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

PHYTOPATHOLOGY

Techniques

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

Greice Amaral Carneiro[1*], Slavica Matić[1*], Giuseppe Ortu[1], Angelo Garibaldi[1], Davide Spadaro[1,2**], and Maria Lodovica Gullino[1,2]

[1]*AGROINNOVA – Centre of Competence for the Innovation in the Agro-environmental Sector, Università di Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy.*

[2]*Dept. Agricultural, Forestry and Food Sciences (DISAFA), Università di Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy.*

* These authors equally contributed to the work.

**Corresponding author.

ABSTRACT

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

Introduction

Bakanae is a rice seedborne disease, which poses a serious threat for 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 the bakanae tillers, which are highly elongated and can be observed at 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 to other plants. Severely infected seedlings show necrotic root tissue, and frequently die either before or after transplanting (Misra et al. 1994). Since the first report of bakanae in Japan (1928), the disease 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 increased in Italy, the major European rice producer (Spadaro et al. 2016). The 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 consist in the application of fungicides, physical treatments, biocontrol agents, antimicrobial plant extracts, and essential oils (Tinivella et al. 2009). All of these methods were reported for *F. fujikuroi* control on rice seeds (Matić et al. 2014; Titone et al. 2004). Despite recent development of new control methods, the management of bakanae disease remains difficult, due to its wide geographical distribution and increasing incidence. The reduction in pesticide availability and the occurrence of fungicide resistance increased the disease severity in Europe (Matić et al. 2016; Spadaro et al. 2016), with serious issues for the seed companies which need to supply the growers with certified rice seeds free from *F. fujikuroi*.

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

(Leslie et al. 2006). There are limitations based on microscope discrimination of *Fusarium* spp. within the GFSC, including *F. fujikuroi*, *Fusarium proliferatum*, *Fusarium verticillioides* and *Fusarium andiyazi*, due to very similar morphological traits (O'Donnell et al. 1998). Accurate detection and identification of *F. fujikuroi* is necessary in the management of bakanae disease and in the evaluation of rice resistance germplasm. Like other disease assessments, the evaluation of bakanae disease is dependent 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 initial stages of disease, and for fungal quantification in infected rice tissues. Pathogen detection based on symptomatology and morphological criteria is possible at later stages, which might result in ineffective disease control and further spread 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 diagnosis of several plant pathogens (Deepak et al. 2007; Mirmajlessi et al. 2015). It has 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: (1) DNA-binding dyes such as SYBR green I; (2) hydrolysis or TaqMan probes, which exploits the 5′-exonuclease activity of *Taq* DNA polymerase; or (3) hybridization probes, which utilize donor and acceptor fluorophores (Valasek and Repa 2005). Although, real time PCR using a SYBR green dye was previously developed for detection of *F. fujikuroi* from different rice tissues (Amatulli et al. 2012), it did not result in quantitative and reliable detection of *F. fujikuroi* from seeds, which prompted for 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 assay was validated determining sensitivity, specificity, selectivity, repeatability and reproducibility. The relationship between symptom

progression and *F. fujikuroi* occurrence was explored in different rice tissues, including 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., and six isolates of two common rice pathogens (*Rhizoctonia solani* and *Magnaporthe oryzae*) were used in this study (Table 1). All the isolates were from the Agroinnova collection (University of Torino, Italy), with exception of *F. fujikuroi* M-1149 (Fusarium Research Centre, Pennsylvania State University, USA), *F. proliferatum* ITEM1720, and *F. verticillioides* ITEM1746 (Institute of Sciences and of Food Production, Bari, Italy). Fungal isolates were long-term stored at -80°C in 20% glycerol.

