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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Accepted for publication 1 April 2017.

ABSTRACT

Bakanae disease, which is caused by the seedborne pathogen *Fusarium fujikuroi*, is found throughout the world on rice. A TaqMan real-time PCR has been developed on the *TEF 1-α* gene to detect *F. fujikuroi* in different rice tissues. Three primer/probe sets were tested. The selected set produced an amplicon of 84 bp and was specific for *F. fujikuroi* with respect to eight *Fusarium* species of rice and six other rice common pathogens. The assay was validated for specificity, selectivity, sensitivity, repeatability, and reproducibility. The detection limit was set at 27.5 fg of DNA, which is approximately equivalent to one haploid genome of *F. fujikuroi*.

The developed TaqMan real-time assay was able to efficiently detect and quantify *F. fujikuroi* from rice culms, leaves, roots, and seeds. At 1 week post-germination (wpg), the pathogen was more diffused in the green tissues, while at 3 wpg it was uniformly spread also in the roots. The highest concentration of *F. fujikuroi* was measured in the M6 cultivar, which showed around 1,450 fungal cells/g. The assay was sufficiently sensitive to detect a few genomic equivalents in the rice seeds, corresponding to 9.89 *F. fujikuroi* cells/g. The assay permitted bakanae disease to be detected in asymptomatic tissues at the early rice development stages.

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

Seed treatments against seedborne fungal pathogens include the application of fungicides, physical treatments, biocontrol agents, antimicrobial plant extracts, and essential oils (Tinivella et al. 2009). All of these methods have been reported for *F. fujikuroi* control on rice seeds (Matić et al. 2014; Titone et al. 2004). Despite the recent development of new control methods, the management of bakanae disease remains difficult, due to its extensive geographical distribution and increasing incidence. The reduction in pesticide availability and the occurrence of fungicide resistance has increased the severity

of the disease in Europe (Matić et al. 2016; Spadaro et al. 2016), with consequent serious issues for seed companies that need to supply growers with rice seeds that are certified to be free from *F. fujikuroi*.

Identification of *Fusarium* spp. on the basis of an observation of the visible symptoms or morphological characteristics after fungal isolation and on the basis of the culture is complicated and time-consuming (Leslie and Summerell 2006). The microscope discrimination of *Fusarium* spp. within the GFSC, which includes *F. fujikuroi*, *F. proliferatum*, *F. verticillioides*, and *F. andiyazi*, can lead to certain limitations due to their very similar morphological traits (O'Donnell et al. 1998). An accurate detection and identification of *F. fujikuroi* is necessary for the management of bakanae disease and to evaluate the rice resistance germplasm. Like other disease assessments, the evaluation of bakanae disease depends on the presence of visible symptoms (Hwang et al. 2013; Ou 1985). Although this method is useful during bakanae development in the field, it is not useful for early disease detection and control at the initial disease stages or for fungal quantification in infected rice tissues. Pathogen detection, based on symptomatology and morphological criteria, is possible at later stages, but this might result in an ineffective disease control and a further spreading of the pathogen. On the other hand, molecular diagnostics of plant pathogenic fungi is characterized by high specificity, sensitivity and early detection (Capote et al. 2012).

Real-time PCR has been used in plant pathology for DNA quantification and the diagnosis of several plant pathogens (Deepak et al. 2007; Mirmajlessi et al. 2015). It offers many advantages over conventional PCR, including speed, sensitivity, specificity, reproducibility, and quantification (Smith and Osborn 2009). Real-time PCR exploits different fluorescence-measurement approaches for DNA amplification: (i) DNA-binding dyes, such as SYBR green I; (ii) hydrolysis or TaqMan probes, which exploit the 5'-exonuclease activity of *Taq* DNA polymerase; and (iii) hybridization probes, which utilize donor and acceptor fluorophores (Valasek and Repa 2005). Although real-time PCR using an SYBR green dye was previously developed for the detection of *F. fujikuroi* from different rice tissues

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(Amatulli et al. 2012), it was not able to quantitatively and reliably detect *F. fujikuroi* from seeds, and this led to the development of a more sensitive molecular diagnostic tool.

The aim of this study was to develop a TaqMan real-time PCR for *F. fujikuroi* identification and quantification in infected rice materials. The sensitivity, specificity, selectivity, repeatability, and reproducibility of the assay were validated. The relationship between symptom progression and *F. fujikuroi* occurrence was explored in different rice tissues, including the leaves with culms, roots, and naturally infected seeds from six different cultivars.

