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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). *Fusarium fujikuroi* is predominantly a seed borne pathogen, but 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 and colleagues (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. The potential for pathogenicity in soil rapidly decreases, going from 93% of infection of rice planted immediately after artificial inoculation of the soil to 0.7 % for rice planted 90 days after soil inoculation, with no disease at all occurring after 180 days from the inoculation. However, the fungus can survive as hyphae on infested seeds for much longer, lasting 4-10 months at room temperature and more than 3 years at 7°C (Kanjanasoon 1965).

Bakanae disease is increasing in the main rice-producing areas worldwide. 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), 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 important 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 the Green Fluorescent Protein (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 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),

143 they were: Ff 192, Ff 297, Ff 364 and Ff 1550. After transformation by the *gfp* vector, as described
144 below, four isolates named Ff 192-GFP, Ff 297-GFP, Ff 364-GFP and Ff 1550-GFP were obtained,
145 and they are all listed in Online Resource 1. *Fusarium fujikuroi* isolates were grown on potato
146 dextrose agar (PDA) or potato dextrose broth (PDB) at 23˚C, in the case of transformed isolates
147 hygromycin (Hyg) at concentration of 100 µg ml^{-1} was added to the media.
148
149 **Pathogenicity assay**
150 To test pathogenicity and virulence of GFP transformants, compared to the wild type isolates, they
151 were grown on PDA or PDB at 23˚C for conidia production. Conidia were harvested and resuspended
152 in water at the concentration of 10^6 ml^{-1}. Thirty-two seeds of the susceptible rice cultivar Galileo were
153 inoculated with each fungal isolate by adding 2 ml of the conidial suspension to each seed, before
154 sowing in soil. The seeds of control plants (mock) were treated in the same way but inoculating them
155 with sterile dH_2O. A complete randomized block design with three replicates was used. Plants were
156 kept in the greenhouse at 25–28°C under fluorescent lights, with a 12 h photoperiod. After 30 days,
157 seedlings were evaluated for symptoms. Disease severity was evaluated using a scale from 0 to 4 as
158 described by Zainudin et al. (2008) and modified by Valente et al. (2016). The scale includes 5
159 classes: 0 = no symptoms; 1 = normal growth but leaves beginning to show yellowish–green and/or
160 small necrotic lesions localized at the crown level; 2 = abnormal growth, elongated, thin and
161 yellowish-green leaves, stunted seedlings, necrotic lesions on main root and crown; 3 = abnormal
162 growth, elongated stems, chlorotic, thin and brownish leaves, larger leaf angle, seedlings also shorter
163 or taller than normal, reduced root system with necrotic lesions on secondary roots and on basal stem;
164 4 = dead plants before or after emergence. One or more of the described symptoms, for each class,
165 could be present on the infected plants. Evaluation of virulence of the isolates was performed as
166 described in Scherm et al. (2019) and infection severity was calculated by the McKinney index
167 (McKinney, 1923), here named disease index (DI). Analysis of variance (ANOVA) was performed
168 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 ($P < 0.05$).

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 *Agrobacterium 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 ug 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 Hyg588U and Hyg588L, 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.

Root infection assay

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 cultivar from 138 diverse Italian rice accessions screened for evaluation of rice bakanae disease resistance (Volante et al., 2017). Seeds of both cultivars were inoculated by the wild type isolate Ff 297 and the derived transformant Ff 297-GFP. 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, young emerged roots were inoculated 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 (CLSM) analysis or *chitinase* expression analysis.

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).

Confocal microscopy analysis of infected roots

After 48 hours after inoculation (hai), 72 hai and 8 days after inoculation (dai) by Ff 297-GFP strain, 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

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) 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 (Ff 192, Ff 297, Ff 364 and Ff 1550) were transformed with the plasmid pCAM*gfp* containing the *sgfp* gene. The transformed isolates almost retained the colony morphology typical of the wild-type isolates indicating that *gfp* expression did not affect the growth phenotype, in online resource 3 is showed an image of the Ff297-GFP isolate and the parental Ff297, selected for microscope 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. 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). 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). However, protoplast production is time consuming and, even for the same isolates, strictly dependent on the batch of lysing enzymes used, so 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). For the first time we transformed the conidia of four *F. fujikuroi* isolates by the pCAM*gfp* plasmid introduced into the *A. tumefaciens* AGL-1 strain. This was previously and successfully used for transforming the rice leaf blast pathogen *M. oryzae* (Sesma and Ousborn, 2004; Campos-Soriano and San Segundo, 2009). Among the *F. fujikuroi* transformants obtained one retained virulence similar to the parental strain, showed stable integration of the transforming vector into a single site of the genome and stable fluorescence after plant inoculation.

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 was the most virulent (disease index, DI, = 68.0), but in the corresponding transformant, Ff 192-GFP, DI was 28.0, suggesting that, in this isolate, *sgfp* gene insertion affected fungal virulence, in a direct or indirect way. Ff 364-GFP and Ff 1550-GFP

showed virulence comparable to parental isolates, but the DI values were lower than Ff 297-GFP. Ff 297 WT strain showed to be highly virulent (DI=60) and its virulence was not significantly affected in the corresponding GFP-tagged isolate (Table 1), so that Ff 297-GFP was selected for root infection and subsequent microscopic analyses. In figure 2 is illustrated the phenotype of Ff 297-GFP and of the parental strain, in the middle and on the right, respectively. 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 this figure 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 *F. fujikuroi* inoculation, showing a susceptible and resistant profile, respectively (Matic et al. 2016; Siciliano et al. 2015; Volante et al, 2017).

