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(Article begins on next page)

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

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30 **Concise title:** Rice roots infection by gfp-tagged *Fusarium fujikuroi*

31
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38

39 **Author contributions**

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

47

48

49 **ABSTRACT**

50

51 *Fusarium fujikuroi* (teleomorph *Gibberella fujikuroi*) is the main seed-borne pathogen of rice, the
52 causal agent of bakanae, a disease that in the last years has become of increasing economical concern
53 in many Italian rice growing areas. A virulent *F. fujikuroi* isolate was tagged with the green
54 fluorescent protein (GFP) gene, using *Agrobacterium tumefaciens* mediated transformation, and the
55 virulence of the GFP isolate has been confirmed. Little is known about the early interaction of the
56 pathogen with its host, in this work fungal development during the *F. fujikuroi*/root interaction was
57 analysed by LASER scanning confocal microscopy (LSCM), by using the GFP isolate obtained. The
58 infection of rice roots was investigated from 48 h to 8 days post-inoculation both in resistant and
59 susceptible cultivars. Roots of resistant genotype seem to trigger a hypersensitive response at the
60 infection site and LSCM analysis of root sections allowed the visualization of fungal growth within
61 host tissues. Fungal growth occurred both in the resistant and the susceptible cultivar, even if it was
62 less abundant in the resistant one. Expression analysis of *Chitinase1*, a gene involved in fungal

63 pathogenesis, was investigated by qPCR on the *F. fujikuroi* infected rice roots. *Chitinase1* expression
64 increased greatly upon infection in the resistant cultivar Selenio,

65

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

68

69

70 INTRODUCTION

71

72 Bakanae is a rice disease caused by the hemibiotrophic fungal pathogen *Fusarium fujikuroi*. It was
73 originally observed in Japan in 1928 (Ito and Kimura, 1931), but it is now present in several countries
74 in America, Europe, Asia, Oceania and Africa (Amatulli et al. 2010; Carter et al. 2008; Chen et al.
75 2016; Desjardins et al. 2000; Jeon et al. 2013; Karov et al. 2005; Khan et al. 2000; Kim et al. 2015;
76 Zainudin et al. 2008). The meaning of Bakanae is “foolish seedling”, and it is due to the main
77 symptom of the disease: the elongation and thinning of internodes, inducing frail stems and abnormal
78 height, thin leaves, and grains entirely or partially empty. The altered plant morphology is due to the
79 production of gibberellic acids (GAs) by *F. fujikuroi*, the only *Fusarium* species capable of GAs
80 biosynthesis (Ou 1985). GAs are not essential for fungal growth and development but, controlling
81 jasmonic acid-mediated plant immune responses, they probably contribute to the virulence of *F.*
82 *fujikuroi* (Wiemann et al. 2013; Siciliano et al. 2015). *Fusarium fujikuroi* is predominantly a seed
83 borne pathogen, but also survives in soil and diseased plant debris (Ou 1985). Seeds can become
84 infested when conidia, produced on diseased plants, use the wind and water splash to reach
85 neighbouring panicles at flowering. In a recent work Sunani and colleagues (2019), studying the
86 infectious structures, penetration and colonization of *F. fujikuroi* in seeds and seedlings of rice,
87 showed that infection through floret is the dominant pathway to seed infection. The localization of
88 the pathogen could be both inside and on the outside of the seed, being predominant in the lemma
89 and palea, followed by embryo (Kumar et al. 2015). Seeds can also be contaminated by the fungus at

90 harvest, when they can be reached by conidia produced on diseased and dead plants. A third source
91 of seedborne infection is represented by spores and mycelium contaminating the water used to
92 stimulate germination in soaked seeds (Karov et al. 2009). Both ascospores and conidia can also infect
93 seedlings through the roots and crown, colonising both the intracellular and intercellular spaces of
94 the rice root: the fungus invades the plant without producing visible symptoms, so that *F. fujikuroi*
95 can be found in apparently healthy seeds. The potential for pathogenicity in soil rapidly decreases,
96 going from 93% of infection of rice planted immediately after artificial inoculation of the soil to 0.7
97 % for rice planted 90 days after soil inoculation, with no disease at all occurring after 180 days from
98 the inoculation. However, the fungus can survive as hyphae on infested seeds for much longer, lasting
99 4-10 months at room temperature and more than 3 years at 7°C (Kanjanasoon 1965).