DNA extraction from fungal cultures. Total DNA of all fungal species was extracted with the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Darmstadt, Germany) from 0.1 g of mycelium grown on potato dextrose agar (PDA, Merck KGaA) following the manufacturer's instructions. The quality and concentration of DNA were measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE), and 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 *TEF 1-α* gene 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) were selected and aligned. The portions of the sequences specific to *F. fujikuroi*, such as those including the six-nucleotides deletion, were used to select potential primers

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

Real time PCR optimization. Real time PCR assay was performed in a 20-µl reaction in triplicates. Various concentrations of primers (0.2-0.8 µM) and probe (0.1-0.3 µM) were tested in different combinations. Amplification and detection were performed in 96-well Optical Reaction Plates (Applied Biosystems) sealed with MicroAmp^{TM} Optical Adhesive Film, on StepOnePlus Real Time PCR system (Applied Biosystems). Optimized real time PCR mixture and conditions consisted in the application of TaqMan Master Mix (2×) (Applied Biosystems), two primers at 0.5 µM each, TaqMan probe at 0.25 µM, and 2 µl DNA with an incubation at 50°C for 2 min and 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 *R. solani* and *M. oryzae* 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 for presence or absence of amplification signal. The potential influence of the matrix, including 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, 27.5 fg). Each sample

was amplified in triplicates, and the entire experiment was repeated eight times (EPPO 2014). The experiments were reproduced in two independent laboratories.

Detection of *F. fujikuroi* from rice plants. Six cultivars of rice (*Oryza sativa* L.; 'Selenio', 'Graldo', 'Greppi', 'Handao 297', 'M6', and 'Dorella') were selected for *F. fujikuroi* detection. For the artificial inoculation of rice, a conidial suspension (10^6 conidia/ml) of highly pathogenic *F. fujikuroi* strain M-1149 grown in potato dextrose broth (PDB, Merck KGaA, Darmstadt, Germany) was used. 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 group of 40 seeds were soaked in a 100 ml-spore suspension in triplicates, and shaken for 30 min at room temperature. 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%) in three replicates, and the plants were grown in greenhouse under controlled temperature conditions (25°C day:17°C night) with 3-times watering per 24 h. Germination rate and disease index were evaluated 3 weeks post germination (wpg) using a scale of five distinct groups of 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). For molecular experiments, culm with leaves, and roots were collected at 1 and 3 wpg. DNA extraction from 0.1 g of fresh plant material was ground with liquid nitrogen. The resulting powder after grinding was transferred into a 1.5 ml microcentrifuge tube, where 600 µl P1 buffer of E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek) was added, and then it was vortexed and incubated at 65°C for 10 min. Extracted DNA from rice tissues was finally eluted in 30 µl elution buffer. The quality and concentration of DNA were measured as previously described.

Quantification of *F. fujikuroi*. Pathogen quantification in plants was measured by extracting DNA from aerial and underground parts of six rice cultivars, which showed different levels of

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

$$\text{Number of cells/µl} = \text{DNA quantity}/0.000048$$

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

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

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

Results

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

primers/probe were designed (Table 2). Combination of probe and primers sets were tested in quantitative PCR (qPCR) with different cycling conditions and probe/primers concentrations to evaluate the ability to specifically detect *F. fujikuroi*. To increase the stringency and to avoid false positive results, the temperature of annealing/extension was optimized at 62°C. After examination of amplification results, probe FfujiPq and primer pair TqF2 and TqR were selected as the best candidates for the TaqMan real time PCR assay.

TaqMan probe specificity, selectivity, sensitivity, repeatability, and reproducibility. Regarding specificity of Taqman qPCR, the probe/primers combination (FfujiPq and TqF2/TqR) repeatedly and reliably amplified the 84-bp DNA fragment from different strains of *F. fujikuroi*, while not from eight other species of *Fusarium* and two additionally tested rice pathogens. The presence of plant or soil microbial DNA together with the fungal DNA did not influence the selectivity of the TaqMan probe (FfujiPq) in qPCR assays.

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). 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 five orders of magnitude of DNA concentration, which exhibited a linear dynamic range of amplification. *F. fujikuroi* DNA (from both pure fungal cultures and rice tissues) was accurately quantified until the concentration of 27.5 fg with a threshold cycle (C_T) range between 31-34. The detection limit of 27.5 fg DNA is lower than a single haploid genome of *F. fujikuroi* (48 fg DNA).

