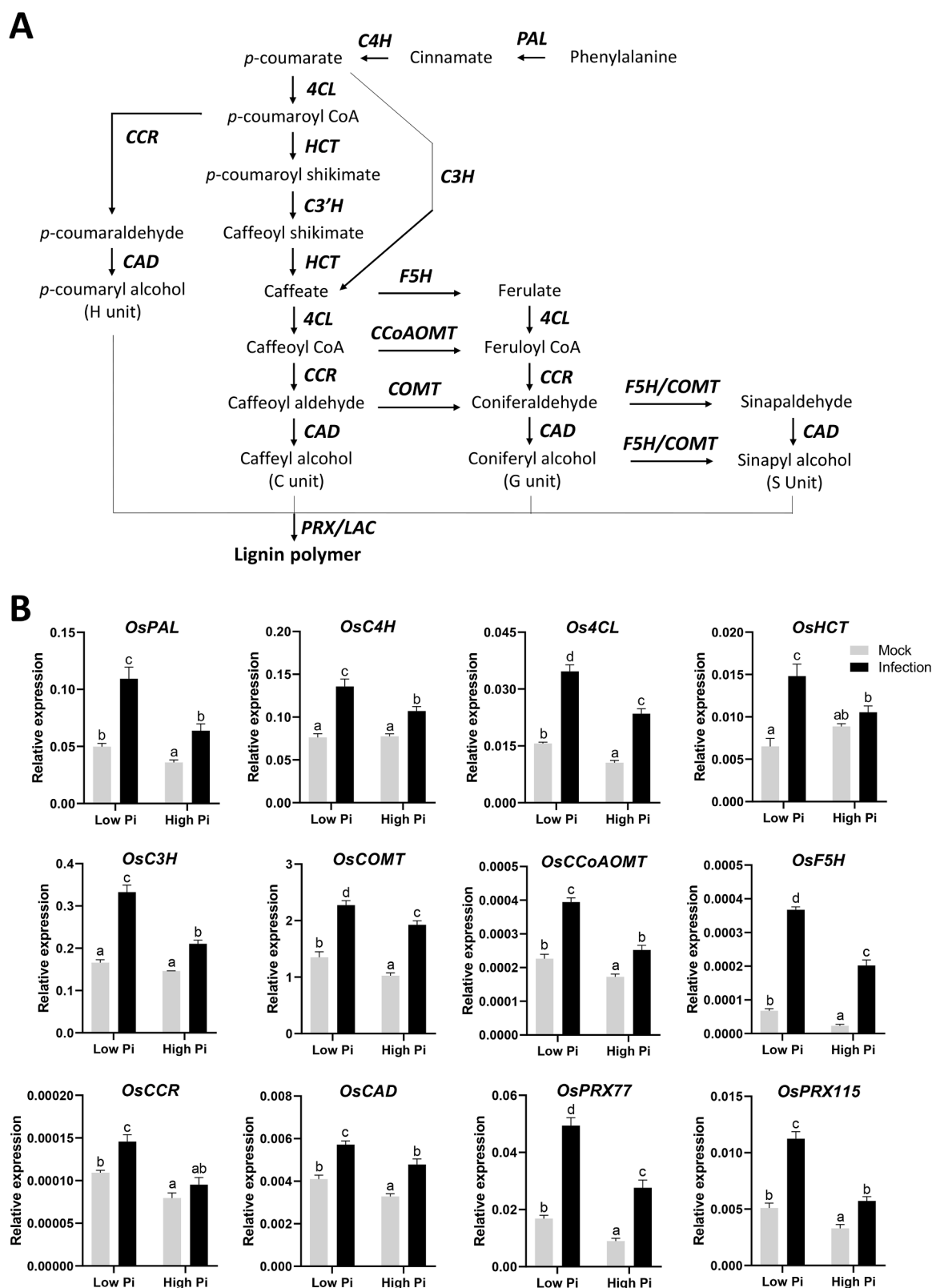


Fig. 7. Expression of lignin biosynthesis genes in roots of rice (cv Nipponbare) plants grown under low Pi or high Pi supply, and inoculated with *F. fujikuroi* spores, or mock-inoculated. (A) Lignin biosynthesis pathway. Genes whose expression is shown in B are highlighted in bold. (B) Expression of lignin biosynthesis genes at 7 dpi. RT-qPCR was carried out using the *OsUbi1* rice gene as the internal control. Bars correspond to mean $\pm$ SEM of 4 biological replicates, each one from a pool from three different plants. The following genes were analyzed: *OsPAL* (*OsPAL04*), *OsC4H* (*OsC4H2*), *Os4CL* (*Os4CL3*), *OsHCT* (*Os08g0543400*), *OsC3H* (*OsC3H1*), *OsCOMT* (*OsCOMT1*), *OsCCoAOMT* (*OsCCoAOMT-1*), *OsF5H* (*OsF5H2*), *OsCCR* (*OsCCR17*), *OsCAD* (*OsCAD8a*), *OsPRXs* (*OsPRX77* and *OsPRX115*). Statistically significant differences were determined by two-way ANOVA followed by Tukey's HSD test.

Genes implicated in suberin biosynthesis, transport of suberin precursors to the apoplast or suberin polymerization (e.g., *OsKCS6*, *OsCYP86A7*, *OsCYP86A9*, *OsGPATs*, *OsABCGs*, *OsLTP14* and α/β -Hydrolase) also showed higher expression levels in Low-Pi plants compared to High-Pi plants, these genes also exhibiting higher level of expression in response to pathogen infection in Low-Pi plants (Fig. 8).

Collectively, results obtained on the expression of lignin and suberin-related genes in roots of Pi-treated, infected with *F. fujikuroi*, or not, were essentially in agreement with those obtained by histochemical analysis of lignin and suberin accumulation in the rice roots (see Fig. 6). Growing rice plants under low Pi conditions is accompanied by higher lignin and suberin accumulation