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Development and validation of a TaqMan real time PCR assay for the specific detection and quantification of Fusarium fujikuroi in rice plants and seeds

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26 April 2024

1 **PHYTOPATHOLOGY**

2 **Techniques**

3

4 **Development and validation of a TaqMan real time PCR assay for the specific detection and**
5 **quantification of *Fusarium fujikuroi* in rice plants and seeds**

6

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27 ABSTRACT

28 Bakanae disease, caused by the seed-borne pathogen *Fusarium fujikuroi*, is worldwide distributed
29 on rice. A TaqMan real time PCR was developed on the *TEF 1-α* gene for the detection of *F.*
30 *fujikuroi* from different rice tissues. Three sets of primers/probe were tested. The set selected
31 produced an amplicon of 84 bp and was specific for *F. fujikuroi* with respect to eight *Fusarium*
32 species of rice and two other rice fungal pathogens. The assay was validated for specificity,
33 selectivity, sensitivity, repeatability, and reproducibility. The detection limit was 27.5 fg of DNA
34 approximately equivalent to one haploid genome of *F. fujikuroi*. The developed TaqMan real time
35 assay efficiently detected and quantified *F. fujikuroi* from rice culms, leaves, roots and seeds. At 1
36 weeks post germination (wpg), the pathogen was more diffused in the green tissues, while at 3 wpg
37 it was uniformly spread also into the roots. The highest concentration of *F. fujikuroi* was measured
38 in the cultivar M6, with around 1,450 fungal cells/g. The assay was sensitive enough to detect a few
39 genomic equivalents in rice seeds, corresponding to 9.89 *F. fujikuroi* cells/g. The assay permitted to
40 detect bakanae disease at early stages of rice development in asymptomatic tissues.

41 **Introduction**

42 Bakanae is a rice seedborne disease, which poses a serious threat for rice cultivation 43 (Webster and Gunnell 1992). The disease is caused by *Fusarium fujikuroi* Nirenberg [teleomorph 44 *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura], a member of the polyphyletic taxon *G.* 45 *fujikuroi* species complex (GFSC; Carter et al. 2008; Desjardins et al. 1997; O'Donnell et al. 1998). 46 The most common symptoms are the bakanae tillers, which are highly elongated and can be 47 observed at a distance in fields and seedbeds, due to the high level of gibberellins released by the 48 pathogen (Stowe and Yamaki 1957). Diseased plants appear to be abnormally tall, thin, and 49 yellowish compared to other plants. Severely infected seedlings show necrotic root tissue, and 50 frequently die either before or after transplanting (Misra et al. 1994). Since the first report of 51 bakanae in Japan (1928), the disease spread widely across Asia causing important yield losses in 52 Pakistan, Malaysia, Indonesia, Bangladesh, India, South Korea, Taiwan, Philippines, and Thailand 53 (Gupta et al. 2015; Jeon et al. 2013). Yield losses increased in Italy, the major European rice 54 producer (Spadaro et al. 2016). The bakanae disease has also been reported in California (Carter et 55 al. 2008), Russia (Jeon et al. 2013), and Macedonia (Karov et al. 2009).

56 Seed treatments against seedborne fungal pathogens consist in the application of fungicides, 57 physical treatments, biocontrol agents, antimicrobial plant extracts, and essential oils (Tinivella et 58 al. 2009). All of these methods were reported for *F. fujikuroi* control on rice seeds (Matić et al. 59 2014; Titone et al. 2004). Despite recent development of new control methods, the management of 60 bakanae disease remains difficult, due to its wide geographical distribution and increasing 61 incidence. The reduction in pesticide availability and the occurrence of fungicide resistance 62 increased the disease severity in Europe (Matić et al. 2016; Spadaro et al. 2016), with serious issues 63 for the seed companies which need to supply the growers with certified rice seeds free from *F.* 64 *fujikuroi*.