MATERIALS AND METHODS

Fungal isolates. Thirty-five isolates of *F. fujikuroi* and of eight other *Fusarium* spp. were used in this study. Furthermore, 11 isolates of six common rice pathogenic fungi (*Magnaporthe oryzae*, *M. salvinii*, *Rhizoctonia solani*, *Cochliobolus miyabeanus*, *Gaeumannomyces graminis*, and *Alternaria padwickii*) (Table 1). All isolates of *F. fujikuroi* were from the Agroinnova collection (University of Torino, Italy), with the exception of *F. fujikuroi* M-1149 (*Fusarium* Research Centre, Pennsylvania State University), *F. proliferatum* ITEM1720, and *F. verticillioides* ITEM1746

(Institute of Sciences and of Food Production, Bari, Italy). The fungal isolates were stored long term at -80°C in 20% glycerol.

DNA extraction from fungal cultures. The total DNA of all the fungal species was extracted with an E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-Tek, Darmstadt, Germany) from 0.1 g of mycelium grown on potato dextrose agar (Merck KGaA), according to the manufacturer's instructions. The quality and concentration of DNA were measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE), and the DNA was serially diluted to concentrations ranging from 1 ng to 1 fg per μl .

Sequence analyses, *TEF 1- α* amplification, and probe design. Sequences of the *TEF 1- α* gene were selected and aligned for nine *Fusarium* spp. available in GenBank (*F. fujikuroi* GQ848522, GQ848524, and HM243234; *F. proliferatum* GQ848533; *F. verticillioides* GQ848532; *F. andiyazi* HM243238; *F. napiforme* GQ848547; *F. culmorum* JN092353; *F. graminearum* GQ848545; *F. equiseti* GQ848541; and *F. oxysporum* GQ848538). The portions of the sequences that were specific to *F. fujikuroi*, that is those including six-nucleotide deletion, were used to select potential primers and a TaqMan probe for real-time PCR. The primers and probe were designed by means of Primer Express 3.0 software (Applied Biosystems, Foster City, CA). Three sets of primers paired with a probe were selected to predict amplicons ranging from 84 to

TABLE 1. Representative *Fusarium* spp. and other fungal species tested by means of TaqMan real-time PCR