In this study a virulent *F. fujikuroi* strain constitutively expressing the *gfp* reporter gene was obtained, enabling us to study the early stages of *F. fujikuroi* infection of rice roots in the resistant and the susceptible cultivar. Until now, most 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). 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 after 48 and 72 hai and 8 dai. 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 (Figure 4). 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 4). 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 than in the resistant one (Figure 4, transverse sections). 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,* when measured by qPCR, were reported between the roots of susceptible and resistant cultivars (Carneiro et al. 2017; Cheng et al. 2020). Confocal analysis of transverse sections also showed colonization of the xylem vessels in both genotypes, though the fluorescence is restricted to

313 the vessels in Selenio while in Galileo there is also a more generalized labelling around the vessels.
314 At 8 dai the roots were completely covered by the fungal hyphae and the diffuse fluorescence didn't
315 allow any microscopic analysis (data not shown).
316 Another phytopathogenic *Fusarium* spp., such as a *Fusarium oxysporum* f.sp. *cubense* race 4 isolate
317 tagged by GFP, showed the capacity of invading epidermal cells of host roots directly, and spores
318 were produced in the root system. However, in this case, roots of susceptible banana plants were
319 colonized, but not those of the resistant cultivar, probably due to the production of host exudates that
320 inhibited the germination and growth of pathogenic isolate (Li et al., 2011). Similarly, in lettuce, the
321 spread of a GFP transformed virulent isolate of *Verticillium dahliae* has been hampered in two
322 resistant varieties, limiting the fungus to lateral roots and prevented systemic spread to the taproot
323 (Vallad and Subbarao, 2008). In conclusion, fungal colonization occurred in both the resistant cultivar
324 Selenio and the susceptible Galileo, though the fungal presence was less abundant in the former one.
325 This suggests that *F. fujikuroi* is able to colonize the root tissues of both varieties, as previously shown
326 by Carneiro et al. (2017) on the roots of six rice cultivars, though Selenio proves to be resistant when
327 seeds are inoculated.
328 In our experience, *F. fujikuroi* was always detected in the basal roots, and we chose this tissue as the
329 target for pathogen infection and investigation of direct interaction between *F. fujikuroi* and rice. We
330 observed that the earliest infectious structures were represented by the infection hyphae, as recently
331 reported by Sunani and colleagues (2019) by scanning electron microscope analysis. The infection
332 hyphae penetrated the epidermal cells of rice roots after 48-72 hai, and at those times the mycelium
333 was found intra and intercellularly and was able to colonize the vascular bundles. Intercellular and
334 intracellular growth in roots has been documented for other phytopathogenic *Fusarium* spp.,
335 including *F. culmorum* on rye root tissue (Jaroszuk-Ściseł et al. 2008) and *F. oxysporum* f. sp. *radicis*
336 *lycopersici* on tomato (Lagopodi et al. 2002).
337
338 **Chitinase expression analysis**
339 In this work, we tested the expression of *chitinase1* at root level upon *F. fujikuroi* infection. Chitinases
340 are proteins involved in the plant defence against pathogens because of their ability to hydrolyse
341 chitin in the cell wall of fungi (Sharma et al. 2011). We observed that Selenio and Galileo expressed
342 *chitinase1* at similar levels in the not inoculated roots, but the expression increased greatly upon
343 pathogen challenge in the resistant cultivar Selenio (Figure 3). It has been suggested that in
344 filamentous fungi, chitinases may act during hyphal growth (Kumar et al., 2018), therefore, the
345 induction of chitinase1 in Selenio may be involved in the control of hyphal growth during the
346 infection, and correlates well with the phenotype of resistance observed in this cultivar. Up-regulation

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 sample. We repeated the assay three times and always observed that, after 72 hai, in Galileo many germinated seeds showed shorter root length than 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. We hypothesized that *F. fujikuroi* inoculation could have also effect on root growth of the susceptible cultivar, compared to the resistant one, but these preliminary observations need further 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 a seedborne fungal pathogen as *F. fujikuroi*. We clarified that *F. fujikuroi* spreads both in the roots of resistant and susceptible rice plants, although there was a reduction in fungal colonization in the resistant variety. This suggests that *F. fujikuroi* is able to survive and grow inside root tissue even when not causing symptoms. Visualizing *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 the behaviour of GFP-transformed *F. fujikuroi* isolates present within the seeds of susceptible and resistant varieties after artificially inoculations 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, bars: 20 µm (B, C, D and F) and 10 µm (E); (F) Confocal image of fluorescent mycelium on PDA plates; (G) Epifluorescent image of mycelium on the surface of a rice seed.

Fig. 2 Phenotype of the rice susceptible variety Galileo at 30 days post inoculation 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.

Fig. 3 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

Fig. 4 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

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 Ff297-GFP selected for microscopical analyses and the parental strain Ff297, 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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