than in roots of High-Pi plants. This increase results from a higher expression of genes involved in lignin and suberin biosynthesis. Under infection conditions, the expression of these genes was also higher in roots of Low-Pi plants than in High-Pi plants. These results support that lignin and suberin accumulation in roots of Low-Pi plants enhances the capability of rice plants to prevent root colonization by this fungus.

4. Discussion

P is a vital nutrient required for plant growth and development. It is acquired in the form of inorganic P (Pi) that once inside the cell is incorporated into various molecules such as phospholipids, phosphoproteins, nucleic acids (DNA and RNA), numerous sugar phosphates, and ATP (the energy currency of the cell) (Poirier et al., 2022). Although evidence supports that the plant's Pi status might influence disease resistance, the molecular mechanisms underpinning this process only recently begun to be characterized. The complexity in cross-talk between Pi signaling and immune signaling is illustrated by observations in which Pi imbalances can either weaken or reinforce resistance to pathogen infection in plants (e.g., *Arabidopsis* and rice) (Campos-Soriano et al., 2020; Val-Torregrosa et al., 2021, 2022b).

In this study we provide evidence that Pi accumulation enhances susceptibility to infection by *F. fujikuroi*, the causal agent of bakanae in rice plants. Susceptibility to *F. fujikuroi* infection is observed in plants grown under high Pi supply as well as in miR399 overexpressor plants, the later ones also over-accumulating Pi. Several pieces of evidence support that Pi accumulation increases susceptibility against *F. fujikuroi* in rice. Firstly, bakanae symptomatology was more severe in rice plants