Species	Isolate	Host	Origin
<i>F. fujikuroi</i>	GCE1	<i>Oryza sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	8-6	<i>O. sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	11-6	<i>O. sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	4-10	<i>O. sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	SO9-34	<i>O. sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	7/4	<i>O. sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	14-22	<i>O. sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	I 1.3	<i>O. sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	I 2-4 A	<i>O. sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	M-1149	<i>O. sativa</i>	Taiwan
<i>F. fujikuroi</i>	SO9-21	<i>O. sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	11-93	<i>O. sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	TVS2	<i>O. sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	CSV1	<i>O. sativa</i>	Italy (Piedmont)
<i>F. fujikuroi</i>	VE13	<i>O. sativa</i>	Italy (Piedmont)
<i>F. proliferatum</i>	19-113	<i>O. sativa</i>	Italy (Piedmont)
<i>F. proliferatum</i>	19-109	<i>O. sativa</i>	Italy (Piedmont)
<i>F. proliferatum</i>	ITEM 1720	<i>Zea mays</i>	Italy (Sardinia)
<i>F. verticillioides</i>	19-116	<i>O. sativa</i>	Italy (Piedmont)
<i>F. verticillioides</i>	19-42	<i>O. sativa</i>	Italy (Piedmont)
<i>F. verticillioides</i>	ITEM 1746	<i>Z. mays</i>	Italy (Sardinia)
<i>F. oxysporum</i>	11-47	<i>O. sativa</i>	Italy (Piedmont)
<i>F. oxysporum</i>	11-77	<i>O. sativa</i>	Italy (Piedmont)
<i>F. oxysporum</i>	19-9	<i>O. sativa</i>	Italy (Piedmont)
<i>F. graminearum</i>	2-1	<i>O. sativa</i>	Italy (Piedmont)
<i>F. graminearum</i>	SO9-49	<i>O. sativa</i>	Italy (Piedmont)
<i>F. graminearum</i>	9-4	<i>O. sativa</i>	Italy (Piedmont)
<i>F. equiseti</i>	15-23	<i>O. sativa</i>	Italy (Piedmont)
<i>F. equiseti</i>	15-95	<i>O. sativa</i>	Italy (Piedmont)
<i>F. equiseti</i>	11-17	<i>O. sativa</i>	Italy (Piedmont)
<i>F. andiyazi</i>	SO9-6	<i>O. sativa</i>	Italy (Piedmont)
<i>F. andiyazi</i>	SO9-7	<i>O. sativa</i>	Italy (Piedmont)
<i>F. andiyazi</i>	SO9-8	<i>O. sativa</i>	Italy (Piedmont)
<i>F. culmorum</i>	1706	<i>O. sativa</i>	Italy (Piedmont)
<i>F. napiforme</i>	2-130	<i>O. sativa</i>	Italy (Piedmont)
<i>Magnaporthe oryzae</i>	AG121	<i>O. sativa</i>	Italy (Piedmont)
<i>M. oryzae</i>	AG149	<i>O. sativa</i>	Italy (Piedmont)
<i>M. oryzae</i>	AG150	<i>O. sativa</i>	Italy (Piedmont)
<i>M. salvinii</i>	MS1	<i>O. sativa</i>	Italy (Piedmont)
<i>M. salvinii</i>	MS5	<i>O. sativa</i>	Italy (Piedmont)
<i>Rhizoctonia solani</i>	DB240712	<i>O. sativa</i>	Italy (Piedmont)
<i>R. solani</i>	DB14OTT25	<i>O. sativa</i>	Italy (Piedmont)
<i>R. solani</i>	DB070712	<i>O. sativa</i>	Italy (Piedmont)
<i>Cochliobolus miyabeanus</i>	CM1	<i>O. sativa</i>	Italy (Piedmont)
<i>Gaeumannomyces graminis</i>	GGR1	<i>O. sativa</i>	Italy (Piedmont)
<i>Alternaria padwickii</i>	ALT-PR	<i>O. sativa</i>	Italy (Piedmont)

116 bp (Table 2). The TaqMan probe was labeled with the 6-carboxy-fluorescein dye (6-FAM) fluorescent reporter at the 5'-end, and the nonfluorescent quencher-minor groove binder (NF-MGB) at the 3'-end (Applied Biosystems). A primer-probe combination, targeting a highly conserved region of the fungal 18S ribosomal DNA, was used as the exogenous control.

Real-time PCR optimization. A real-time PCR assay was performed in a 20-μl reaction in triplicate. Various concentrations of the primers (0.2 to 0.8 μM) and probe (0.1 to 0.3 μM) were tested in different combinations. Amplification was performed in 96-well Optical Reaction Plates (Applied Biosystems) sealed with a Micro-Amp Optical Adhesive Film, on a StepOnePlus real-time PCR system (Applied Biosystems). The optimized real-time PCR mixture and conditions involved the application of a TaqMan Master Mix (2×) (Applied Biosystems), two 0.5 μM primers, the TaqMan probe at 0.25 μM, and 2 μl of DNA with an incubation at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 62°C.

Specificity, selectivity, sensitivity, repeatability, and reproducibility of the real-time PCR. Genomic DNA from nine different species of *Fusarium* and from six rice pathogens was analyzed to evaluate the specificity of the real-time PCR assay for *F. fujikuroi*. After 40 cycles, each real-time PCR reaction was evaluated to establish the presence or absence of an amplification signal. The potential influence of the matrix (rice and soil microbiota) on the selectivity of real-time PCR amplifications was evaluated in different experiments. Three strains of *F. fujikuroi* (14-22, 11-93, and M-1149 with accession numbers GQ848524, GQ848522, and HM243234) were used to determine the sensitivity and linear range of the assay. Serial dilutions of genomic DNA from these strains were used (275 ng, 27.5 ng, 2.75 ng, 275 pg, 27.5 pg, 2.75 pg, 275 fg, and 27.5 fg). Each sample was amplified in triplicate, and the entire experiment was repeated eight times (EPPO 2014). The experiments were reproduced in two distinct laboratories.