100 Bakanae disease is increasing in the main rice-producing areas worldwide. Losses in rice production
101 caused by bakanae depend on climate, rice cultivars and pathogen strain, ranging from 3% to 15% in
102 Thailand (Kanjanasoon 1965), 2% - 20% in Macedonia (Karov et al. 2005), 20% - 50% in Japan (Ito
103 and Kimura, 1931), and up to 75% in Iran (Saremi et al. 2008). The most common Bakanae
104 management is based on thermal seed treatment and the use of fungicides, but *F. fujikuroi* resistance
105 to various fungicides has been reported (Chen et al., 2016). The need of developing new control
106 measures is therefore increasing. The identification of new sources of resistance to *F. fujikuroi* was
107 based on the screening of large collections of rice germplasm and allowed to map several quantitative
108 trait loci (QTLs) on rice chromosomes (Chen et al., 2019; Volante et al., 2017). The development of
109 simple sequence repeat (SSR) markers and mating type analysis allowed to detect *F. fujikuroi* genetic
110 variability at population level (Valente et al., 2016), which is important for screening of resistance.
111 In this work we focused on the analysis of early stages of root infection by a fluorescent *F. fujikuroi*
112 isolate, with the aim to unravel the differences between the susceptible and the resistant rice cultivars
113 facing pathogen infection and colonization. Interaction between pathogens and host plants have been
114 extensively studied using fluorescent reporter proteins. Organisms that express genes encoding
115 fluorescent reporter proteins are frequently used to monitor pathogen behaviours in plant tissues under

116 various physiological conditions (Lagopodi et al. 2002; Oren et al. 2003). The advantage of the Green
117 Fluorescent Protein (GFP) as a reporter is that it allows *in vivo* imaging of fungal hyphae during its
118 interaction with the host plant. Hyphae of *gfp*-expressing fungal strains can be visualized in living
119 tissue in real time, using fluorescence microscopy without extensive manipulation. Compared to
120 many fungal pathogens, such as *Aspergillus* spp. and other *Fusarium* species, the lack of efficient
121 technologies for genetic manipulation has become a major obstacle for the development of *F.*
122 *fujikuroi* molecular research (Cen et al. 2020). However, a polyethylene glycol (PEG)-mediated
123 transformation of protoplasts has been used to introduce the *gfp* and the *red fluorescent protein (rfp)*
124 gene into *F. fujikuroi* for visualizing interaction with biocontrol agents (Watanabe et al. 2007; Kato
125 et al. 2012) and the early root colonization of a GA-producing wild-type and a GA-deficient mutant
126 strain (Wiemann et al. 2013). Recently, a *gfp*-expressing *F. fujikuroi* isolate, obtained by PEG
127 transformation, has been used to analyse rice infection at the basal stem level by confocal microscopy
128 analysis (Lee et al. 2018).

129 No study so far, an *A. tumefaciens*-based method has been developed for transformation of *F.*
130 *fujikuroi*. By this way we transformed four virulent *F. fujikuroi* strains by using a *gfp*-expressing
131 vector, and one of the GFP-tagged isolates obtained was used to visualize and analyse the infection
132 and colonization processes at root level in susceptible and resistant rice cultivars, by confocal
133 microscopy. Quantification of expression in the rootlets of *chitinase 1*, a gene related to the response
134 to bakanae disease, was also performed.