65 Identification of *Fusarium* species (spp.) based on the observation of visual symptoms or 66 morphological characteristics after fungal isolation and culture is complicated and time-consuming

67 (Leslie et al. 2006). There are limitations based on microscope discrimination of *Fusarium* spp.
68 within the GFSC, including *F. fujikuroi, Fusarium proliferatum*, *Fusarium verticillioides* and
69 *Fusarium andiyazi,* due to very similar morphological traits (O'Donnell et al. 1998). Accurate
70 detection and identification of *F. fujikuroi* is necessary in the management of bakanae disease and
71 in the evaluation of rice resistance germplasm. Like other disease assessments, the evaluation of
72 bakanae disease is dependent on the presence of visible symptoms (Hwang et al. 2013; Ou 1985).
73 Although this method is useful during bakanae development in the field, it is not useful for early
74 disease detection and control at initial stages of disease, and for fungal quantification in infected
75 rice tissues. Pathogen detection based on symptomatology and morphological criteria is possible at
76 later stages, which might result in ineffective disease control and further spread of the pathogen. On
77 the other hand, molecular diagnostics of plant pathogenic fungi is characterized by high specificity,
78 sensitivity, and early detection (Capote et al. 2012).

79 Real time PCR has been used in plant pathology for DNA quantification and diagnosis of
80 several plant pathogens (Deepak et al. 2007; Mirmajlessi et al. 2015). It has many advantages over
81 conventional PCR including speed, sensitivity, specificity, reproducibility, and quantification
82 (Smith and Osborn 2009). Real time PCR exploits different fluorescence-measurement approaches
83 for DNA amplification: (1) DNA-binding dyes such as SYBR green I; (2) hydrolysis or TaqMan
84 probes, which exploits the 5′-exonuclease activity of *Taq* DNA polymerase; or (3) hybridization
85 probes, which utilize donor and acceptor fluorophores (Valasek and Repa 2005). Although, real
86 time PCR using a SYBR green dye was previously developed for detection of *F. fujikuroi* from
87 different rice tissues (Amatulli et al. 2012), it did not result in quantitative and reliable detection of
88 *F. fujikuroi* from seeds, which prompted for the development of a more sensitive molecular
89 diagnostic tool.

90 The aim of this study was to develop a TaqMan real time PCR for *F. fujikuroi* identification
91 and quantification in infected rice materials. The assay was validated determining sensitivity,
92 specificity, selectivity, repeatability and reproducibility. The relationship between symptom

93 progression and *F. fujikuroi* occurrence was explored in different rice tissues, including leaves with
94 culms, roots and naturally infected seeds from six different cultivars.
95
96 **Materials and methods**
97
98 **Fungal isolates**. Thirty-five isolates of *F. fujikuroi* and of eight other *Fusarium* spp., and six
99 isolates of two common rice pathogens (*Rhizoctonia solani* and *Magnaporthe oryzae*) were used in
100 this study (Table 1). All the isolates were from the Agroinnova collection (University of Torino,
101 Italy), with exception of *F. fujikuroi* M-1149 (Fusarium Research Centre, Pennsylvania State
102 University, USA), *F. proliferatum* ITEM1720, and *F. verticillioides* ITEM1746 (Institute of
103 Sciences and of Food Production, Bari, Italy). Fungal isolates were long-term stored at -80°C in
104 20% glycerol.
105
106 **DNA extraction from fungal cultures**. Total DNA of all fungal species was extracted with the
107 E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Darmstadt, Germany) from 0.1 g of mycelium
108 grown on potato dextrose agar (PDA, Merck KGaA) following the manufacturer's instructions. The
109 quality and concentration of DNA were measured using a NanoDrop 2000 Spectrophotometer
110 (Thermo Scientific, Wilmington, DE), and DNA was serially diluted to concentrations ranging from
111 1 ng to 1 fg per µl.
112
113 **Sequence analyses, *TEF 1-α* amplification, and probe design**. Sequences of *TEF 1-α* gene for
114 nine *Fusarium* spp. available in GenBank (*F. fujikuroi* GQ848522, GQ848524 and HM243234, *F.*
115 *proliferatum* GQ848533, *F. verticillioides* GQ848532, *F. andiyazi* HM243238, *F. napiforme*
116 GQ848547, *F. culmorum* JN092353, *F. graminearum* GQ848545, *F. equiseti* GQ848541, and *F.*
117 *oxysporum* GQ848538) were selected and aligned. The portions of the sequences specific to *F.*
118 *fujikuroi*, such as those including the six-nucleotides deletion, were used to select potential primers

