Imaging the invasion of rice roots by the bakanae agent *Fusarium fujikuroi* using a GFP-tagged isolate

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Author contributions

Maria Aragona designed the project, performed the root infection and analysis and wrote the manuscript; Lidia Campos-Soriano performed the genetic transformation of *F. fujikuroi*; Edoardo Piombo performed the expression analyses and wrote the manuscript; Elena Romano performed LSCM analysis; Alessandro Infantino contributed to design the research work and cared the mycological part; Davide Spadaro and Blanca San Segundo contributed to design the research work and writing the manuscript. All authors commented on previous versions of the manuscript, read and approved the final manuscript.

ABSTRACT

Fusarium fujikuroi (teleomorph *Gibberella fujikuroi*) is the main seed-borne pathogen of rice, the causal agent of bakanae, a disease that in the last years has become of increasing economical concern in many Italian rice growing areas. A virulent *F. fujikuroi* isolate was tagged with the green fluorescent protein (*gfp*) gene using *Agrobacterium tumefaciens* mediated transformation, and the virulence of the GFP isolate has been confirmed. Little is known about the early interaction of the pathogen with its host, in this work fungal development during the *F. fujikuroi*/root interaction was analysed by Laser Scanning Confocal Microscopy (LSCM), by using the GFP isolate obtained. The infection of rice roots was investigated from 48 h to 8 days post-inoculation both in resistant and susceptible cultivars. Roots of resistant genotype seem to trigger a hypersensitive response at the infection site and LSCM analysis of root sections allowed the visualization of fungal growth within host tissues. Fungal growth occurred both in the resistant and the susceptible cultivar, even if it was less abundant in the resistant one. Expression analysis of *Chitinase1*, a gene involved in fungal pathogenesis, was investigated by qPCR on the *F. fujikuroi* infected rice roots. *Chitinase1* expression increased greatly upon infection in the resistant cultivar Selenio.

Keywords: genetic transformation, *Agrobacterium tumefaciens*, confocal laser scanning microscopic analysis, gene expression

INTRODUCTION

Bakanae is a rice disease caused by the hemibiotrophic fungal pathogen *Fusarium fujikuroi*. It was originally observed in Japan in 1928 (Ito and Kimura 1931), but it is now present in several countries in America, Europe, Asia, Oceania and Africa (Amatulli et al. 2010; Carter et al. 2008; Chen et al. 2016; Desjardins et al. 2000; Jeon et al. 2013; Karov et al. 2005; Khan et al. 2000; Kim et al. 2015; Zainudin et al. 2008). The meaning of Bakanae is “foolish seedling”, and it is due to the main symptom of the disease: the elongation and thinning of internodes, inducing frail stems and abnormal

height, thin leaves, and grains entirely or partially empty. The altered plant morphology is due to the production of gibberellic acids (GAs) by *F. fujikuroi,* the only *Fusarium* species capable of GAs biosynthesis (Ou 1985). GAs are not essential for fungal growth and development but, controlling jasmonic acid-mediated plant immune responses, they probably contribute to the virulence of *F. fujikuroi* (Wiemann et al. 2013; Siciliano et al. 2015). Although *F. fujikuroi* is predominantly a seed borne pathogen, it also survives in soil and diseased plant debris (Ou 1985). Seeds can become infested when conidia, produced on diseased plants, use the wind and water splash to reach neighbouring panicles at flowering. In a recent work Sunani et al. (2019), studying the infectious structures, penetration and colonization of *F. fujikuroi* in seeds and seedlings of rice, showed that infection through floret is the dominant pathway to seed infection. The localization of the pathogen could be both inside and on the outside of the seed, being predominant in the lemma and palea, followed by embryo (Kumar et al. 2015). Seeds can also be contaminated by the fungus at harvest, when they can be reached by conidia produced on diseased and dead plants. A third source of seedborne infection is represented by spores and mycelium contaminating the water used to stimulate germination in soaked seeds (Karov et al. 2009). Both ascospores and conidia can also infect seedlings through the roots and crown, colonising both the intracellular and intercellular spaces of the rice root: the fungus invades the plant without producing visible symptoms, so that *F. fujikuroi* can be found in apparently healthy seeds (Kanjanasoon 1965).

Bakanae disease is increasing in the main rice-producing areas worldwide (Infantino et al. 2017; Amatulli et al. 2010). Losses in rice production caused by bakanae depend on climate, rice cultivars and pathogen strain, ranging from 3% to 15% in Thailand (Kanjanasoon 1965), 2% - 20% in Macedonia (Karov et al. 2005), 20% - 50% in Japan (Ito and Kimura 1931), up to 52% in Bangladesh (Hossain et al. 2013), and up to 75% in Iran (Saremi et al. 2008). The most common Bakanae management is based on thermal seed treatment and the use of fungicides, but *F. fujikuroi* resistance to various fungicides has been reported (Chen et al. 2016). The need of developing new control measures is therefore increasing. The identification of new sources of resistance to *F. fujikuroi* was