135

136

137 **MATERIALS AND METHODS**

138

139

140 **Fungal strains and growth conditions**

141 Four virulent *F. fujikuroi* strains were selected inside a collection of more than 300 isolates stocked
142 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 10⁶ 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 dH₂O. 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

169 index data. Data were arcsine-transformed prior to ANOVA analysis. The means were separated
170 using Student–Newman–Keuls multiple-range tests ($P < 0.05$).

171

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

173 The four selected *F. fujikuroi* isolates were transformed with the plasmid pCAM*gfp* (kindly provided
174 by A. Sesma, John Innes Center, UK) (Sesma and Osbourn, 2004). The pCAM*gfp* plasmid contains
175 the *sgfp* gene (Chiu et al. 1996) under the control of the *ToxA* promoter from *Pyrenophora tritici-*
176 *repentis* (Lorang et al. 2001) and the hygromycin phosphotransferase (*hph*) gene as the selectable
177 marker gene. The pCAM*gfp* plasmid was introduced into the *Agrobacterium tumefaciens* AGL-1
178 strain, the virulent strain required for fungal transformation. *F. fujikuroi* transformation was carried
179 out using the *A. tumefaciens* AGL-1-transformed strain following the protocol previously described
180 (Campos Soriano and San Segundo 2009; Campos-Soriano et al. 2013) with minor modifications.
181 Co-cultivation was performed at 25°C and selection was done at 28°C. PDA medium plus hygromycin
182 B (250 µg ml⁻¹ final concentration) was used as selective medium to grow the *F. fujikuroi* transformed
183 isolates. Fungal colonies were transferred to 24-well plates containing the selective medium to test
184 the effective transformation. A stereomicroscope (Olympus SZX16) with 480-nm excitation and 500
185 to 550-nm emission filter block was used to verify GFP-transformed fungal colonies. The stability of
186 transgene integration and *gfp* expression of transformants were tested by sub-culturing them for five
187 generations on PDA medium and then transferring them again on selective PDA medium containing
188 100 µg ml⁻¹ hygromycin B. The number of pCAM*gfp* copies integrated into the genome of
189 transformants has been assessed by qReal Time-PCR, using the primers Hyg588U and Hyg588L,
190 listed in Online Resource 2. The PCR mix was composed of 10 µl of SensiMix 2x (Bioline), 2 µl of
191 primer mix (forward and reverse, 5 µM of each primer) and 4 µl of nuclease free water. To each
192 sample 2 µl of fungal genomic DNA and 2 µl of known amounts of the plasmid pAN7-1 were added.
193 The thermal cycler protocol was the following: 95°C for 10 min and 40 cycles with the following
194 steps: 95°C for 30 s; 55°C for 30 s and 72°C for 45 s.

195

196 **Root infection assay**

197 Two rice varieties, the bakanae disease resistant *japonica* variety Selenio and the susceptible *japonica*
198 variety Galileo, were used in this study. Selenio was selected as one of the most resistant rice cultivar
199 from 138 diverse Italian rice accessions screened for evaluation of rice bakanae disease resistance
200 (Volante et al., 2017). Seeds of both cultivars were inoculated by the wild type isolate Ff 297 and the
201 derived transformant Ff 297-GFP. Seeds were surface sterilized in 2% NaOCl for 2 min and rinsed
202 in sterile H₂O before plating on sterile wet paper for germination. After 5 days at 30°C in the dark,
203 young emerged roots were inoculated by applying 100 µl of a suspension at 10⁶ spores ml⁻¹ in the
204 middle of the rootlets, seedlings were allowed continuing the growth at 30°C in the dark until
205 confocal laser scanning microscopic (CLSM) analysis or *chitinase* expression analysis.

206

207 **Epi-fluorescence microscopic analysis**

208 GFP-labelled *F. fujikuroi* mycelium and spores, grown on PDA plates or inoculated roots, were
209 photographed using an epifluorescence microscope (Axioscope, Zeiss) equipped with a GFP filter
210 and a camera to capture images of GFP fluorescence (excitation at 455 to 490 nm and emission at
211 515 to 560 nm).

