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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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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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9

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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- $\alpha$*  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- $\mu$ l reaction in  
128 triplicates. Various concentrations of primers (0.2-0.8  $\mu$ M) and probe (0.1-0.3  $\mu$ M) were tested in  
129 different combinations. Amplification and detection were performed in 96-well Optical Reaction  
130 Plates (Applied Biosystems) sealed with MicroAmp<sup>TM</sup> 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 $\times$ ) (Applied Biosystems), two primers at 0.5  
133  $\mu$ M each, TaqMan probe at 0.25  $\mu$ M, and 2  $\mu$ l 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:

$$174 \quad \text{Number of cells}/\mu\text{l} = \text{DNA quantity}/0.000048$$

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  $\mu\text{l}$  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  $\mu\text{l}$ , 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- $\alpha$*  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- $\alpha$*   
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  $C_t$  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<sub>T</sub> 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
<i>Fusarium fujikuroi</i>	GCE1	<i>Oryza sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	8-6	<i>O. sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	11-6	<i>O. sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	4-10	<i>O. sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	SO9-34	<i>O. sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	7/4	<i>O. sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	14-22	<i>O. sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	I 1.3	<i>O. sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	I 2-4 A	<i>O. sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	M-1149	<i>O. sativa</i>	Taiwan
<i>F. fujikuroi</i>	S09-21	<i>O. sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	11-93	<i>O. sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	TVS2	<i>O. sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	CSV1	<i>O. sativa</i>	Italy (Piemonte)
<i>F. fujikuroi</i>	VE13	<i>O. sativa</i>	Italy (Piemonte)
<i>F. proliferatum</i>	19-113	<i>O. sativa</i>	Italy (Piemonte)
<i>F. proliferatum</i>	19-109	<i>O. sativa</i>	Italy (Piemonte)
<i>Fusarium proliferatum</i>	ITEM 1720	<i>Zea mays</i>	Italy (Sardinia)
<i>Fusarium verticillioides</i>	19-116	<i>O. sativa</i>	Italy (Piemonte)
<i>F. verticillioides</i>	19-42	<i>O. sativa</i>	Italy (Piemonte)
<i>F. verticillioides</i>	ITEM 1746	<i>Z. mays</i>	Italy (Sardinia)
<i>Fusarium oxysporum</i>	11-47	<i>O. sativa</i>	Italy (Piemonte)
<i>F. oxysporum</i>	11-77	<i>O. sativa</i>	Italy (Piemonte)
<i>F. oxysporum</i>	19-9	<i>O. sativa</i>	Italy (Piemonte)
<i>Fusarium graminearum</i>	2-1	<i>O. sativa</i>	Italy (Piemonte)
<i>F. graminearum</i>	S09-49	<i>O. sativa</i>	Italy (Piemonte)
<i>F. graminearum</i>	9-4	<i>O. sativa</i>	Italy (Piemonte)
<i>Fusarium equiseti</i>	15-23	<i>O. sativa</i>	Italy (Piemonte)
<i>F. equiseti</i>	15-95	<i>O. sativa</i>	Italy (Piemonte)
<i>F. equiseti</i>	11-17	<i>O. sativa</i>	Italy (Piemonte)
<i>Fusarium andiyazi</i>	S09-6	<i>O. sativa</i>	Italy (Piemonte)
<i>F. andiyazi</i>	S09-7	<i>O. sativa</i>	Italy (Piemonte)
<i>F. andiyazi</i>	S09-8	<i>O. sativa</i>	Italy (Piemonte)
<i>Fusarium culmorum</i>	1706	<i>O. sativa</i>	Italy (Piemonte)
<i>Fusarium napiforme</i>	2-130	<i>O. sativa</i>	Italy (Piemonte)
<i>Magnaporthe oryzae</i>	AG121	<i>O. sativa</i>	Italy (Piemonte)
<i>M. oryzae</i>	AG149	<i>O. sativa</i>	Italy (Piemonte)
<i>M. oryzae</i>	AG150	<i>O. sativa</i>	Italy (Piemonte)
<i>Rhizoctonia solani</i>	DB240712	<i>O. sativa</i>	Italy (Piemonte)
<i>R. solani</i>	DB14OTT25	<i>O. sativa</i>	Italy (Piemonte)
<i>R. solani</i>	DB070712	<i>O. sativa</i>	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	84	GGCGCGTTTTGCCCTTTCCT
TqR		AGCGGCTTCCTATTGTCGAA
FfujiPq		[FAM]-TCACGTGTCAAATAAA-[NF-MGB]
TqF1	93	CGAGTGATGGGCGCGTTTTG
TqR		AGCGGCTTCCTATTGTCGAA
TaqFuji		[FAM]-CGCATTGTCACGTGTCAAAC-[NF-MGB]
FfujiFq	116	CACGTGTCAAATAAACATTTCG
FfujiRq		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<sup>a</sup>.

<b>Cultivar<sup>b</sup></b>	<b>Disease index (%)</b>	<b>Germination (%)</b>
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 <sup>a</sup>Forty seeds per variety were tested in three replicates per treatment. Experiments were carried out three times.

471 <sup>b</sup>Three resistant ('Selenio', 'Graldo' and 'Greppi') and three susceptible ('Dorella', 'Handao 297' and 'M6') rice

472 cultivars were evaluated.