based on the screening of large collections of rice germplasm and allowed to map several quantitative trait loci (QTLs) on rice chromosomes (Chen et al. 2019; Volante et al. 2017). The development of simple sequence repeat (SSR) markers and mating type analysis allowed to detect *F. fujikuroi* genetic variability at population level (Valente et al. 2016), which is of fundamental importance for screening of resistance. In this work, we focused on the analysis of early stages of root infection by a fluorescent *F. fujikuroi* isolate, with the aim to unravel the differences between the susceptible and the resistant rice cultivars facing pathogen infection and colonization. Interaction between pathogens and host plants have been extensively studied using fluorescent reporter proteins. Organisms that express genes encoding fluorescent reporter proteins are frequently used to monitor pathogen behaviours in plant tissues under various physiological conditions (Lagopodi et al. 2002; Oren et al. 2003). The advantage of GFP as a reporter is that it allows *in vivo* imaging of fungal hyphae during its interaction with the host plant. Hyphae of *gfp*-expressing fungal strains can be visualized in living tissue in real time, using fluorescence microscopy without extensive manipulation. Compared to many fungal pathogens, such as *Aspergillus* spp. and other *Fusarium* species, the lack of efficient technologies for genetic manipulation has become a major obstacle for the development of *F. fujikuroi* molecular research (Cen et al. 2020). However, a polyethylene glycol (PEG)-mediated transformation of protoplasts has been used to introduce the *gfp* and the red fluorescent protein (*rfp*) gene into *F. fujikuroi* for visualizing interaction with biocontrol agents (Watanabe et al. 2007; Kato et al. 2012) and the early root colonization of a GA-producing wild-type and a GA-deficient mutant strain (Wiemann et al. 2013). Recently, a *gfp*-expressing *F. fujikuroi* isolate, obtained by PEG transformation, has been used to analyse rice infection of susceptible and resistant cultivars at the basal stem level, by confocal microscopy analysis (Lee et al. 2018).

No study so far, an *A. tumefaciens*-based method has been developed for transformation of *F. fujikuroi*. By this way we transformed four virulent *F. fujikuroi* strains by using a *gfp*-expressing vector, and one of the GFP-tagged isolates obtained was used to visualize and analyse the infection and colonization processes at root level in susceptible and resistant rice cultivars, by confocal

microscopy. Quantification of expression in the rootlets of *chitinase 1*, a gene related to the response to bakanae disease, was also performed.

MATERIALS AND METHODS

Fungal strains and growth conditions

Four virulent *F. fujikuroi* strains were selected inside a collection of more than 300 isolates stocked at CREA-DC and previously used for a study of population structure analysis (Valente et al. 2016). The Ff 192, Ff 297, Ff 364 and Ff 1550 isolates were chosen among the most virulent ones and belonging to different haplotypes. After transformation by the *gfp* vector, as described below, four isolates named Ff 192-GFP, Ff 297-GFP, Ff 364-GFP and Ff 1550-GFP were obtained, and they are all listed in Online Resource 1. *Fusarium fujikuroi* isolates were grown on potato dextrose agar (PDA) or potato dextrose broth (PDB) at 23˚C, in the case of transformed isolates hygromycin (Hyg) at concentration of 100 µg ml^{-1} was added to the media.

Generation of *F. fujikuroi* strains expressing the *gfp* gene

The four selected *F. fujikuroi* isolates were transformed with the plasmid pCAM*gfp* (kindly provided by A. Sesma, John Innes Center, UK) (Sesma and Osbourn 2004). The pCAMgfp plasmid contains the *sgfp* gene (Chiu et al. 1996) under the control of the *ToxA* promoter from *Pyrenophora tritici-repentis* (Lorang et al. 2001) and the hygromycin phosphotransferase (*hph*) gene as the selectable marker gene. The pCAM*gfp* plasmid was introduced into the *A. tumefaciens* AGL-1 strain, the virulent strain required for fungal transformation. *F. fujikuroi* transformation was carried out using the *A. tumefaciens* AGL-1-transformed strain following the protocol previously described (Campos-Soriano and San Segundo 2009; Campos-Soriano et al. 2013) with minor modifications. Co-cultivation was performed at 25˚C and selection was done at 28˚C. PDA medium plus hygromycin B (250 µg ml^{-1} final concentration) was used as selective medium to grow the *F. fujikuroi* transformed isolates. Fungal colonies were transferred to 24-well plates containing the selective medium to test

the effective transformation. A stereomicroscope (Olympus SZX16) with 480-nm excitation and 500 to 550-nm emission filter block was used to verify GFP-transformed fungal colonies. The stability of transgene integration and *gfp* expression of transformants were tested by sub-culturing them for five generations on PDA medium and then transferring them again on selective PDA medium containing 100 µg ml^{-1} hygromycin B. The number of pCAM*gfp* copies integrated into the genome of transformants has been assessed by qReal Time-PCR, using the primers Hyg588F and Hyg588R, listed in Online Resource 2. The PCR mix was composed of 10 µl of SensiMix 2x (Bioline), 2 µl of primer mix (forward and reverse, 5 µM of each primer) and 4 µl of nuclease free water. To each sample 2 µl of fungal genomic DNA and 2 µl of known amounts of the plasmid pAN7-1 were added. The thermal cycler protocol was the following: 95°C for 10 min and 40 cycles with the following steps: 95°C for 30 s; 55°C for 30 s and 72°C for 45 s.