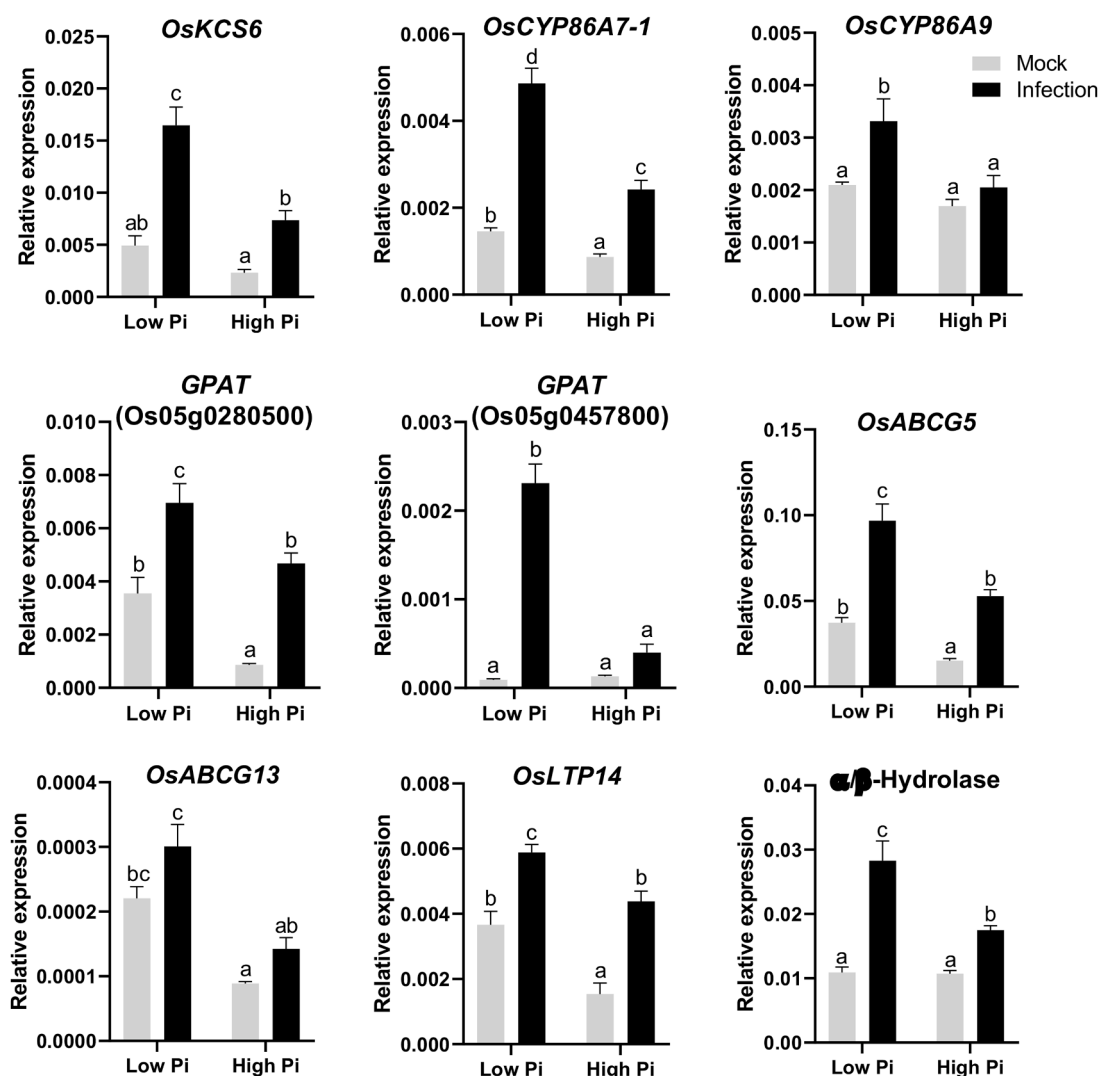


Fig. 8. Expression of suberin-related genes in rice (cv. Nipponbare) roots of rice plants grown under low Pi or high Pi supply, and inoculated with *F. fujikuroi* spores, or mock-inoculated. RT-qPCR analysis was carried out at 7 dpi using the *OsUbi1* rice gene as the internal control. Genes analyzed were: *OsKCS6*, *OsCYP86A7*, *OsCYP86A9*, *GPAT* genes (*Os05g0280500*, *Os05g0457800*), *OsABCG* genes (*OsABCG5*, *OsABCG13*), *OsLTP14*, and α/β -Hydrolase (*Os01g0317800*). Bars represent mean $\pm$ SEM of 4 biological replicates, each one from a pool from three different plants. Statistically significant differences were determined by two-way ANOVA followed by Tukey's HSD test.