119 and TaqMan probe for real time PCR. The primers and probe were designed with Primer Express
120 3.0 software (Applied Biosystems, Foster City, CA). Three sets of primers paired with a probe were
121 selected predicting amplicons ranging from 84 to 116 bp (Table 2). TaqMan probe was labelled
122 with the fluorescent reporter 6-carboxy-fluorescein dye (6-FAM) at the 5'-end, and the
123 nonfluorescent quencher-minor groove binder (NF-MGB) at the 3'-end (Applied Biosystems). As
124 exogenous control, a primer-probe combination targeting a highly conserved region of the fungal
125 18S ribosomal DNA was used.

126

127 **Real time PCR optimization**. Real time PCR assay was performed in a 20-µl reaction in
128 triplicates. Various concentrations of primers (0.2-0.8 µM) and probe (0.1-0.3 µM) were tested in
129 different combinations. Amplification and detection were performed in 96-well Optical Reaction
130 Plates (Applied Biosystems) sealed with MicroAmp™ Optical Adhesive Film, on StepOnePlus
131 Real Time PCR system (Applied Biosystems). Optimized real time PCR mixture and conditions
132 consisted in the application of TaqMan Master Mix (2×) (Applied Biosystems), two primers at 0.5
133 µM each, TaqMan probe at 0.25 µM, and 2 µl DNA with an incubation at 50°C for 2 min and 95°C
134 for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 62°C.

135

136 **Specificity, selectivity, sensitivity, repeatability, and reproducibility of the real time PCR**.
137 Genomic DNA from nine different species of *Fusarium* and from *R. solani* and *M. oryzae* was
138 analyzed to evaluate the specificity of the real time PCR assay for *F. fujikuroi*. After 40 cycles, each
139 real time PCR reaction was evaluated for presence or absence of amplification signal. The potential
140 influence of the matrix, including rice and soil microbiota, on the selectivity of real time PCR
141 amplifications was evaluated in different experiments. Three strains of *F. fujikuroi* (14-22, 11-93,
142 and M-1149 with Accession Numbers GQ848524, GQ848522 and HM243234) were used to
143 determine the sensitivity and linear range of the assay. Serial dilutions of genomic DNA from these
144 strains were used (275 ng, 27.5 ng, 2.75 ng, 275 pg, 27.5 pg, 2.75 pg, 275 fg, 27.5 fg). Each sample

145 was amplified in triplicates, and the entire experiment was repeated eight times (EPPO 2014). The
146 experiments were reproduced in two independent laboratories.
147
148 **Detection of *F. fujikuroi* from rice plants.** Six cultivars of rice (*Oryza sativa* L.; 'Selenio',
149 'Graldo', 'Greppi', 'Handao 297', 'M6', and 'Dorella') were selected for *F. fujikuroi* detection. For
150 the artificial inoculation of rice, a conidial suspension (10^6 conidia/ml) of highly pathogenic *F.*
151 *fujikuroi* strain M-1149 grown in potato dextrose broth (PDB, Merck KGaA, Darmstadt, Germany)
152 was used. Rice inoculation was carried out as previously described (Matić et al. 2013). Rice seeds
153 of each cultivar were briefly surface-disinfected in 1% sodium hypochlorite and then rinsed in
154 sterile distilled water. A group of 40 seeds were soaked in a 100 ml-spore suspension in triplicates,
155 and shaken for 30 min at room temperature. Control seeds were soaked in sterile distilled water.
156 The seeds were sown in plastic pots in a sterile mixture of peat and sand (60%:40%) in three
157 replicates, and the plants were grown in greenhouse under controlled temperature conditions (25°C
158 day:17°C night) with 3-times watering per 24 h. Germination rate and disease index were evaluated
159 3 weeks post germination (wpg) using a scale of five distinct groups of symptoms: (0)
160 asymptomatic plants, (1) plants with yellow leaves, (2) plants with long internodes, (3) necrotic
161 plants, and (4) dead plants (Amatulli et al. 2010). For molecular experiments, culm with leaves, and
162 roots were collected at 1 and 3 wpg. DNA extraction from 0.1 g of fresh plant material was ground
163 with liquid nitrogen. The resulting powder after grinding was transferred into a 1.5 ml
164 microcentrifuge tube, where 600 µl P1 buffer of E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek) was
165 added, and then it was vortexed and incubated at 65°C for 10 min. Extracted DNA from rice tissues
166 was finally eluted in 30 µl elution buffer. The quality and concentration of DNA were measured as
167 previously described.
168
169 **Quantification of *F. fujikuroi*.** Pathogen quantification in plants was measured by extracting DNA
170 from aerial and underground parts of six rice cultivars, which showed different levels of