Epi-fluorescence microscopic analysis

GFP-labelled *F. fujikuroi* mycelium and spores, grown on PDA plates or inoculated roots, were photographed using an epifluorescence microscope (Axioscope, Zeiss) equipped with a GFP filter and a camera to capture images of GFP fluorescence (excitation at 455 to 490 nm and emission at 515 to 560 nm).

Pathogenicity assay

To test pathogenicity and virulence of GFP transformants, compared to the wild type isolates, they were grown on PDA or PDB at 23˚C for conidia production. Conidia were harvested and resuspended in water at the concentration of 10^6 conidia ml^{-1}. The Italian rice cultivar Galileo was used, based on its susceptibility to Bakanae showed in several experiments performed by our group (Valente et al. 2016; Volante et al. 2017). Rice seeds were surface sterilized in 70% ethanol for 1 min, then in 2% sodium hypochlorite for 3 min and subsequently rinsed 3 times in sterile water, finally were allowed to dry at room temperature before sowing. Thirty-two seeds were inoculated with each fungal isolate

by pipetting 2 ml of the conidial suspension to each seed. The seeds of control plants (mock) were treated in the same way but inoculating them with sterile deionized H_2O. The inoculated seeds were transplanted into 32-cell plastic pots filled with Radicom soil (Vigorplant). A complete randomized block design with three replicates was used. Plants were kept in the greenhouse at 25–28°C under fluorescent lights, with a 12 h photoperiod. After 30 days, seedlings were evaluated for symptoms. Disease severity was evaluated using a scale from 0 to 4 as described by Zainudin et al. (2008) and modified by Valente et al. (2016). The scale includes 5 classes: 0 = no symptoms; 1 = normal growth but leaves beginning to show yellowish–green and/or small necrotic lesions localized at the crown level; 2 = abnormal growth, elongated, thin and yellowish-green leaves, stunted seedlings, necrotic lesions on main root and crown; 3 = abnormal growth, elongated stems, chlorotic, thin and brownish leaves, larger leaf angle, seedlings also shorter or taller than normal, reduced root system with necrotic lesions on secondary roots and on basal stem; 4 = dead plants before or after emergence. One or more of the described symptoms, for each class, could be present on the infected plants. Evaluation of virulence of the isolates was performed as described in Scherm et al. (2019) and infection severity was calculated by the McKinney index (McKinney 1923), here named disease index (DI). Analysis of variance (ANOVA) was performed using COSTAT (version 6.311.; CoHort Software, Monterey, CA, USA) to evaluate the McKinney index data. Data were arcsine-transformed prior to ANOVA analysis. The means were separated using Student–Newman–Keuls multiple-range tests ($P < 0.05$).

Root infection assay and confocal microscopy analysis

Two rice varieties, the bakanae disease resistant *japonica* variety Selenio and the susceptible *japonica* variety Galileo, were used in this study. Selenio was selected as one of the most resistant rice cultivars from 138 diverse Italian rice accessions screened for evaluation of rice bakanae disease resistance (Volante et al. 2017). Seeds were surface sterilized in 2% NaOCl for 2 min and rinsed in sterile H_2O before plating on sterile wet paper for germination. After 5 days at 30°C in the dark, the young emerged roots were inoculated by the Ff 297-GFP strain, by applying 100 µl of a suspension at 10^6

spores ml^{-1} in the middle of the rootlets, seedlings were allowed continuing the growth at 30°C in the dark until confocal laser scanning microscopic analysis or *chitinase* expression analysis.

After 48 hours after inoculation (hai), 72 hai and 8 days after inoculation (dai), infected rice roots were stained with propidium iodide (0.2 µg ml^{-1}) for 3 min before microscope observation, both unaltered and hand-sectioned roots were analysed. Images of GFP-labelled *F. fujikuroi* strain in host roots were captured using a confocal laser scanning microscope FV1000 Olympus (Tokyo, Japan) equipped with inverted microscope IX 81. Images were acquired in z stack with objective 10x (N.A. 0,40), using 488nm (argon Ion, emission 520nm) for GFP fluorescence, and 543nm (HeNe, emission 570 nm) laser for propidium iodide staining of root bark. Subsequently they were processed using Imaris 6.2.1 software (Bitplane, Switzerland).

Expression analysis of rice *Chitinase 1*

Total RNA was extracted using the RNeasy kit (Qiagen, Germany) from root tissues (0.1 g) at 72 hai with the selected GFP-tagged *F. fujikuroi* strain Ff 297-GFP. RNA was treated with TURBO DNA-free kit to remove contaminating DNA (Ambion, Foster City, California, United States). The absence of DNA contamination in RNA samples was further assessed by PCR using the rice elongation factor 1-alpha gene (Manosalva et al. 2009). Total RNA was quantified by Nanodrop (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Reverse transcription reaction was performed using the iScript cDNA synthesis kit (Biorad, Hercules, California, United States). cDNA was then used for expression analysis by quantitative PCR (Applied Biosystem StepOnePlus, Foster City, California, United States) using the specific primers CHIT1-FW (TACTCGTGGGGCTACTGCTT) and CHIT1-RV (CGGGCCGTAGTTGTAGTTGT) for the quantification of the *Chitinase 1* rice gene. The primers were designed using the Primer3Plus software (Untergasser et al. 2007). The PCR mix was composed of 5 µl of SYBR Green Power Mix (Applied Biosystem), 2 µl of cDNA, 0.15 µl of each primer (10 µM each) and 2.4 µl of nuclease free water. The thermal cycler protocol was the following: 95°C for 10 min, followed by 40 cycles (95°C for 15 s; 60°C for 60 s) and 95°C for 15 s.