Detection of *F. fujikuroi* from rice plants. Six rice cultivars (*Oryza sativa* L. 'Selenio', 'Graldo', 'Greppi', 'Handao 297', 'M6', and 'Dorella') were selected for *F. fujikuroi* detection. A conidial suspension (10⁶ conidia/ml) of a highly pathogenic *F. fujikuroi* strain (M-1149) grown in potato dextrose broth (Merck KGaA, Darmstadt, Germany) was used for the artificial inoculation of the rice. Rice inoculation was carried out as previously described (Matić et al. 2013). Rice seeds of each cultivar were briefly surface-disinfected in 1% sodium hypochlorite and then rinsed in sterile distilled water. A set of 40 seeds was soaked in a 100-ml spore suspension in triplicate, and shaken for 30 min at room temperature. The control seeds were soaked in sterile distilled water. The seeds were sown in plastic pots in a sterile mixture of peat and sand (60%:40%) with three replicates, and the plants were grown in a greenhouse under controlled temperature conditions (25°C day:17°C night) with watering three times in 24 h. The germination rate and disease index were evaluated 3 weeks post-germination (wpg), using a five-point scale of the symptoms: (0) asymptomatic plants, (1) plants with yellow leaves, (2) plants with long internodes, (3) necrotic plants, and (4) dead plants (Amatulli et al. 2010). Culms with leaves, and roots were collected at 1 and 3 wpg for the molecular experiments. The DNA was extracted from 0.1 g of fresh plant material that was ground with liquid nitrogen. The powder resulting from the grinding was transferred into a 1.5-ml microcentrifuge tube, to which a 600 μl of P1 buffer of E.Z.N.A. Plant DNA Kit (Omega Bio-Tek) was added, and was then vortexed and incubated at 65°C for 10 min. Finally, the extracted DNA from rice tissues was eluted in a 30-μl elution buffer. The quality and concentration of DNA were measured as previously described.

Quantification of *F. fujikuroi*. Quantification of the pathogen in the plants was measured by extracting DNA from aerial and roots of six rice cultivars that showed different levels of susceptibility to *F. fujikuroi*. The DNA quantity of the target (from three technical replicates per sample) was used to calculate the approximate number

of *F. fujikuroi* cells present in each sample, through the following formula: number of cells/μl = DNA quantity/0.000048. The number 0.000048 refers to the weight of a *F. fujikuroi* genome expressed in ng (Jeong et al. 2013; Wiemann et al. 2013), and the number of cells to haploid *Fusarium* cells (Ruiz-Roldán et al. 2010). This formula allows the number of fungal cells per microliter of reaction mix to be found. However, the initial weight of the sample was also taken into consideration (e.g., 0.1 g), and the number of *F. fujikuroi* cells per milligram of rice tissue was therefore calculated by multiplying the cells by the volume of extracted DNA in microliters, and dividing the value by the initial weight of the rice sample (0.1 g).

Detection of *F. fujikuroi* from rice seeds. The TaqMan real-time PCR assay was conducted on naturally infected seed lots of the six tested rice cultivars. Each lot contained 1,000 seeds (divided in two technical replicates) and that quantity is higher than the standard working sample (400 seeds) for rice seed health testing (ISTA 1985; Mojica and Gomez 1994). The DNA extraction from the seeds was performed on a 0.1 g fraction of 1,000 seeds and ground with liquid nitrogen. The resulting powder was extracted with the E.Z.N.A. Plant DNA Kit, as previously described.

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

RESULTS

Probe and primer development. The *TEF 1-α* gene was chosen as a specific region, due to the deletion of six nucleotides that had previously only been found in *F. fujikuroi* (Amatulli et al. 2012). *TEF 1-α* sequences from different isolates of nine *Fusarium* spp. were aligned and three primer/probe sets were designed (Table 2). The combination of probe and primer sets was tested in quantitative PCR (qPCR), under different cycling conditions and probe/primer concentrations to evaluate their ability to specifically detect *F. fujikuroi*. In order to increase the stringency and to avoid false positive results, the annealing/extension temperature was optimized to 62°C. After examination of the amplification results, the FfujiPq probe and TqF2/TqR primer pair were selected as the best candidates for the TaqMan real-time PCR assay.

TaqMan probe specificity, selectivity, sensitivity, repeatability, and reproducibility. As far as the specificity of Taqman qPCR is concerned, the FfujiPq and TqF2/TqR probe/primer combination was able to repeatedly and reliably amplify the 84-bp DNA fragment from different strains of *F. fujikuroi*, but not from eight other species of *Fusarium* or from six additionally tested rice pathogens. The presence of plant and/or soil microbial DNA together with the fungal DNA did not influence the selectivity of the TaqMan probe (FfujiPq) in the qPCR assays.