212

213 **Confocal microscopy analysis of infected roots**

214 After 48 hours after inoculation (hai), 72 hai and 8 days after inoculation (dai) by Ff 297-GFP strain,
215 infected rice roots were stained with propidium iodide (0.2 µg ml⁻¹) for 3 min before microscope
216 observation, both unaltered and hand-sectioned roots were analysed. Images of GFP-labelled *F.*
217 *fujikuroi* strain in host roots were captured using a confocal laser scanning microscope FV1000
218 Olympus (Tokyo, Japan) equipped with inverted microscope IX 81. Images were acquired in z stack
219 with objective 10x (N.A. 0,40), using 488nm (argon Ion, emission 520nm) for GFP fluorescence, and

220 543nm (HeNe, emission 570 nm) laser for propidium iodide staining of root bark. Subsequently they
221 were processed using Imaris 6.2.1 software (Bitplane, Switzerland).

222

223 **Expression analysis**

224 Total RNA was extracted using the RNeasy kit (Qiagen, Germany) from root tissues (0.1 g) at 72 hai
225 with the selected GFP-tagged *F. fujikuroi* strain Ff 297-GFP. RNA was treated with TURBO DNA-
226 free kit to remove contaminating DNA (Ambion, Foster City, California, United States). The absence
227 of DNA contamination in RNA samples was further assessed by PCR using the rice elongation factor
228 1-alpha gene (Manosalva et al. 2009). Total RNA was quantified by Nanodrop (Thermo Fisher
229 Scientific, Waltham, Massachusetts, United States). Reverse transcription reaction was performed
230 using the iScript cDNA synthesis kit (Biorad, Hercules, California, United States). cDNA was then
231 used for expression analysis by quantitative PCR (Applied Biosystem StepOnePlus, Foster City,
232 California, United States) using the specific primers CHIT1-FW (TACTCGTGGGGCTACTGCTT)
233 and CHIT1-RV (CGGGCCGTAGTTGTAGTTGT) for the quantification of the *chitinase 1* rice gene.
234 The primers were designed using the Primer3Plus software (Untergasser et al. 2007). The PCR mix
235 was composed of 5 µl of SYBR Green Power Mix (Applied Biosystem), 2 µl of cDNA, 0.15 µl of
236 each primer (10 µM) and 2.4 µl of nuclease free water. The thermal cycler protocol was the following:
237 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
238 elongation factor 1-alpha was used as housekeeping gene with primers EF1α1F and EF1α1R
239 (Manosalva *et al.*, 2009), listed in Online Resource 2. The efficiency of the primers was tested with
240 a standard curve built upon five serial dilutions (1:10) in three technical replicates. After calculating
241 the fold change values, significant differential expression was evaluated with the Duncan's Post Hoc
242 test, using SPSS v.25.

243

244

245

246 **RESULTS AND DISCUSSION**

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

248 Four different *F. fujikuroi* isolates (Ff 192, Ff 297, Ff 364 and Ff 1550) were transformed with the
249 plasmid pCAM*gfp* containing the *sgfp* gene. The transformed isolates almost retained the colony
250 morphology typical of the wild-type isolates indicating that *gfp* expression did not affect the growth
251 phenotype, in online resource 3 is showed an image of the Ff297-GFP isolate and the parental Ff297,
252 selected for microscope analyses, grown on PDA plates. Approximately, 80-85% of the transformants
253 showed strong fluorescent signal, furthermore, strong fluorescence could be visualized in fungal
254 spores and mycelium (Figure 1).