The rice elongation factor 1-alpha was used as housekeeping gene with primers EF1α1F and EF1α1R (Manosalva et al. 2009), listed in Online Resource 2. The efficiency of the primers was tested with a standard curve built upon five serial dilutions (1:10) in three technical replicates. After calculating the fold change values, significant differential expression was evaluated with the Duncan's Post Hoc test, using SPSS v.25.

RESULTS AND DISCUSSION

Development of *gfp*-expressing *Fusarium fujikuroi* isolates

Four different *F. fujikuroi* isolates, named Ff 192, Ff 297, Ff 364 and Ff 1550, were transformed with the vector pCAM*gfp* introduced into the *A. tumefaciens* AGL-1 strain. Up to now, *F. fujikuroi* transformation methods have all been based on the use of protoplasts (Watanabe et al. 2007; Kato et al. 2012; Lee et al. 2018; Chen et al. 2020). However, protoplast production is time consuming and, even for the same isolates, strictly dependent on the batch of lysing enzymes used. Since several years, the *Agrobacterium tumefaciens*-mediated transformation (ATMT) systems successfully overcame the protoplast-based ones in fungi. Moreover, ATMT-based methods facilitate vector DNA integration in a single site of the recipient genome, and are applicable at different developmental stages, such as conidia, mycelium and fruiting bodies, but germinating conidia are preferred in most of cases, if available (Michielse et al. 2005; Lakshman et al. 2012). Colony morphology and growth rate of *F. fujikuroi* transformed isolates were similar of those of wild-type isolates, suggesting that *gfp* expression did not affect the pathogen phenotype. Online resource 3 showed an image of the Ff 297-GFP and the parental Ff 297 isolates selected for microscopy analyses, grown on PDA plates. Approximately, 80-85% of the transformants showed strong fluorescent signal, furthermore, strong fluorescence could be visualized in fungal spores and mycelium (Figure 1).

The fluorescence of GFP in transformed *F. fujikuroi* strains remained stable through subsequent cultivation onto PDA medium without antibiotic, indicating the stable integration of the transforming

plasmid in the genome. The number of pCAM*gfp* copies integrated into the transformant genomes varied from 1 to 2 in the different isolates, Ff 297-GFP had only one copy (data not shown).

Pathogenicity of GFP transformants

All the four *gfp*-expressing isolates were found to be pathogenic in infection assays of seeds, but they showed different virulence (Table 1). Ff 192 WT and Ff 297 WT were the most virulent (disease index, DI = 68.0 and 60.0, respectively). Considering the corresponding transformants, Ff 192-GFP DI was 28.0, suggesting that, in this isolate, *sgfp* gene insertion affected fungal virulence, in a direct or indirect way. On the contrary, Ff 297 WT virulence was not, or little, affected in the corresponding GFP-tagged isolate (DI = 60.0 and 47.0, respectively) and no statistical differences were found between the Disease Indices from Ff 297 WT and Ff 297-GFP. Ff 364-GFP and Ff 1550-GFP showed virulence comparable to parental isolates, but the DI values were lower than Ff 297-GFP (Table 1), so that Ff 297-GFP was selected for root infection and subsequent microscopic analyses. In figure 2A the phenotype of Ff 297-GFP and of the parental strain is illustrated. We already mentioned that *F. fujikuroi* causes different symptoms on rice, as pre-emergence seedling death, elongated and thinner leaves, chlorosis, stunting, crown rot and root rot and even death of seedlings (Ou 1985; Sunani et al. 2019; Piombo et al. 2020). In figure 2A and B more than one of these symptoms are visible in the seedlings inoculated by Ff 297-GFP and Ff 297: the number of plants is lower than in the mock test (T, on the left), indicating a pre-emergence seedling death; many leaves and stems are elongated and thinner than in the control and have a larger leaf angle; some of them show stunted growth.

Infection of susceptible and resistant rice cultivars with one *gfp*-expressing *F. fujikuroi* strain

The two cultivars, Galileo and Selenio, were previously tested for their response to seed inoculation by *F. fujikuroi*, showing a susceptible and resistant profile, respectively (Matic et al. 2016; Siciliano et al. 2015; Volante et al. 2017). In our experience and as confirmed by Chen et al. (2020), *F. fujikuroi*

was always detected in the basal roots, so we chose this tissue as the target for pathogen infection and investigation of direct interaction between *F. fujikuroi* and rice.