TABLE 2. Primer and probe sets used in TaqMan PCR for *Fusarium fujikuroi* detection

Primers and TaqMan probe	Amplicon (nt)	Sequence (5'-3')
TqF2	84	GGCGCGTTTGTCCCTTTCCT
TqR		AGCGGCTTCTATTGTGCGAA
FfujiPq		[FAM]-TCACGTGTCAAACATAA-[NF-MGB]
TqF1	93	CGAGTGATGGGCGCGTTTGT
TqR		AGCGGCTTCTATTGTGCGAA
TaqFuji		[FAM]-CGCATTGTACGTGTCAAAC-[NF-MGB]
FfujiFq	116	CACGTGTCAAACATAAATTTCG
FfujiRq		GATGGTGATACACGCTCAC
BakanPq		[FAM]-GCCGTGAGCTCGGTAAGGG-[NF-MGB]

The detection limit and dynamic range of the qPCR reaction were determined from the standard curve using DNA extracted from a pure culture of three *F. fujikuroi* strains (14-22, 11-93, and M-1149). A standard regression line was generated for each DNA standard curve, using a range of DNA from 10 ng to 0.1 pg. High sensitivity was achieved over 5 orders of magnitude of DNA concentration that exhibited a linear dynamic range of amplification. *F. fujikuroi* DNA (from both the pure fungal cultures and rice tissues) was quantified accurately up to a concentration of 27.5 fg, with a C_T range of between 31 and 34. The detection limit of 27.5 fg of DNA is lower than a weight of a single haploid genome of *F. fujikuroi* (48 fg of DNA).

The correlation coefficients between the C_T value and the amount of genomic DNA of all three strains were high ($R^2 > 0.99$), and the regression slopes were -3.34 , -3.14 , and -3.12 (for the strains 11-93, 14-22, and M-1149, respectively). The corresponding slopes were within a slope range of between -3.58 and -3.10 , which approved good qPCR efficiency (between 90 and 110%; Adams, 2006). As expected, the three standard curves overlapped almost completely with a high degree of repeatability (Fig. 1) and the slight differences could be related to the DNA extraction that could be influenced by the secondary metabolite profile (Zhang et al. 2010). The assay was performed on various days by two different operators in two different laboratories in order to confirm its reproducibility.

Detection and quantification of *F. fujikuroi* in rice culms, leaves, and roots. One experiment was performed in vivo on six rice cultivars to evaluate the presence of *F. fujikuroi* on rice culms with leaves and roots at different points of time. At 1 wpg, none of the cultivars showed visible symptoms. The typical symptoms of bakanae, such as abnormal stem elongation and increase of the leaf insertion angle started to occur on the Dorella, Handao 297, and M6 rice cultivars later on. These latter cultivars showed the most severe symptoms at 3 wpg (Fig. 2), while most plants were withered and dead at 4 wpg, and showed drastically inhibited root growth. All the plants of the M6 cultivar were already dead at 3 wpg (Table 3). The Dorella, Handao 297, and M6 cultivars were considered susceptible. On the

contrary, the Selenio, Galdo, and Greppi cultivars did not show any typical bakanae symptoms throughout the whole experiment, and only a small number of yellowish plants could be observed (Fig. 2).

F. fujikuroi was detected at 1 wpg (asymptomatic period) by the TaqMan qPCR assay in both susceptible and resistant cultivars, and its presence was higher in the aerial parts than in the roots (Fig. 3). At 3 wpg, when the most notable bakanae symptoms occurred, the concentration was higher in both the aerial and root parts of the susceptible cultivars, while it was lower in the culms and leaves of the resistant cultivars (Fig. 3; Table 3). Interestingly, cultivars Handao 297 and Dorella exhibited slightly higher concentrations of *F. fujikuroi* in the aerial parts of the plant, while M6 exhibited a higher concentration in the roots. The highest concentration of *F. fujikuroi* was measured in the roots of the most susceptible cultivar M6, with around 1,450 fungal cells per gram of tissue (Fig. 3). The resistant cultivars (Greppi, Galdo, and Selenio) showed a much lower presence of *F. fujikuroi* (from 279 to 0.3 cells per gram of plant tissue), mainly in the aerial parts of the plants, than the susceptible cultivars, in the initial infection period.