accumulating Pi (Nipponbare, TN67 grown under high Pi supply, miR399 overexpressor plants). Plants accumulating Pi exhibited less root biomass and higher DSI values than plants grown under low Pi supply. Greater root colonization by *F. fujikuroi* in High-Pi plants was confirmed by fungal biomass quantification and visualization of the *in vivo* invasion of *F. fujikuroi* in root tissues using a GFP-tagged *F. fujikuroi* isolate. Equally, *in vivo* imaging of *F. fujikuroi*-infected roots revealed higher colonization in MIR399 overexpressor plants compared to wild-type plants.

Secondly, a weaker induction of PR genes (*OsPR1a*, *OsPBZ1*) occurs in *F. fujikuroi*-infected roots of High-Pi plants compared to infected roots of Low-Pi plants, supporting that Pi accumulation negatively affects the expression of PR genes during infection. Previous studies in rice plants accumulating Pi also revealed a weaker induction of PR genes in leaves during infection with the foliar pathogen *Magnaporthe oryzae* (Campos-Soriano et al., 2020; Martín-Cardoso et al., 2024). In other studies, a relationship between defense gene expression and resistance/susceptibility to *F. fujikuroi* was described in shoots of rice cultivars showing different levels of bakanae resistance (Ji et al., 2016; Matić et al., 2016; Cheng et al., 2020; Bashyal et al., 2022). Adding further complexity to the effect of Pi nutrition on plant immunity, an opposite regulation of defense reactions has been described in Pi-treated *Arabidopsis* plants. Thus, stronger expression of PR genes occurs during pathogen infection in *Arabidopsis* plants overaccumulating Pi which leads to a phenotype of disease resistance. Clearly, further research is needed to better understand how Pi supply affects defense reactions in different plant/pathogen interactions.

Another finding of this study is that, upon *F. fujikuroi* infection, there is a redistribution of ROS, in particular H₂O₂, in the rice root. The generation of H₂O₂ is a widely recognized defense mechanism utilized by plants to mitigate pathogen infections, including vascular pathogens (Wang et al., 2024). Thus, a delayed or weaker accumulation of ROS is usually observed in compatible plant/pathogen interactions, which generally culminates in susceptibility to infection, while a quick and strong accumulation of ROS occurs at the sites of infection in incompatible interactions which then prevents host colonization. Here, histochemical analysis of rice roots revealed ROS (e.g., H₂O₂) accumulation in *F. fujikuroi*-infected roots, this analysis also showing a localized accumulation of ROS, presumably at the sites of pathogen infection. Cells accumulating H₂O₂ were however less abundant in High-Pi plants than in Low-Pi plants. Quantification of H₂O₂ in rice roots also indicated lower levels of H₂O₂ in High-Pi plants in response to pathogen infection, which can be explained by a lower induction of *OsRBOH* expression during infection. From these results, it appears that growing rice under high Pi supply hampers pathogen-inducible ROS production at the infected tissues. Conversely, growing rice under a low Pi regime promotes local accumulation of H₂O₂, a mechanism that resembles the hypersensitive response (HR) used by plants to prevent pathogen infection. This would explain the observed enhanced resistance to *F. fujikuroi* infection in Low-Pi plants compared to High-Pi plants. A similar reaction of local accumulation of H₂O₂ has been recently described during infection of rice leaves by the pathogen *M. oryzae* in plants grown under a high Pi regime (Martín-Cardoso et al., 2024).