In this study, a virulent *F. fujikuroi* strain constitutively expressing the *gfp* reporter gene was obtained *via Agrobacterium* transformation of conidia, enabling us to study the early stages of *F. fujikuroi* infection of rice roots in one resistant and one susceptible cultivar. The infection process in root tissues of the rice cultivars Galileo and Selenio was followed by using the GFP-tagged *F. fujikuroi* strain Ff 297-GFP and visualized by confocal microscopy at 24 hai, 48 hai, 72 hai, 96 hai, 8 dai and 12 dai of roots. In figure 3, the images captured at 48 and 72 hai were showed, which better illustrated the differences between susceptible and resistant cultivars. Hyphae growing longitudinally along the root surface and in the root hairs were primarily observed (48 hai), and penetration into the epidermal root cells was clearly observed at 72 hai. By this time, most epidermal cells were invaded by the fungus in the susceptible cv Galileo. A similar pattern of hyphal colonization was observed in the roots of the resistant cultivar Selenio, although host cell colonization was much lower in Selenio than that on Galileo (Figure 3). Confocal imaging of transverse sections of the roots showed that the fungus penetrated the stele in both varieties and was more abundant in the susceptible variety Galileo than in the resistant one, Selenio. These results are in agreement with the observations of Lee et al. (2018) at the basal stem level of susceptible and resistant rice cultivars: after ten days from seed inoculation by *F. fujikuroi* the pathogen was present in the stele and was more abundant in the susceptible one. We cannot exclude that this evidence was due to the major amount of fungal biomass in the susceptible cultivar. However, in literature no significant differences of the amount of *F. fujikuroi*, measured by qPCR, were reported between the roots of the resistant cultivar Selenio and the susceptible cultivar Dorella (Carneiro et al. 2017). Moreover, Chen et al. (2020) and Cheng et al. (2020) observed, in other rice cultivars, that *F. fujikuroi* biomasses were similar, both in the stem and roots, in resistant and susceptible cultivars. Confocal analysis of root transverse sections showed colonization of the xylem vessels in both genotypes at 72 hai. The fluorescence was restricted to the vessels in Selenio, while in Galileo there was a more generalized labelling around the vessels. In other

words, fungal colonization occurred in both the cultivars, though the fungal presence was less abundant in the resistant one (Figure 3). At 8 dai the roots were completely covered by the fungal hyphae and the diffuse fluorescence did not allow any microscopic analysis (data not shown). This confirmed that fluorescence of *F. fujikuroi* transformants was stable over time even inside the host tissues. In conclusion, *F. fujikuroi* is able to colonize the root tissues of both varieties, though Selenio proves to be resistant when seeds are inoculated. These results are in agreement with that observed by Carneiro et al. (2017), which detected the pathogen by qRT-PCR analysis on both the roots of susceptible and resistant cultivars after seed artificial inoculation. Other phytopathogenic *Fusarium* spp., such as a *Fusarium oxysporum* f.sp. *cubense* race 4 isolate tagged by GFP, showed the capacity of invading epidermal cells of host roots directly, and spores were produced in the root system. However, in this case, roots of susceptible banana plants were colonized, but not those of the resistant cultivar, probably due to the production of host exudates that inhibited the germination and growth of pathogenic isolate (Li et al. 2011).

We observed that the earliest infectious structures were represented by the infection hyphae, as recently reported by Sunani et al. (2019) by scanning electron microscope analysis. The infection hyphae penetrated the epidermal cells of rice roots at 48-72 hai, and at those times the mycelium was found intra and intercellularly and was able to colonize the vascular bundles. Intercellular and intracellular growth in roots has been documented for other phytopathogenic *Fusarium* spp., including *F. culmorum* on rye root tissue (Jaroszuk-Ściseł et al. 2008) and *F. oxysporum* f. sp. *radicis lycopersici* on tomato (Lagopodi et al. 2002).

Until now, several studies on the rice-*F. fujikuroi* interaction have been carried out on the aerial part of plants and at several weeks after inoculation (Ji et al. 2016; Ji et al. 2019; Matić et al. 2016). Very recently, the process of infection by *F. fujikuroi* in rice seeds, seedlings and adult plants was documented in the literature (Sunani et al. 2019; Chen et al. 2020). In the present study we evaluated, for the first time, the progress of infection and colonization in rice roots after direct inoculation of roots by a GFP-tagged isolate of *F. fujikuroi.* Sunani et al. (2019) addressed details of root

colonization by this pathogen after seedling inoculation of a susceptible cultivar. By scanning electron microscopy, they observed the intra and intercellular movement of mycelium at 24 hai and the fully colonization of root tissue at 72 hai of rice seeds. These observations were confirmed in our work after root inoculation, by using a GFP isolate of *F. fujikuroi* combined with confocal microscopical analysis. Chen et al. (2020) examined the infection in susceptible and resistant seedlings and adult plants by using a GFP-tagged isolate for seed inoculation. In agreement with that observed by Chen et al. (2020), we found that in root tissues the *F. fujikuroi* infection process was similar in susceptible and resistant cultivars. The fungus, in the first steps of infection, grew predominantly in the root-hair region and was associated with large production of hyphae which directly penetrated the epidermis. The vascular bundles were already colonized at 72 hai, while Chen et al. (2020) observed the hyphae penetration of exodermis and the central cylinder after 6-8 dai, this discrepancy could be due to the use of confocal microscopy, in our case, which is a more powerful technique. We did not observe any appressorium-like structure, in agreement with that reported by Chen et al. (2020), though appressoria formation was reported in several pathogenic *Fusarium* species, and Sunani et al. (2019) observed them on rice roots after 12 h after seed inoculation by *F. fujikuroi*. We could hypothesize that at 24 hai, the first time point we considered in our work, the appressoria had already penetrated the host tissue and mycelium was developed.