Detection and quantification of *F. fujikuroi* in rice seeds.

The TaqMan qPCR assay was also used to detect and quantify *F. fujikuroi* in naturally contaminated rice seed lots of six rice cultivars. Mixed populations of *Fusarium* spp. could occur in rice seeds, but the TaqMan Ffujipq PCR amplification was specific and was therefore not influenced by other *Fusarium* species or by the presence of rice genomic DNA. This qPCR was sufficiently sensitive to detect 9.89 *F. fujikuroi* cells/g of naturally infected rice seed lots (Fig. 4). The *F. fujikuroi* DNA from the seeds was quantified precisely up to the same concentration of the other rice tissues (27.5 fg) and a C_T range between 34 and 36.

DISCUSSION

The method developed in this study has allowed a fast *F. fujikuroi* identification at the early stages of the disease, and a reliable

Fig. 1. Standard curve showing the quantification of genomic DNA of the M-1149 (accession no. HM243234), 14-22 (accession no. GQ848524), and 11-93 (accession no. GQ848522) strains of *Fusarium fujikuroi* (fg). The C_T values are plotted against *F. fujikuroi* DNA amounts. The linear regression equation and its R^2 value are shown in the graph.

Fig. 2. Pathogenicity tests on rice plants of six cultivars artificially inoculated with *Fusarium fujikuroi* at 3 weeks post-germination.

quantification from different rice materials, including the seeds, where the fungus is present at a lower concentration (Dieter 2002). It could be applied to DNA extracted directly from rice seeds or fresh plant tissues, and it is not affected by a nonspecific background from coextracted plant DNA, soil microbial DNA, or from other *Fusarium* spp. associated with bakanae disease or commonly present on rice (Desjardins et al. 2000; Wulff et al. 2010). The probe and primers were evaluated by amplifying the DNA from pure culture isolates of *Fusarium* spp., and the specificity was assessed by means of a specific amplification of DNA from *F. fujikuroi*, and not from other *Fusarium* spp. The method was also tested on six common pathogenic species of rice, without any amplification signal. Most of the tested *Fusarium* spp. are members of the GFSC and can be distinguished morphologically from *F. fujikuroi* according to the microconidia production and absence of chlamydospores. However, other *Fusarium* species, such as *F. proliferatum* and *F. verticilloides*, would need expertise in *Fusarium* taxonomy to be differentiated from *F. fujikuroi* (Leslie et al. 2004). The increased stringency of the amplification reaction developed in the present study, which was obtained by increasing the annealing temperature to 62°C, avoided the problem of cross-amplification and allowed *F. fujikuroi* to be specifically detected.

TABLE 3. Disease index and germination of six rice cultivars inoculated (+) or not inoculated (–) with *Fusarium fujikuroi* at 3 weeks post-germination^a

Cultivar ^b	Disease index (%)	Germination (%)
Selenio +	7.5	93.2
Selenio –	0.0	95.8
Graldo +	7.5	87.7
Graldo –	0.0	96.7
Greppi +	12.5	83.3
Greppi –	0.0	89.2
Dorella +	87.5	40.2
Dorella –	0.0	95.4
Handao 297 +	72.5	55.0
Handao 297 –	0.0	98.3
M6 +	100.0	41.1
M6 –	17.2	90.4

^a Forty seeds per variety were tested in three replicates per treatment. The experiments were carried out three times.

^b Three resistant (Selenio, Graldo, and Greppi) and three susceptible (Dorella, Handao 297, and M6) rice cultivars were evaluated.