Results here presented also provide evidence that Pi accumulation stimulates lignin and suberin accumulation in specific cell types in the rice root. It is generally accepted that H₂O₂ induces polymerization of the lignin subunits and promotes lignin deposition. In addition to a reduction in H₂O₂ levels, Pi accumulation is accompanied by a weaker induction of lignin biosynthesis genes in High-Pi plants compared to Low-Pi plants during *F. fujikuroi* infection (the former ones showing susceptibility to bakanae). As for suberin, this polymer is deposited at the endodermal cells, and the suberized endodermis prevents pathogen entry into the vascular tissue and subsequent spread throughout the plant (Kashyap et al., 2021; Chen et al., 2022). The observation that lignin and suberin accumulate at a lower level in High-Pi plants during pathogen infection is in agreement with results previously reported in

barley plants in which greater lignin and suberin accumulation was linked to resistance towards *Fusarium graminearum* infection (Basheer et al., 2022). Reduced accumulation of suberin in rice roots of High-Pi plants, as revealed by histochemical analysis of rice roots, is also consistent with a lower expression of suberin-related genes during *F. fujikuroi* infection in High-Pi plants compared to Low-Pi plants.

It is noteworthy to mention that our knowledge on the expression of genes involved in lignin and/or in suberin biosynthesis in response to either Pi availability or pathogen infection is still very limited. The expression pattern of genes involved in the synthesis of the various building blocks of the lignin polymer (e.g., G, S, C and H units) in response to Pi treatment and pathogen infection has been examined in detail in this work. Similarly, the expression of genes involved in different processes related to suberin accumulation is presented in this work. They included genes involved in the formation of suberin precursors (e.g., KCS, β -ketoacyl-CoA synthase; CYP, cytochrome P450, and GPAT, glycerol-3-phosphate acyltransferase), suberin export to the apoplast (ABC, ATP-binding cassette transporter; and LTP, lipid transfer protein), and polymerization of oligomers and monomers of the suberin macromolecule ($\alpha\beta$ HD, α,β -hydrolase), as well as peroxidase (PRX) genes (Shiono et al., 2014). The observed reduction in the accumulation of both polymers, lignin and suberin in High-Pi plants relative to Low-Pi plants, also during pathogen infection, correlates well with a weaker induction of genes involved in lignin and suberin formation. The combination of these factors might well contribute to weaken the strength and structure of the cell walls in rice roots to facilitate colonization by *F. fujikuroi*. Then, reinforcement of cell walls through the accumulation of lignin and suberin would contribute to immunity against *F. fujikuroi* in rice. Taken together, the information presented in this study enhances our understanding of the influence of Pi on processes related to cell wall reinforcement in rice roots, and the relevance of these processes in disease resistance. From this, it appears that rice plants adapt to changes in Pi availability by increasing or decreasing lignin accumulation and suberization which might also have an impact on disease resistance.

Lastly, the data derived from analyzing expression phosphate transporter genes demonstrated that, not only Pi availability, but also pathogen infection, modulates the expression of these genes. A differential regulation of Pi transporter genes from the PHT1 family in rice roots in response to either Pi availability or *F. fujikuroi* infection is here reported. Among PHT1 family members, only *OsPT10* was found to be regulated by Pi treatment (e.g., up-regulation in High-Pi plants relative to Low-Pi plants, mock conditions in both cases). In response to pathogen infection, the expression of phosphate transporter genes was either up-regulated (*OsPT1*, *OsPT3*, *OsPT4*) or down-regulated (*OsPT2*, *OsPT6*, *OsPT10*), whereas *OsPT5* was up-regulated by pathogen infection only in low Pi conditions. This differential regulation of Pi transporter genes might be relevant in integrating responses to Pi and plant defense. In favor of this possibility, although PTs primarily function in nutrient absorption and transport, there are examples of transporters that function in disease resistance, such as the *Arabidopsis AtPHT4;1* gene (Wang et al., 2011). In rice, the analysis of *OsPT8* overexpressor and RNAi transgenic plants revealed a role of PT8 in resistance against fungal (*M. oryzae*) and bacterial (*Xanthomonas oryzae* pv. *oryzae*) pathogens by negatively regulating the expression of PR genes (Dong et al., 2019). On the other hand, although PHT1 family members are generally involved in the acquisition and redistribution of Pi in plants (Victor Roch et al., 2019), evidence also support that some PHT1 members might participate in diverse biological processes involving Pi transport (Kopriva and Chu, 2018). For instance, *OsPT7* has been shown to play a role in Pi redistribution and anther development, and *>OsPT8* regulates plant growth and resistance to infection by foliar pathogens (*X. oryzae*, *M. oryzae*) (Dong et al., 2019; Dai et al., 2022). *OsPT11* and *OsPT13* expression is associated with Pi uptake in mycorrhizal rice plants (Paszowski et al., 2002; Yang et al., 2012). However, several open questions remain regarding the role of Pi transporters in modulating Pi distribution in biological processes in plants. It has been proposed that