Chitinase expression analysis

Chitinases are proteins involved in the plant defence against pathogens because of their ability to hydrolyse chitin in the cell wall of fungi (Sharma et al. 2011). Previous studies revealed that rice leaves expressed chitinases at 2-3 weeks after seed inoculation by *F. fujikuroi* (Matic et al. 2016). Based on these evidences, in preliminary experiments we tested expression in roots of three genes: *Chitinase 1, Gibberellin 20 oxidase 1* and *Peroxidase P7.* After inoculation by the four wild type *F. fujikuroi* isolates, only *Chitinase 1* was differentially expressed in inoculated rice roots, compared to uninoculated ones. For this reason, we selected this gene for evaluating the expression with the Ff

297-GFP transformed isolate. Results showed that *Chitinase 1* was expressed in roots also after root inoculation by the Ff 297-GFP isolate. We observed that Selenio and Galileo expressed *Chitinase1* at similar levels in the non-inoculated roots, but the expression increased greatly upon pathogen challenge in the resistant cultivar Selenio (Figure 4). The results were in agreement with those obtained with the wildtype isolate (data not shown), excluding that transformation was responsible of the increased chitinase expression in inoculated roots. It has been suggested that in filamentous fungi, chitinases may act during hyphal growth (Kumar et al. 2018), therefore, the induction of *Chitinase1* in Selenio may be involved in the control of hyphal growth during the infection, and correlates well with the phenotype of resistance observed in this cultivar. Up-regulation of *Chitinase 1* during incompatible interaction between rice and *M. oryzae* has also been reported (Kawahara et al. 2012). We cannot draw any conclusion regarding the susceptible cultivar Galileo because standard deviation (SD) values of the fold change were too high in Galileo inoculated samples. We repeated the assay three times and always observed that, at 72 hai, in Galileo many germinated seeds showed reduced root length compared to the same not inoculated cultivar. In conclusion, the Galileo inoculated sample was not homogeneous, and this could be a possible explanation of high SD when analyzing gene expression. The reduced root length observed was in agreement with that reported by Zainudin et al. (2008) after seed inoculation. They included the "reduction of root system" among the symptoms of class 3 of the disease scale we used for the pathogenicity test of transformed and untransformed *F. fujikuroi* isolates. We strongly believe that expression of more and different genes deserves to be explored in the root system, in future studies.

CONCLUSIONS

Roots represent the first specialized tissue emerging from seeds upon germination, so it might represent an easy tool to study the early stages and the mechanisms performed for rice infection by the seedborne fungal pathogen *F. fujikuroi*. A *F. fujikuroi* isolate, constitutively expressing the *gfp* reporter gene, was successfully obtained *via Agrobacterium* transformation of a virulent strain. The

GFP isolate did not show significant reduction in virulence and was used for studying the early stages of *F. fujikuroi* infection of rice roots in both resistant and susceptible rice cultivars, by confocal microscope analysis. Though *F. fujikuroi* is mainly a seedborne pathogen, we found several analogies in infection and colonization of roots after direct root inoculation and seed inoculation, recently well documented in literature. In both cases, the infection process is similar in susceptible and resistant cultivars. The stele is colonized by the pathogen at both basal stem and root level and *F. fujikuroi* is able to spread both in resistant and susceptible rice plants, although a reduction in fungal colonization in the resistant variety has been observed. This suggests that *F. fujikuroi* is able to survive and grow inside root tissue even when not causing symptoms. Overall, these results suggest that visualization of *F. fujikuroi* in roots will help in investigating the early stages of the infection process by this fungal pathogen in rice, while representing a useful tool for the screening of rice cultivars for resistance/susceptibility to *F. fujikuroi.* Further research is in progress to evaluate and compare the behaviour of *F. fujikuroi* within the seeds of susceptible and resistant varieties after artificially inoculations, by a GFP-tagged isolate, of floret, which represents the main route of entry of this pathogen.

Figure captions

Fig. 1. Morphological characteristics of transformed isolates of *F. fujikuroi.* (A) Typical growth of *gfp*-expressing *F. fujikuroi* isolates; (B, C, D, E) Fluorescent and transmission micrographs of *gfp*-expressing *F. fujikuroi* spores; (F) Confocal image of fluorescent mycelium on PDA plates; (G) Epifluorescent image of mycelium on the surface of a rice seed. Bars: 20 µm (B, C, D, F), 10 µm (E).

Fig. 2. (A) Phenotype of the rice susceptible variety Galileo at 30 day with the *gfp*-expressing *F. fujikuroi* isolate Ff 297-GFP (in the middle) and wild type Ff 297 (on the right). T, in the left, represents the control mock-inoculated with dH_2O. (B) A closer image of symptomatic plants inoculated by Ff 297. Some of the typical symptoms of Bakanae are visible in the inoculated plants:

a lower number of plants, indicating pre-emergence seedling death; many leaves and stems elongated and thinner than in the control and with a larger leaf angle; stunted growth.

Fig. 3. Rootlets of rice cv. Galileo (susceptible) and Selenio (resistant), inoculated with the *gfp*-expressing *F. fujikuroi* isolate Ff 297-GFP. Root surface and transverse sections at the indicated time after inoculation are shown. Bars: 70 µm for transverse sections, 150 µm for the other pictures.