171 susceptibility to *F. fujikuroi*. The DNA quantity of the target (in three technical replicates per
172 sample) was used to calculate the approximate number of *F. fujikuroi* cells present in each sample,
173 using the following formula:

174 Number of cells/µl = DNA quantity/0.000048

175 The number 0.000048 refers to the weight of a *F. fujikuroi* genome expressed in ng (Jeong et al.
176 2013; Wiemann et al. 2013). This formula allows to find the number of fungal cells per µl of
177 reaction mix. However, the initial weight of the sample was also taken in consideration (e.g. 0.1 g),
178 therefore the number of *F. fujikuroi* cells per mg of rice tissue was calculated by multiplying the
179 cells by the volume of extracted DNA in µl, and dividing the value by the initial weight of the rice
180 sample (0.1 g).

181

182 **Detection of *F. fujikuroi* from rice seeds.** TaqMan real time PCR assay was conducted on the seed
183 lots of six tested rice cultivars, each one containing 1,000 naturally infected seeds divided in two
184 technical replicates. DNA extraction from seeds was performed from 0.1 g fraction of 1,000 seeds,
185 ground with liquid nitrogen. The resulting powder was extracted with E.Z.N.A.® Plant DNA Kit, as
186 previously described.

187

188 **Data analysis.** Baseline range, C_T values and real time PCR standard curves were automatically
189 generated using the StepOne™ software. Statistical analyses of data were performed with the
190 Statistical Package for Social Science (SPSS, IBM, Chicago, IL, USA) version 21.0.

191

192 Results

193

194 **Probe and primers development.** The *TEF 1-α* gene was chosen as a specific region due to the
195 deletion of six nucleotides previously found uniquely in *F. fujikuroi* (Amatulli et al. 2012). *TEF 1-α*
196 sequences from different isolates of nine *Fusarium* spp. were aligned and three sets of

197 primers/probe were designed (Table 2). Combination of probe and primers sets were tested in
198 quantitative PCR (qPCR) with different cycling conditions and probe/primers concentrations to
199 evaluate the ability to specifically detect *F. fujikuroi*. To increase the stringency and to avoid false
200 positive results, the temperature of annealing/extension was optimized at 62°C. After examination
201 of amplification results, probe FfujiPq and primer pair TqF2 and TqR were selected as the best
202 candidates for the TaqMan real time PCR assay.
203
204 **TaqMan probe specificity, selectivity, sensitivity, repeatability, and reproducibility.** Regarding
205 specificity of Taqman qPCR, the probe/primers combination (FfujiPq and TqF2/TqR) repeatedly
206 and reliably amplified the 84-bp DNA fragment from different strains of *F. fujikuroi*, while not
207 from eight other species of *Fusarium* and two additionally tested rice pathogens. The presence of
208 plant or soil microbial DNA together with the fungal DNA did not influence the selectivity of the
209 TaqMan probe (FfujiPq) in qPCR assays.
210 The detection limit and dynamic range of the qPCR reaction were determined from the standard
211 curve using DNA extracted from a pure culture of three *F. fujikuroi* strains (14-22, 11-93 and M-
212 1149). Standard regression line was generated for each DNA standard curve using a range of DNA
213 from 10 ng to 0.1 pg. High sensitivity was achieved over five orders of magnitude of DNA
214 concentration, which exhibited a linear dynamic range of amplification. *F. fujikuroi* DNA (from
215 both pure fungal cultures and rice tissues) was accurately quantified until the concentration of 27.5
216 fg with a threshold cycle (C_T) range between 31-34. The detection limit of 27.5 fg DNA is lower
217 than a single haploid genome of *F. fujikuroi* (48 fg DNA).
218 The correlation coefficients between the Ct value and the amount of genomic DNAs of all the three
219 strains were high (R^2 >0.99), and the regression slopes were -3.34, -3.14 and -3.12, respectively.
220 Corresponding slopes were within a slope range between -3.58 and -3.10, that approved good qPCR
221 efficiency (between 90% and 110%; Adams, 2006). As expected, the three standard curves almost
222 completely overlapped with a high degree of repeatability (Fig. 1).