the spatio-temporal expression pattern of Pi transporters is a major determinant of their physiological function (Dai et al., 2022). Results here presented on the differential expression pattern of rice *PHT1* family members in response to Pi supply and *F. fujikuroi* infection suggest specialized functions of this family of Pi transporters in processes related to Pi acquisition and response to pathogen infection in rice roots.

Based on the above data, a model summarizing the effect of *F. fujikuroi* infection in rice plants grown under contrasting Pi regimes is showcased in Fig. 9. According to the model here proposed, Pi accumulation has a negative impact on root development and chlorophyll content in shoots. Higher disease severity index and root colonization by *F. fujikuroi* occurs in rice plants accumulating Pi. In plant defense, Pi accumulation compromises the expression of defense-related genes (*OsPR1a*, *OsPBZ1*) and provokes a localized accumulation of H_2O_2 which is less frequently observed in roots of Pi-accumulating plants. Pi accumulation also reduces lignin and suberin accumulation in rice roots which would make the High-Pi plants more susceptible to pathogen colonization.

Together, the results here presented support that Pi content shapes

immune responses and that Pi is an important factor in controlling bakanae disease by negatively regulating immune responses in rice roots. Previously, we reported that increasing Pi content in rice increases susceptibility to infection by the foliar pathogen *M. oryzae* (Campos-Soriano et al., 2020; Martín-Cardoso et al., 2024). Nevertheless, the negative effect of Pi accumulation on disease resistance cannot be extended to other plant-pathogen interactions. Contrary to what is observed in rice, an increase in Pi content has been shown to increase resistance towards fungal pathogens in *Arabidopsis* (Val-Torregrosa et al., 2022a, 2022b), while in cotton, Pi deficiency enhances resistance to *Verticillium dahlia* (Luo et al., 2021). It is then reasonable to assume that the extent to which a plant's nutritional condition influences disease resistance might be related to the lifestyle of the pathogen (necrotrophic, biotrophic or hemibiotrophic pathogen). Further investigation is needed to clarify this aspect. On the other hand, Pi starvation was reported to modulate immune responses in *Arabidopsis* further supporting a link between Pi homeostasis and immunity in plants (Castrillo et al., 2017; Chan et al., 2021). Clearly, there are still many open questions regarding the molecular mechanisms by which plants