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

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

After optimizing the PCR conditions, in order to achieve the highest specificity, the protocol was implemented for quantitative purposes. After the quantification of *F. fujikuroi* DNA from a pure culture, the utility of the quantification protocol was checked in different rice tissues. It was found that the sensitivity of the qPCR protocol was not affected by the nature of the sample, since the fungus could be detected and quantified in complex naturally infected matrices of diverse origin, including different plant tissues and soil residues. Our results showed fungal presence in both the aerial tissues and roots of infected rice plants, without any visible symptoms during the early stage of bakanae disease. Furthermore, a successful amplification of *F. fujikuroi* was obtained by TaqMan qPCR from naturally infected rice seeds with a high starch level (one of the main polysaccharides), insoluble phenolic compounds, and other interfering substances (Tian et al. 2004; Wang et al. 2012). Polysaccharides can be especially troublesome inside DNA extracts, since their presence is inhibitory to *Taq* polymerase activity (Fang et al. 1992). Polyphenolic compounds may bind to DNA during cell lysis, and may be recalcitrant to further enzymatic manipulations (John 1992). In the present study, no inhibition of *Taq* DNA polymerase was observed after the application of the developed TaqMan real-time PCR assay. Hence, the purity of the DNA extracts from rice seeds obtained with the E.Z.N.A. DNA Kit had a higher $A_{260/280}$ ratio than 1.8 and a higher $A_{260/230}$ ratio than 2, thus indicating little or no DNA contamination by proteins, polyphenolics, or polysaccharides. Moreover, it was above the amenable DNA purity limit for molecular studies (Sambrook and Russell 2001).

Fig. 3. Quantification of *Fusarium fujikuroi* with TaqMan real-time PCR in rice culms with leaves, and roots from six cultivars at 3 weeks post-germination. The values are the means $\pm$ standard deviation ($n = 9$ independent analyses).

Fig. 4. Quantification of *Fusarium fujikuroi* with TaqMan real-time PCR on naturally contaminated seed lots of six rice cultivars. The values are the means $\pm$ standard deviation ($n = 9$ independent analyses).

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

The most abundant presence of *F. fujikuroi* during disease development was found in the roots and green tissues of the highly susceptible M6 cultivar, while the pathogen was present at lower concentrations in the roots of the other two susceptible cultivars. The higher fungal presence in M6 cultivar could be associated with a natural infection with *F. fujikuroi* (17.2%) in the M6 seed lot, which was not found in the other tested cultivars. At 1 wpg, the pathogen was found to be more diffused in the green tissues and in the roots to a lesser extent, while at 3 wpg the fungus was uniformly spread in the plant tissues and the difference between the roots and green tissues became negligible. The high presence of *F. fujikuroi* in the roots of rice plants should be considered to obtain more detailed knowledge on the epidemiology of bakanae disease. The results of the fungal quantification in this study match the results of a transcriptomic study of two cultivars (Dorella and Selenio) infected with *F. fujikuroi* (Matić et al. 2016), which indicated a higher presence of *F. fujikuroi* and its systemic distribution throughout the whole rice plants of susceptible cultivar Dorella. On the other hand, a lower concentration and local (aerial) distribution of *F. fujikuroi* was found in the resistant Selenio cultivar, without any visible damage to the plants.

In this study, it was possible to quantify *F. fujikuroi* DNA accurately over a large range of concentrations, using real-time PCR in complex biological matrices, including naturally contaminated and artificially inoculated rice samples of diverse origin, in which various microorganisms may coexist with the target species. In the case of naturally infected seeds, it is not possible to accurately distinguish the target pathogen from other *Fusarium* spp. associated with bakanae disease through the exclusive use of discriminative techniques, such as plating on culture media. Such a procedure is time consuming and delays the identification of *F. fujikuroi* until detailed microscopic observations of the morphological traits have been made (Munkvold 2009). Consequently, the

qPCR protocol developed to quantify *F. fujikuroi* may be considered a useful tool for the early detection of *F. fujikuroi* in rice seeds. The treatment of rice seeds is currently the most valuable tool to control bakanae disease. For small quantities, seed companies could choose to use thermal treatments with hot water (60 to 65°C for 15 min) or chemical treatments (Matić et al. 2014; Park et al. 2008). A chemical treatment is commonly used for larger seed quantities, but in recent years, the most important active ingredient (prochloraz) has been withdrawn from the market, and the effectiveness of other fungicides is not satisfactory. Therefore, it is of utmost importance to take into consideration possible alternatives (Matić et al. 2014). Seed companies could use this molecular tool before and after seed treatments against *F. fujikuroi* in order to test the efficacy of the treatments and to certify that the seed lots are free from *F. fujikuroi*.

ACKNOWLEDGMENTS

The research that has led to these results has received funding from the Seventh Framework Program (FP7/2007-2013) under Grant Agreement No 311875 “Seed health: development of seed treatment methods, evidence for seed transmission and assessment of seed health” (TESTA) and from the European Union Horizon 2020 research and innovation program under grant agreement number 634179 “Effective Management of Pests and Harmful Alien Species-Integrated Solutions” (EMPHASIS).

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