255 The fluorescence of GFP in transformed *F. fujikuroi* strains remained stable through subsequent
256 cultivation onto PDA medium without antibiotic, indicating the stable integration of the transforming
257 plasmid. The number of pCAM*gfp* copies integrated into the transformant genomes varied from 1 to
258 2 in the different isolates, Ff 297-GFP had only one copy (data not shown). Up to now, *F. fujikuroi*
259 transformation methods have all been based on the use of protoplasts (Watanabe et al., 2007; Kato et
260 al., 2012; Lee et al., 2018). However, protoplast production is time consuming and, even for the same
261 isolates, strictly dependent on the batch of lysing enzymes used, so since several years, the
262 *Agrobacterium tumefaciens*-mediated transformation (ATMT) systems successfully overcame the
263 protoplast-based ones in fungi. Moreover, ATMT-based methods facilitate vector DNA integration
264 in a single site of the recipient genome, and are applicable at different developmental stages, such as
265 conidia, mycelium and fruiting bodies, but germinating conidia are preferred in most of cases, if
266 available (Michielse et al. 2005; Lakshman et al. 2012). For the first time we transformed the conidia
267 of four *F. fujikuroi* isolates by the pCAM*gfp* plasmid introduced into the *A. tumefaciens* AGL-1 strain.
268 This was previously and successfully used for transforming the rice leaf blast pathogen *M. oryzae*
269 (Sesma and Ousborn, 2004; Campos-Soriano and San Segundo, 2009). Among the *F. fujikuroi*
270 transformants obtained one retained virulence similar to the parental strain, showed stable integration
271 of the transforming vector into a single site of the genome and stable fluorescence after plant
272 inoculation.

273

274 **Pathogenicity of GFP transformants**

275 All the four *gfp*-expressing isolates were found to be pathogenic in infection assays of seeds, but they
276 showed different virulence (Table 1). Ff 192 WT was the most virulent (disease index, DI, = 68.0),
277 but in the corresponding transformant, Ff 192-GFP, DI was 28.0, suggesting that, in this isolate, *sgfp*
278 gene insertion affected fungal virulence, in a direct or indirect way. Ff 364-GFP and Ff 1550-GFP

279 showed virulence comparable to parental isolates, but the DI values were lower than Ff 297-GFP. Ff
280 297 WT strain showed to be highly virulent (DI=60) and its virulence was not significantly affected
281 in the corresponding GFP-tagged isolate (Table 1), so that Ff 297-GFP was selected for root infection
282 and subsequent microscopic analyses. In figure 2 is illustrated the phenotype of Ff 297-GFP and of
283 the parental strain, in the middle and on the right, respectively. We already mentioned that *F. fujikuroi*
284 causes different symptoms on rice, as pre-emergence seedling death, elongated and thinner leaves,
285 chlorosis, stunting, crown rot and root rot and even death of seedlings (Ou 1985; Sunani et al. 2019;
286 Piombo et al. 2020). In this figure more than one of these symptoms are visible in the seedlings
287 inoculated by Ff 297-GFP and Ff 297: the number of plants is lower than in the mock test (T, on the
288 left), indicating a pre-emergence seedling death; many leaves and stems are elongated and thinner
289 than in the control and have a larger leaf angle; some of them show stunted growth.

290

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

292 The two cultivars, Galileo and Selenio, were previously tested for their response to *F. fujikuroi*
293 inoculation, showing a susceptible and resistant profile, respectively (Matic et al. 2016; Siciliano et
294 al. 2015; Volante et al, 2017).

295 In this study a virulent *F. fujikuroi* strain constitutively expressing the *gfp* reporter gene was obtained,
296 enabling us to study the early stages of *F. fujikuroi* infection of rice roots in the resistant and the
297 susceptible cultivar. Until now, most studies on the rice-*F. fujikuroi* interaction have been carried out
298 on the aerial part of plants and at several weeks after inoculation (Ji et al. 2016; Ji et al. 2019; Matic
299 et al. 2016). The infection process in root tissues of the rice cultivars Galileo and Selenio was followed
300 by using the GFP-tagged *F. fujikuroi* strain Ff 297-GFP, and visualized by confocal microscopy after
301 48 and 72 hai and 8 dai. Hyphae growing longitudinally along the root surface and in the root hairs
302 were primarily observed (48 hai), and penetration into the epidermal root cells was clearly observed
303 at 72 hai (Figure 4). By this time, most epidermal cells were invaded by the fungus in the susceptible
304 cv Galileo. A similar pattern of hyphal colonization was observed in the roots of the resistant cultivar
305 Selenio, although host cell colonization was much lower in Selenio than that on Galileo (Figure 4).
306 Confocal imaging of transverse sections of the roots showed that the fungus penetrated the stele in
307 both varieties, and was more abundant in the susceptible variety than in the resistant one (Figure 4,
308 transverse sections). We cannot exclude that this evidence was due to the major amount of fungal
309 biomass in the susceptible cultivar, however, in literature no significant differences of the amount of
310 *F. fujikuroi*, when measured by qPCR, were reported between the roots of susceptible and resistant
311 cultivars (Carneiro et al. 2017; Cheng et al. 2020). Confocal analysis of transverse sections also
312 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-Ścisiel 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