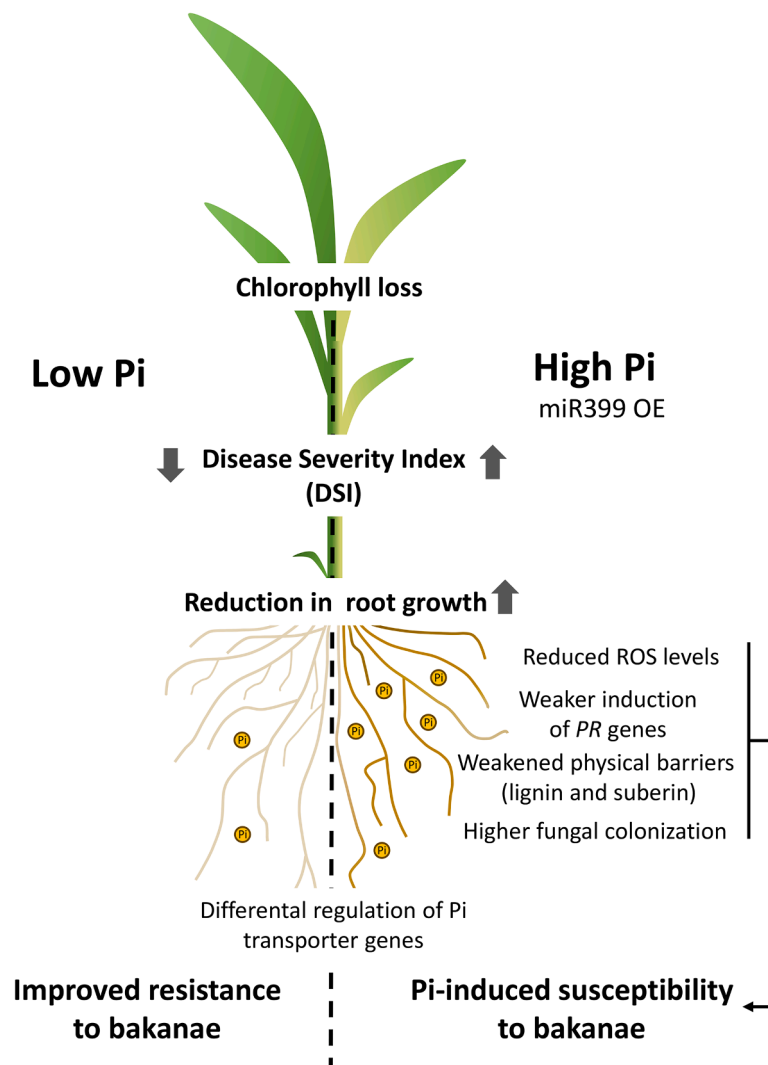


Fig. 9. Proposed model to explain mechanisms responsible of Pi-induced susceptibility to bakanae disease. Treatment of rice plants with high Pi, and miR399 overexpression, causes Pi accumulation in rice tissues. Upon pathogen infection, a reduction in chlorophyll content occurs in shoots of *F. fujikuroi*-infected rice plants. *F. fujikuroi* infection also results in root growth reduction, with a higher reduction in root growth being observed in High-Pi plants compared to Low-Pi plants. Higher levels of root colonization by *F. fujikuroi* and bakanae disease symptoms (Disease Severity Index) are observed in rice plants accumulating Pi. *MIR399* overexpression mimics the disease phenotype of rice plants grown under a high Pi regime. The induction of immune responses (e.g., expression of defense-related, ROS production) is compromised during infection by *F. fujikuroi* in High-Pi plants. Lignin and suberin accumulation is also reduced in *F. fujikuroi*-infected roots of High-Pi plants compared to Low-Pi plants. Same responses to Pi treatment and pathogen infection were observed in Nipponbare and TN67 rice plants.

integrate nutrient responses and immune responses, and how plants respond to pathogen infection under nutrient stress conditions. The current picture is that the effect of Pi availability on disease resistance must be evaluated on a case-by-case basis.

Finally, the observation that Pi accumulation in rice tissues enhances susceptibility to *F. fujikuroi* infection has important implications in management of bakanae disease, a disease of increasing economic importance. Currently, high doses of Pi fertilizers are applied in rice farming to keep optimal rice production, while pesticides are utilized to mitigate losses caused by pathogen infection. Paradoxically, growing rice under high Pi supply is likely to increase susceptibility to bakanae. Furthermore, the overuse of Pi fertilizers and pesticides is unsustainable as it adversely affects the environment and human/animal health. Given that Pi accumulation stimulates root colonization by *F. fujikuroi* and diminishes the rice plant's defensive capabilities, it is crucial to address the challenges associated with increased demand of rice by the global population using sustainable rice production systems. In this direction, a better understanding of mechanisms involved in rice defense against *F. fujikuroi* infection will help in developing strategies to protect rice against bakanae disease.