The correlation coefficients between the Ct value and the amount of genomic DNAs of all the three strains were high (R^2 >0.99), and the regression slopes were -3.34, -3.14 and -3.12, respectively. Corresponding slopes were within a slope range between -3.58 and -3.10, that approved good qPCR efficiency (between 90% and 110%; Adams, 2006). As expected, the three standard curves almost completely overlapped with a high degree of repeatability (Fig. 1).

The assay was performed on different days by two operators in two laboratories, confirming the 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 time points. At 1 wpg, all cultivars did not show visible symptoms. Later on, typical symptoms of bakanae started to occur on rice cultivars 'Dorella', 'Handao 297' and 'M6', such as abnormal stem elongation, and increase of the leaf insertion angle. At 3 wpg (Fig. 2), these latter cultivars showed the most severe symptoms, while at 4 wpg most plants were withered and mainly dead, with drastically inhibited root growth. All the plants of cultivar 'M6' already died at 3 wpg (Table 3). The cultivars 'Dorella', 'Handao 297' and 'M6' were considered susceptible. On the contrary, the three cultivars 'Selenio', 'Graldo', and 'Greppi' did not show typical bakanae symptoms during the whole experiment, and only a small proportion of yellowish plants could be observed (Fig. 2).

At 1 wpg (asymptomatic period), *F. fujikuroi* was detected by TaqMan qPCR assay both in susceptible and resistant cultivars, and its presence was higher in aerial parts compared to roots (Fig. 3). At 3 wpg, when the most striking bakanae symptoms occurred, the concentration was the highest in both aerial and root parts of the susceptible cultivars, while it was lower in culms and leaves of resistant cultivars (Fig. 3 and Table 3). Interestingly, 'Handao 297' and 'Dorella' exhibited a slightly higher concentration of *F. fujikuroi* in the aerial parts of the plant, while 'M6' 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). Resistant cultivars ('Greppi', 'Graldo', and 'Selenio') showed much less presence of *F. fujikuroi* (from 279 to 0.3 cells per gram of the plant tissue), mainly in aerial plant parts compared to the susceptible cultivars and to 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 seeds lots of six rice cultivars. On rice seeds, mixed populations of *Fusarium* spp. might occur, but the amplification with the TaqMan FfujiPq probe was specific and not influenced by the other *Fusarium* species or with the presence of rice genomic DNA. This qPCR was sensitive enough to detect 9.89 *F. fujikuroi* cells/g of naturally infected rice seed lots (Fig. 4). *F. fujikuroi* DNA from seeds was precisely quantified until the same concentration of other rice tissues (27.5 fg) and a C_T range between 34-36.

Discussion

The method developed in this study allowed a fast *F. fujikuroi* identification at early stages of the disease, and reliable quantification from different rice materials including the seeds where the fungus is present at lower concentration (Dieter 2002). It could be applied on DNA extracted directly from rice seeds or fresh plant tissues, and it is not affected by non-specific background from co-extracted 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 DNA from pure culture isolates of *Fusarium* spp., and the specificity was assessed by specific amplification of DNA from *F. fujikuroi,* and not from other *Fusarium* spp. In addition, the method was also tested on three strains for two pathogenic species of rice *M. oryzae* and *R. solani*, without amplification signal. Most of the tested *Fusarium* spp. are members of the GFSC and can morphologically be distinguished from *F. fujikuroi* based on 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 amplification reaction developed in our study by increasing the annealing temperature to 62°C avoided the problem of cross-amplification and allowed specific *F. fujikuroi* detection. Additionally, sensitivity