223 The assay was performed on different days by two operators in two laboratories, confirming the
224 reproducibility.
225
226 **Detection and quantification of *F. fujikuroi* in rice culms, leaves, and roots.** One experiment
227 was performed *in vivo* on six rice cultivars to evaluate the presence of *F. fujikuroi* on rice culms
228 with leaves and roots at different time points. At 1 wpg, all cultivars did not show visible
229 symptoms. Later on, typical symptoms of bakanae started to occur on rice cultivars 'Dorella',
230 'Handao 297' and 'M6', such as abnormal stem elongation, and increase of the leaf insertion angle.
231 At 3 wpg (Fig. 2), these latter cultivars showed the most severe symptoms, while at 4 wpg most
232 plants were withered and mainly dead, with drastically inhibited root growth. All the plants of
233 cultivar 'M6' already died at 3 wpg (Table 3). The cultivars 'Dorella', 'Handao 297' and 'M6' were
234 considered susceptible. On the contrary, the three cultivars 'Selenio', 'Graldo', and 'Greppi' did not
235 show typical bakanae symptoms during the whole experiment, and only a small proportion of
236 yellowish plants could be observed (Fig. 2).
237 At 1 wpg (asymptomatic period), *F. fujikuroi* was detected by TaqMan qPCR assay both in
238 susceptible and resistant cultivars, and its presence was higher in aerial parts compared to roots
239 (Fig. 3). At 3 wpg, when the most striking bakanae symptoms occurred, the concentration was the
240 highest in both aerial and root parts of the susceptible cultivars, while it was lower in culms and
241 leaves of resistant cultivars (Fig. 3 and Table 3). Interestingly, 'Handao 297' and 'Dorella'
242 exhibited a slightly higher concentration of *F. fujikuroi* in the aerial parts of the plant, while 'M6' in
243 the roots. The highest concentration of *F. fujikuroi* was measured in the roots of the most
244 susceptible cultivar 'M6', with around 1,450 fungal cells per gram of tissue (Fig. 3). Resistant
245 cultivars ('Greppi', 'Graldo', and 'Selenio') showed much less presence of *F. fujikuroi* (from 279 to
246 0.3 cells per gram of the plant tissue), mainly in aerial plant parts compared to the susceptible
247 cultivars and to the initial infection period.
248