Fig. 4. Expression of *Chitinase1* gene in the rootlets of resistant (Selenio) and susceptible (Galileo) rice cultivars. Data obtained by reverse transcriptase real time PCR. The error bar is the standard deviation, and the letters indicate groups not considered to be statistically different using the Duncan test.

Electronic Supplementary Material

Online Resource 1. *Fusarium fujikuroi* strains used in this study.

Online Resource 2. Primers used in this study.

Online Resource 3. Phenotype of the GFP-tagged isolate Ff 297-GFP selected for microscopical analyses and the parental strain Ff 297, both grown on PDA plates.

Compliance with Ethical Standards:

There are no potential conflicts of interest.

This research is not involving human participants and/or animals, therefore, there is no informed consent needed.

All the authors have been informed and consent to publish this work.

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isolate	McKinney index	
Ff 192 WT	68.0 ± 9.75	a
Ff 192 GFP	28.0 ± 9.75	b
Ff 297WT	60.0 ± 15.0	a
Ff 297GFP	47.0 ± 6.71	ab
Ff 364 WT	39.0 ± 17.82	b
Ff 364 GFP	24.0 ± 15.57	b
Ff 1550 WT	27.0 ± 13.51	b
Ff 1550 GFP	31.0 ± 5.48	b

Table 1. Comparison of virulence between the wild type and GFP transformed *F. fujikuroi* isolates. Means ± Standard deviations followed by different letters are significantly different (P≤ 0.05), according to Student-Newman-Keuls test.

Figure 1

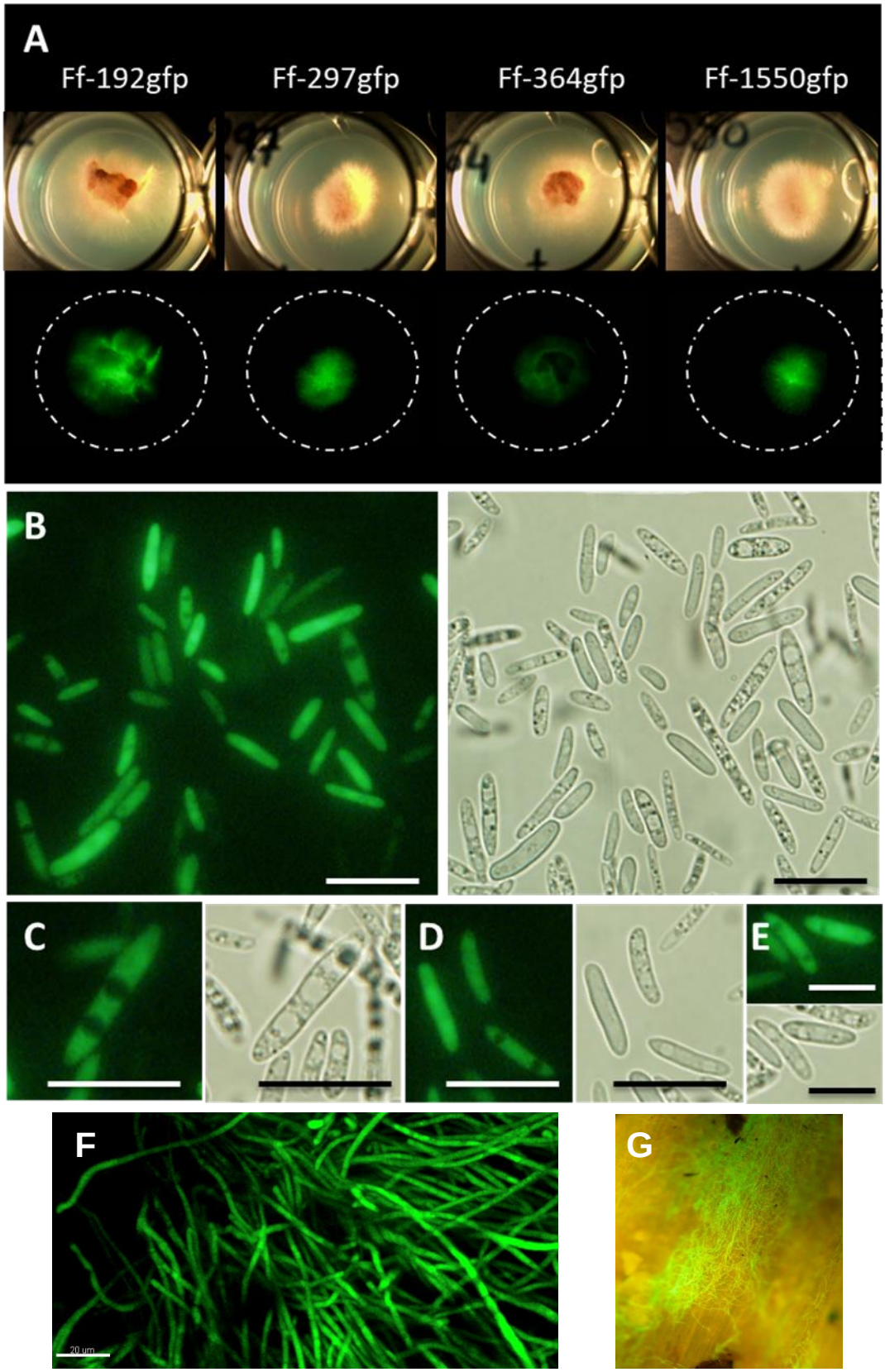
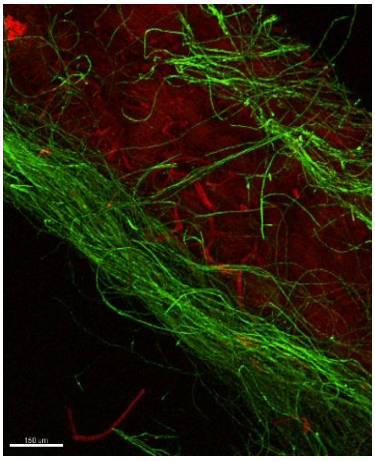