was not impaired by the increased stringency permitting the detection up to 27.5 fg of *F. fujikuroi* in both 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 binds nonspecifically any double-stranded DNA which is present in real time PCR reaction, our protocol was based on a more specific TaqMan probe. Additionally, the *Taq* DNA polymerase can be inhibited by SYBR Green, which limits the fluorescence signal and the assay sensitivity (Kermekchiev et al. 2009). That was also avoided 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 to achieve the highest specificity, the protocol was implemented for quantitative purposes. Followed the quantification of *F. fujikuroi* DNA from pure culture, the utility of the quantification protocol was checked in rice different tissues. We showed 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 the fungal presence in both aerial and underground tissues of infected rice plants without 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 containing 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 the DNA extracts, since their presence is inhibitory on *Taq* polymerase activity (Fang et al. 1992). Polyphenolic compounds may bind to DNA during the cell lysis and result recalcitrant to further enzymatic manipulations (John 1992). In our study, we did not find the inhibition of *Taq* DNA polymerase by the application of developed TaqMan real time PCR assay. Hence, the purity of the DNA extracts from rice seeds 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,

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

We found that the TaqMan real time PCR using 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 targeting other *Fusarium* spp. such as *Fusarium 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 compared to the detection limit of 10 pg of *F. fujikuroi* DNA from rice obtained by the SYBR Green real time PCR assay (Amatulli et al. 2012).

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

In this study, *F. fujikuroi* DNA could be accurately quantified over a large range of concentrations using real time PCR in complex biological matrices, including naturally contaminated and artificially inoculated rice samples from diverse origin, where 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, by exclusive use of discriminative techniques, such as plating on culture media. Such procedure is tedious and delays the identification of *F. fujikuroi*, until detailed microscopic observations of morphological traits are made (Munkvold 2009). Consequently, the availability of the developed qPCR protocol for quantifying *F. fujikuroi* may be a useful tool for early detection of *F. fujikuroi* in rice seeds. Rice seed treatment is currently the most valuable tool in order to control bakanae disease. For small quantities, the seed companies can choose to use thermal treatments with hot water (60-65°C for 15 minutes) or chemical treatments (Matić et al. 2014; Park et al. 2008). For greater seed quantities, a chemical treatment is commonly used, but in the last years, the most important active ingredient – prochloraz – was withdrawn from the market and the effectiveness of other fungicides is not satisfying. Therefore, it is of crucial importance to take in consideration possible alternatives (Matić et al. 2014). Seed companies could use this molecular tool before and after seed treatment against *F. fujikuroi* in order to test the efficacy of the treatment and to certify successfully the seed lots as free from *F. fujikuroi*.

Acknowledgments

The research leading 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 No 634179 "Effective Management of Pests and Harmful Alien Species - Integrated Solutions" (EMPHASIS).

Literature cited

Adams, P. S. 2006. Data analysis and reporting. Pages 39-62 in: Real-time PCR. M. Tevfik Dorak, ed. Taylor & Francis, New York.

Amatulli, M. T., Spadaro, D., Gullino, M. L., and Garibaldi, A. 2010. Molecular identification of *Fusarium* spp. associated with bakanae disease of rice in Italy and assessment of their pathogenicity. Plant Pathol. 59:839-844.

Amatulli, M. T, Spadaro, D., Gullino, M. L., and Garibaldi, A. 2012. Conventional and real-time PCR for the identification of *Fusarium fujikuroi* and *Fusarium proliferatum* from diseased rice tissues and seeds. Eur. J. Plant Pathol. 134:401–408.

Capote, N., Pastrana, A. M., Aguado, A., and Sánchez-Torres, P. 2012. Molecular tools for detection of plant pathogenic fungi and fungicide resistance. Pages 151-202 in: Plant pathology. C. J. Cumagun, Ed., InTech.

Carter, L. L. A., Leslie, L. F., and Webster, R. K. 2008. Population structure of *Fusarium fujikuroi* from California rice and water grass. Phytopathology 9:992-998.

Deepak, S. A., Kottapalli, K. R., Rakwal, R., Oros, G., Rangappa, K. S., Iwahashi, H., Masuo, Y., and Agrawal, G. K. 2007. Real-Time PCR: Revolutionizing Detection and Expression Analysis of Genes. Curr. Genomics 8:234-251.