249 **Detection and quantification of *F. fujikuroi* in rice seeds**. The TaqMan qPCR assay was also
250 used to detect and quantify *F. fujikuroi* in naturally contaminated rice seeds lots of six rice cultivars.
251 On rice seeds, mixed populations of *Fusarium* spp. might occur, but the amplification with the
252 TaqMan FfujiPq probe was specific and not influenced by the other *Fusarium* species or with the
253 presence of rice genomic DNA. This qPCR was sensitive enough to detect 9.89 *F. fujikuroi* cells/g
254 of naturally infected rice seed lots (Fig. 4). *F. fujikuroi* DNA from seeds was precisely quantified
255 until the same concentration of other rice tissues (27.5 fg) and a C_T range between 34-36.
256
257 **Discussion**
258 The method developed in this study allowed a fast *F. fujikuroi* identification at early stages
259 of the disease, and reliable quantification from different rice materials including the seeds where the
260 fungus is present at lower concentration (Dieter 2002). It could be applied on DNA extracted
261 directly from rice seeds or fresh plant tissues, and it is not affected by non-specific background
262 from co-extracted plant DNA, soil microbial DNA, or from other *Fusarium* spp. associated with
263 bakanae disease or commonly present on rice (Desjardins et al. 2000; Wulff et al. 2010). The probe
264 and primers were evaluated by amplifying DNA from pure culture isolates of *Fusarium* spp., and
265 the specificity was assessed by specific amplification of DNA from *F. fujikuroi,* and not from other
266 *Fusarium* spp. In addition, the method was also tested on three strains for two pathogenic species of
267 rice *M. oryzae* and *R. solani*, without amplification signal. Most of the tested *Fusarium* spp. are
268 members of the GFSC and can morphologically be distinguished from *F. fujikuroi* based on
269 microconidia production and absence of chlamydospores. However, other *Fusarium* species, such
270 as *F. proliferatum* and *F. verticilloides,* would need expertise in *Fusarium* taxonomy to be
271 differentiated from *F. fujikuroi*. (Leslie et al. 2004). The increased stringency of amplification
272 reaction developed in our study by increasing the annealing temperature to 62°C avoided the
273 problem of cross-amplification and allowed specific *F. fujikuroi* detection. Additionally, sensitivity

274 was not impaired by the increased stringency permitting the detection up to 27.5 fg of *F. fujikuroi* in
275 both rice plants and seeds.

276 The additional specificity provided by the internal probe is advantageous in overcoming the
277 risks of false positives and negatives. Since SYBR Green binds nonspecifically any double-stranded
278 DNA which is present in real time PCR reaction, our protocol was based on a more specific
279 TaqMan probe. Additionally, the *Taq* DNA polymerase can be inhibited by SYBR Green, which
280 limits the fluorescence signal and the assay sensitivity (Kermekchiev et al. 2009). That was also
281 avoided by using the TaqMan probe, which increased the sensitivity of the assay and reduced the
282 necessary amount of the *Taq* enzyme.

283 After optimizing the PCR conditions to achieve the highest specificity, the protocol was
284 implemented for quantitative purposes. Followed the quantification of *F. fujikuroi* DNA from pure
285 culture, the utility of the quantification protocol was checked in rice different tissues. We showed
286 that the sensitivity of the qPCR protocol was not affected by the nature of the sample, since the
287 fungus could be detected and quantified in complex naturally infected matrices of diverse origin,
288 including different plant tissues and soil residues. Our results showed the fungal presence in both
289 aerial and underground tissues of infected rice plants without visible symptoms during the early-
290 stage of bakanae disease. Furthermore, a successful amplification of *F. fujikuroi* was obtained by
291 Taqman qPCR from naturally infected rice seeds containing high starch level (one of the main
292 polysaccharides), insoluble phenolic compounds, and other interfering substances (Tian et al. 2004;
293 Wang et al. 2012). Polysaccharides can be especially troublesome inside the DNA extracts, since
294 their presence is inhibitory on *Taq* polymerase activity (Fang et al. 1992). Polyphenolic compounds
295 may bind to DNA during the cell lysis and result recalcitrant to further enzymatic manipulations
296 (John 1992). In our study, we did not find the inhibition of *Taq* DNA polymerase by the application
297 of developed TaqMan real time PCR assay. Hence, the purity of the DNA extracts from rice seeds
298 obtained by E.Z.N.A. DNA Kit had an $A_{260/280}$ ratio higher than 1.8 and $A_{260/230}$ ratio higher than 2,

299 indicating little or no DNA contamination with proteins, polyphenolics and polysaccharides, and it
300 was above the amenable DNA purity limit for molecular studies (Sambrook and Russell 2001).

301 We found that the TaqMan real time PCR using FfujiPq and TqF2/TqR primer-probe
302 combination with a detection limit of 27.5 fg of fungal DNA had similar or higher sensitivity than
303 TaqMan PCR assays targeting other *Fusarium* spp. such as *Fusarium virguliforme* (100 fg), *F.*
304 *verticillioides* and *F. proliferatum* (10 pg), and *F. culmorum* and *F. graminearum* (0.9 pg) (Lin et
305 al. 2014; Waalwijk et al. 2004; Wang et al. 2015). Furthermore, our TaqMan real time PCR assay
306 was around 364-fold more sensitive compared to the detection limit of 10 pg of *F. fujikuroi* DNA
307 from rice obtained by the SYBR Green real time PCR assay (Amatulli et al. 2012).