347 during incompatible interaction between rice and *M. oryzae* has also been reported (Kawahara et al.,
348 2012). We cannot draw any conclusion regarding the susceptible cultivar Galileo because standard
349 deviation (SD) values of the fold change were too high in Galileo inoculated sample. We repeated
350 the assay three times and always observed that, after 72 hai, in Galileo many germinated seeds showed
351 shorter root length than the same not inoculated cultivar. In conclusion, the Galileo inoculated sample
352 was not homogeneous, and this could be a possible explanation of high SD when analyzing gene
353 expression. We hypothesized that *F. fujikuroi* inoculation could have also effect on root growth of
354 the susceptible cultivar, compared to the resistant one, but these preliminary observations need further
355 studies.

356

357 CONCLUSIONS

358

359 Roots represent the first specialized tissue emerging from seeds upon germination, so it might
360 represent an easy tool to study the early stages and the mechanisms performed for rice infection by a
361 seedborne fungal pathogen as *F. fujikuroi*. We clarified that *F. fujikuroi* spreads both in the roots of
362 resistant and susceptible rice plants, although there was a reduction in fungal colonization in the
363 resistant variety. This suggests that *F. fujikuroi* is able to survive and grow inside root tissue even
364 when not causing symptoms. Visualizing *F. fujikuroi* in roots will help in investigating the early
365 stages of the infection process by this fungal pathogen in rice, while representing a useful tool for the
366 screening of rice cultivars for resistance/susceptibility to *F. fujikuroi*. Further research is in progress
367 to evaluate the behaviour of GFP-transformed *F. fujikuroi* isolates present within the seeds of
368 susceptible and resistant varieties after artificially inoculations of floret, which represents the main
369 route of entry of this pathogen.

370

371

372 Figure captions

373

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

379

380 **Fig. 2** Phenotype of the rice susceptible variety Galileo at 30 days post inoculation with the *gfp*-
381 expressing *F. fujikuroi* isolate Ff 297-GFP (in the middle) and wild type Ff 297(on the right). T, in
382 the left, represents the control mock-inoculated with dH₂O.

383

384 **Fig. 3** Expression of *Chitinase1* gene in the rootlets of resistant (Selenio) and susceptible (Galileo)
385 rice cultivars. Data obtained by reverse transcriptase real time PCR. The error bar is the standard
386 deviation, and the letters indicate groups not considered to be statistically different using the Duncan
387 test

388
389 **Fig. 4** Rootlets of rice cv. Galileo (susceptible) and Selenio (resistant), inoculated with the *gfp*-
390 expressing *F. fujikuroi* isolate Ff 297-GFP. Root surface and transverse sections at the indicated time
391 after inoculation are shown. Bars:70 µm for transverse sections, 150 µm for the other pictures
392
393

394

395 **Electronic Supplementary Material**

396

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

398 **Online Resource 2.** Primers used in this study.

399 **Online Resource 3.** Phenotype of the GFP-tagged isolate Ff297-GFP selected for microscopical
400 analyses and the parental strain Ff297, both grown on PDA plates.

401

402 **Compliance with Ethical Standards:**

403 There are no potential conflicts of interest.

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

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

407

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