Desjardins, A. E., Plattner, R. D., and Nelson, P. E. 1997. Production of fumonisin B1 and moniliformin by *Gibberella fujikuroi* from rice from various geographic areas. Appl. Environ. Microbiol. 63:1838-1842.

Desjardins, A. E., Manhanadhar, H. K., Plattner, R. D., Manandhar, G. G., Poling, S. M., and Maragos, C.M. 2000. *Fusarium* species from Nepalese rice and production of mycotoxins and gibberellic acid by selected species. Appl. Environ. Microbiol. 66:1020-1025.

Dieter, K. 2002. Quantification using real-time PCR technology: applications and limitations. Trends Mol. Med. 8:257-260.

EPPO. 2014. PM 7/98 (2) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. EPPO Bulletin 44:117-114.

Fang, G., Hammar, S., and Grumet, R. 1992. A quick and inexpensive method for removing polysaccharides from plant genomic DNA. Biotechniques 13:52-54.

Gupta, A. K., Solanki, I. S., Bashyal, B. M., Singh, Y., and Srivastava, K. 2015. Bakanae of rice - an emerging disease in Asia. J. Anim.Plant Sci. 25:1499-1514.

Hwang, I. S., Kang, W. R., Hwang, D. J., Bae, S. C., Yun, S. H., and Ahn, I. P. 2013. Evaluation of bakanae disease progression caused by *Fusarium fujikuroi* in *Oryza sativa* L. J. Microbiol. 51:858-865.

Jeon, Y. A., Yu, S. H., Lee, Y. Y., Park, H. J., Lee, S., Sung, J. S., Kim, Y. G., and Lee, H. S. 2013. Incidence, molecular characteristics and pathogenicity of *Gibberella fujikuroi* species complex associated with rice seeds from Asian countries. Mycobiology 41:225-233.

Jeong, H., Lee, S., Choi, G. J., Lee, T., and Yun. S. H. 2013. Draft genome sequence of *Fusarium fujikuroi* B14, the causal agent of the bakanae disease of rice. Genome Announc. 1:e00035–13.

John, M. E. 1992. An efficient method for isolation of RNA and DNA from plants containing polyphenolics. Nucleic Acids Res. 20(9):2381.

Karov, I. K., Mitrev, S. K., and Kostadinovska, E. D. 2009. *Gibberella fujikuroi* (Sawada) Wollenweber, the new parasitical fungus on rice in the Republic of Macedonia. Proc. Nat. Sci. Matica Srpska Novi Sad 116:175-182.

Kermekchiev, M. B., Kirilova, L., Vail, E. E., and Barnes, W. M. 2009. Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. Nucleic Acids Res. 37:e40.

Leslie, J. F., Zeller, K. A., Wohler, M., and Summerell, B. A. 2004. Interfertility of two mating populations in the *Gibberella fujikuroi* species complex. Eur. J. Plant Pathol. 110:611-618.

Leslie, J. F., and Summerell, B. A., eds. 2006. The *Fusarium* laboratory manual. Oxford, UK.

Lin, Z., Xu, S., Que, Y., Wang, J., Comstock, J. C., Wei, J., McCord, P. H., Chen, B., Chen, R., and Zhang, M. 2014. Species-specific detection and identification of fusarium species complex, the causal agent of sugarcane pokkah boeng in China. PLoS One 9:e104195.

Matić, S., Spadaro, D., Prelle, A., Gullino, M. L., and Garibaldi, A. 2013. Light affects fumonisin production in strains of *Fusarium fujikuroi*, *Fusarium proliferatum*, and *Fusarium verticillioides* isolated from rice. Int. J. Food Microbiol. 166:515-523.

Matić, S., Spadaro, D., Garibaldi, A., and Gullino, M. L. 2014. Antagonistic yeasts and thermotherapy as seed treatments to control *Fusarium fujikuroi* on rice. Biol. Cont. 73:59-67.

Matić, S., Bagnaresi, P., Biselli, C., Orru', L., Amaral Carneiro, G., Siciliano, I., Valé, G., Gullino, M.L., and Spadaro, D. 2016. Comparative transcriptome profiling of resistant and susceptible rice genotypes in response to the seedborne pathogen *Fusarium fujikuroi*. BMC Genomics 17:608.