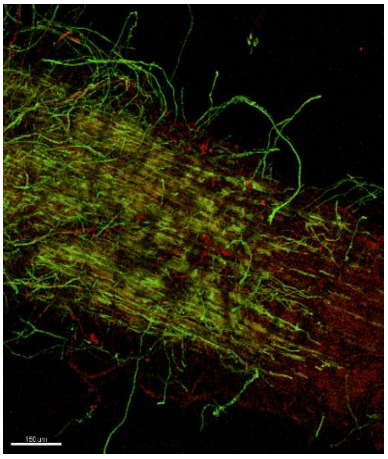
Figure 2

A **B**

Figure 3

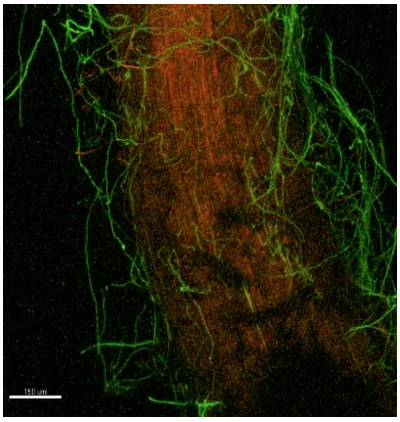


Galileo: 48 hpi

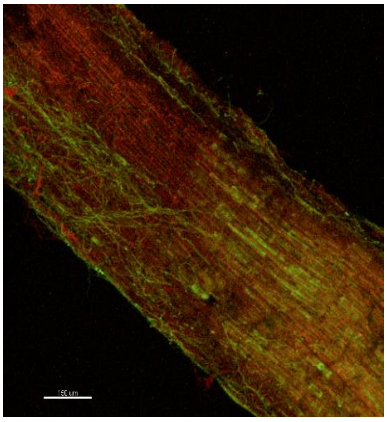


72 hpi

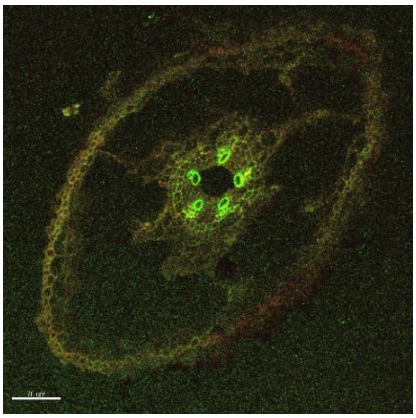
72 hpi transverse section



Selenio: 48 hpi

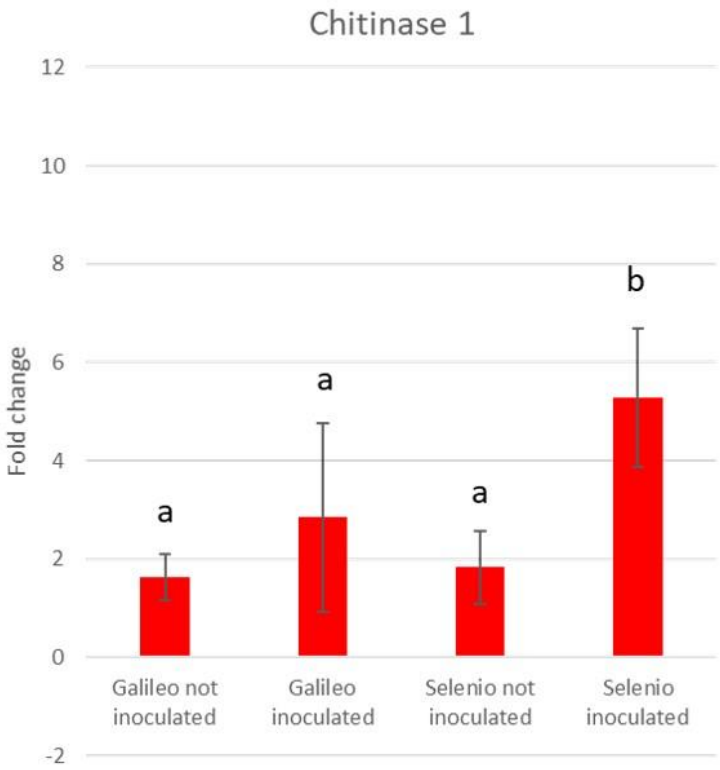


72 hpi



72 hpi transverse section

Figure 4


Chitinase 1
12
10
8
6
4
2
0
-2
Fold change
a
a
a
b
Galileo not inoculated
Galileo inoculated
Selenio not inoculated
Selenio inoculated