308 The most abundant presence of *F. fujikuroi* during the disease development was found in the
309 roots and green tissues of the highly susceptible cultivar 'M6', while the pathogen was present at
310 lower concentrations in the roots of the other two susceptible cultivars. At 1 wpg, the pathogen
311 seems more diffused in the green tissues, and to a lower extent in the roots, while at 3 wpg the
312 fungus was uniformly spread into the plant tissues and the difference between roots and green
313 tissues became negligible. The high presence of *F. fujikuroi* in the roots of rice plants should be
314 considered to deepen the knowledge about the epidemiology of the bakanae disease. The results of
315 the fungal quantification of this study fits with the transcriptomic study of two cultivars ('Dorella'
316 and 'Selenio') infected with *F. fujikuroi* (Matić et al. 2016), indicating the higher presence of *F.*
317 *fujikuroi* and its systemic distribution in the whole rice plants of susceptible 'Dorella'. On the other
318 hand, a lower concentration and local (aerial) distribution of *F. fujikuroi* was found in resistant
319 'Selenio' without any visible damage on the plants.

320 In this study, *F. fujikuroi* DNA could be accurately quantified over a large range of
321 concentrations using real time PCR in complex biological matrices, including naturally
322 contaminated and artificially inoculated rice samples from diverse origin, where various
323 microorganisms may coexist with the target species. In the case of naturally infected seeds, it is not
324 possible to accurately distinguish the target pathogen from other *Fusarium* spp. associated with

325 bakanae disease, by exclusive use of discriminative techniques, such as plating on culture media.
326 Such procedure is tedious and delays the identification of *F. fujikuroi*, until detailed microscopic
327 observations of morphological traits are made (Munkvold 2009). Consequently, the availability of
328 the developed qPCR protocol for quantifying *F. fujikuroi* may be a useful tool for early detection of
329 *F. fujikuroi* in rice seeds. Rice seed treatment is currently the most valuable tool in order to control
330 bakanae disease. For small quantities, the seed companies can choose to use thermal treatments with
331 hot water (60-65°C for 15 minutes) or chemical treatments (Matić et al. 2014; Park et al. 2008). For
332 greater seed quantities, a chemical treatment is commonly used, but in the last years, the most
333 important active ingredient – prochloraz – was withdrawn from the market and the effectiveness of
334 other fungicides is not satisfying. Therefore, it is of crucial importance to take in consideration
335 possible alternatives (Matić et al. 2014). Seed companies could use this molecular tool before and
336 after seed treatment against *F. fujikuroi* in order to test the efficacy of the treatment and to certify
337 successfully the seed lots as free from *F. fujikuroi*.
338