5. Conclusion

This study demonstrates that high phosphate status negatively regulates host defense against *F. fujikuroi* and increases susceptibility to bakanae in rice. During pathogen infection, a weaker activation of PR genes and reduced accumulation of ROS occurs in rice plants accumulating Pi. Increased susceptibility to infection by *F. fujikuroi* also compromises the creation of physical and defense barriers formed by lignin and suberin accumulation in root tissues. Both pathogen infection and Pi supply can modulate the expression of phosphate transporter genes in rice roots. Collectively, this study provides important information on molecular and cellular processes occurring in the response of rice plants against *F. fujikuroi* while demonstrating that Pi supply might affect the outcome of such interaction, which might help in developing strategies to protect rice against bakanae.

Ethical approval

No ethical approval is required.

Supporting information

Supplementary Fig. 1. Effect of infection with the fungus *F. fujikuroi* in rice plants (cv Nipponbare and cv TN67) grown under contrasting Pi regimes, low Pi and high Pi. (A) Experimental design used for Pi treatment. (B, C) Quantification of Pi in shoots (B) and roots (C) of rice seedlings grown under low-Pi and high Pi supply for 5 days. (D) Phenotype of Nipponbare and TN67 cultivars grown under low Pi or high Pi supply and inoculated with *F. fujikuroi* spores, or mock-inoculated.

Supplementary Fig. 2. Effect of Pi treatment and *F. fujikuroi* infection in the susceptible cv Galileo and the resistant cv Selenio. (A) Quantification of Pi in shoots of 5-day old Galileo and Selenio seedlings. (B) Appearance of roots from Galileo and Selenio plants that have been grown under low Pi or high Pi supply, mock-inoculated or inoculated with *F. fujikuroi* spores. (C) Disease Severity Index (DSI) of Galileo and Selenio cultivars at 10 dpi. (D) *F. fujikuroi* biomass in roots of Galileo and Selenio cultivars at 10 dpi.

Supplementary Fig. 3. Chlorophyll content in leaves of the *japonica* rice cultivars Nipponbare, TN67, Galileo, and Selenio inoculated with *F. fujikuroi* spores or mock-inoculated.

Supplementary Fig. 4. Characterization of miR399 overexpressor plants. Wild-type (WT, cv TN67) and miR399 overexpressor plants (lines L2, L4, L20) were grown under sufficient Pi conditions (0.25 mM Pi). (A) Quantification of Pi in shoots of wild-type (TN67) and miR399 OE lines

(L2, L4, L20) prior inoculation with *F. fujikuroi* spores. (B) Chlorophyll content in leaves of wild-type and miR399 overexpressor plants that have been inoculated with *F. fujikuroi* spores (10^6 spores/mL), or mock-inoculated.

Supplementary Fig. 5. Confocal laser scanning microscopy of roots from the rice cultivars Galileo (A) and Selenio (B) (susceptible and resistant checks, respectively) after inoculation with the *gfp*-expressing *F. fujikuroi* isolate.

Supplementary Fig. 6. Expression of lignin-related genes in roots of rice plants grown under low Pi or high Pi, in the presence or absence (mock) of pathogen infection, at 7 dpi. (A) Genes related to lignin biosynthesis. (B) Genes related to formation of the Casparian strip.

Supplementary Table 1. List of oligonucleotides used in this study.

Supplementary Table 2. Statistical analysis for root length, chlorophyll content, H₂O₂ content, and gene expression.

Supplementary Table 3. Statistical analysis for root length and chlorophyll content in wild type (TN67) and miR399 OE plants.

CRedit authorship contribution statement

Héctor Martín-Cardoso: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis. **Gerrit Bucker:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Iratxe Busturia:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Blanca San Segundo:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.stress.2025.100766](https://doi.org/10.1016/j.stress.2025.100766).

Data availability

Data will be made available on request.

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