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460 **TABLES**

461

462 TABLE 1. Representative *Fusarium* spp. and other fungus species tested by TaqMan real time PCR.

Species	Isolate	Host	Origin
Fusarium fujikuroi	GCe1	*Oryza sativa*	Italy (Piemonte)
F. fujikuroi	8-6	*O. sativa*	Italy (Piemonte)
F. fujikuroi	11-6	*O. sativa*	Italy (Piemonte)
F. fujikuroi	4-10	*O. sativa*	Italy (Piemonte)
F. fujikuroi	SO9-34	*O. sativa*	Italy (Piemonte)
F. fujikuroi	7/4	*O. sativa*	Italy (Piemonte)
F. fujikuroi	14-22	*O. sativa*	Italy (Piemonte)
F. fujikuroi	I 1.3	*O. sativa*	Italy (Piemonte)
F. fujikuroi	I 2-4 A	*O. sativa*	Italy (Piemonte)
F. fujikuroi	M-1149	*O. sativa*	Taiwan
F. fujikuroi	S09-21	*O. sativa*	Italy (Piemonte)
F. fujikuroi	11-93	*O. sativa*	Italy (Piemonte)
F. fujikuroi	TVS2	*O. sativa*	Italy (Piemonte)
F. fujikuroi	CSV1	*O. sativa*	Italy (Piemonte)
F. fujikuroi	VE13	*O. sativa*	Italy (Piemonte)
F. proliferatum	19-113	*O. sativa*	Italy (Piemonte)
F. proliferatum	19-109	*O. sativa*	Italy (Piemonte)
Fusarium proliferatum	ITEM 1720	*Zea mays*	Italy (Sardinia)
Fusarium verticillioides	19-116	*O. sativa*	Italy (Piemonte)
F. verticillioides	19-42	*O. sativa*	Italy (Piemonte)
F. verticillioides	ITEM 1746	*Z. mays*	Italy (Sardinia)
Fusarium oxysporum	11-47	*O. sativa*	Italy (Piemonte)
F. oxysporum	11-77	*O. sativa*	Italy (Piemonte)
F. oxysporum	19-9	*O. sativa*	Italy (Piemonte)
Fusarium graminearum	2-1	*O. sativa*	Italy (Piemonte)
F. graminearum	S09-49	*O. sativa*	Italy (Piemonte)
F. graminearum	9-4	*O. sativa*	Italy (Piemonte)
Fusarium equiseti	15-23	*O. sativa*	Italy (Piemonte)
F. equiseti	15-95	*O. sativa*	Italy (Piemonte)
F. equiseti	11-17	*O. sativa*	Italy (Piemonte)
Fusarium andiyazi	S09-6	*O. sativa*	Italy (Piemonte)
F. andiyazi	S09-7	*O. sativa*	Italy (Piemonte)
F. andiyazi	S09-8	*O. sativa*	Italy (Piemonte)
Fusarium culmorum	1706	*O. sativa*	Italy (Piemonte)
Fusarium napiforme	2-130	*O. sativa*	Italy (Piemonte)
Magnaporthe oryzae	AG121	*O. sativa*	Italy (Piemonte)
M. oryzae	AG149	*O. sativa*	Italy (Piemonte)
M. oryzae	AG150	*O. sativa*	Italy (Piemonte)
Rhizoctonia solani	DB240712	*O. sativa*	Italy (Piemonte)
R. solani	DB14OTT25	*O. sativa*	Italy (Piemonte)
R.solani	DB070712	*O. sativa*	Italy (Piemonte)

463

464

465 TABLE 2. Sets of primers and probe used in Taqman PCR for *Fusarium fujikuroi* detection.

Primers and Taqman probe	**Amplicon (nt)**	**Sequence (5'-3')**
TqF2		GGCGCGTTTTGCCCTTTCCT
TqR	84	AGCGGCTTCCTATTGTCGAA
FfujiPq		[FAM]-TCACGTGTCAAACTAAA-[NF-MGB]
TqF1		CGAGTGATGGGCGCGTTTTG
TqR	93	AGCGGCTTCCTATTGTCGAA
TaqFuji		[FAM]-CGCATTGTCACGTGTCAAAC-[NF-MGB]
FfujiFq		CACGTGTCAAACTAAACATTCG
FfujiRq	116	GATGGTGATACCACGCTCAC
BakanPq		[FAM] – GCCGCTGAGCTCGGTAAGGG -[NF-MGB]

466

467 TABLE 3. Disease index and germination of six rice cultivars inoculated (+) or not (-) with *Fusarium*

468 *fujikuroi* at 3 weeks post germination[a].

Cultivar[b]	Disease index (%)	Germination (%)
Selenio +	7.5	93.2
Selenio -	0	95.8
Graldo +	7.5	87.7
Graldo -	0	96.7
Greppi +	12.5	83.3
Greppi -	0	89.2
Dorella +	87.5	40.2
Dorella -	0	95.4
Handao 297 +	72.5	55.0
Handao 297 -	0	98.3
M6 +	100	41.1
M6 -	0	90.4

469

470 [a] Forty seeds per variety were tested in three replicates per treatment. Experiments were carried out three times.

471 [b]Three resistant ('Selenio', 'Graldo' and 'Greppi') and three susceptible ('Dorella', 'Handao 297' and 'M6') rice

472 cultivars were evaluated.