Mirmajlessi, S. M., Loit, E., Mänd, M., and Mansouripour, S. M. 2015. Real-time PCR applied to study on plant pathogens: potential applications in diagnosis - a review. Plant Protect. Sci. 4:177-190.

Misra, J. K., Merca, S. D., and Mew, T. W. 1994. Fungal pathogens. Pages 75-89 in: A manual of rice seed health testing. T. W. Mew, and J. K. Misra, eds. IRRI, Philippines.

Munkvold, G. P. 2009. Seed pathology progress in academia and industry. Annu. Rev. Phytopathol. 47:285-311.

O'Donnell, K., Cigelnik, E., and Nirenberg, H. I. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90:465-493.

Ou, S. H., ed. 1985. Rice Diseases. Commonwealth. Mycological Institute, Kew, UK.

Park, W. S., Yeh, W. H., Lee, S. W., Han, S. S., Lee, J. S., Lim, C. K., and Lee, Y. H. 2008. Electron microscopic study for the influence of soaking in hot water and prochloraz solution on spore and mycelium of *Fusarium fujikuroi* infected in rice seed. Res. Plant Dis. 15:94-100.

Sambrook, J., and Russell, D. W., eds. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, NY, USA.

Smith, C. J., and Osborn, A. M. 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol. Ecol. 67:6-20.

Spadaro, D., Gullino, M. L., and Garibaldi, A. 2016. Recenti acquisizioni sulla malattia del riso provocata da *Fusarium fujikuroi*. Protezione delle Colture 9 (2):11-17.

Stowe, B. B., and Yamaki, T. 1957. The history and physiological action of the gibberellins. Annu. Rev. Plant. Physiol. 8:181-216.

Tian, S., Nakamura, K., and Kayahara, H. 2004. Analysis of phenolic compounds in white rice, brown rice, and germinated brown rice. J. Agric. Food Chem. 52:4808-4813.

Tinivella, F., Hirata, L. M., Celan, M. A., Wright, S. A. I., Amein, T., Schmitt, A., Koch, E., van der Wolf, J. M., Groot, S. P. C., Stephan, D., Garibaldi, A., and Gullino, M. L., 2009. Control of seed-borne pathogens on legumes by microbial and other alternative seed treatments. Eur. J. Plant Pathol. 123:139-151.

Titone, P., Grassi, G., Polenghi, G., Tamborini, L., and Garibaldi, A. 2004. Concia del riso: prodotti chimici e termoterapia a confronto. Informatore Fitopatologico 54 (5):41-49.

Valasek, M. A., and Repa, J. J. 2005. The power of real-time PCR. Adv. Physiol. Educ. 29:151-159.

Waalwijk, C., van der Heide, R., de Vries, I., van der Lee, T., Schoen, C., Costrel-de Corainville, G., Häuser-Hahn, I., Kastelein, P., Köhl, J., Lonnet, P., Demarquet, T., and Kema G. H. J. 2004. Quantitative detection of *Fusarium* species in wheat using TaqMan. Eur. J. Plant Pathol. 110:481-494.

Wang, G., Wang, G., Zhang, X., Wang, F., and Song, R. 2012. Isolation of high quality RNA from cereal seeds containing high levels of starch. Phytochem. Anal. 23:159-163.

Wang, J,, Jacobs, J. L., Byrne, J. M., and Chilvers, M. I. 2015. Improved diagnoses and quantification of *Fusarium virguliforme*, causal agent of soybean sudden death syndrome. Phytopathology 105:378-387.

Webster, R. K., and Gunnell P. S., eds. 1992. Compendium of rice diseases. The American Phytopathological Society Press, St. Paul, Minnesota, USA.

Wiemann, P., Sieber, C. M. K., von Bargen, K. W., Studt, L., Niehaus, E., Espino, M., J. J., Huß, K., Michielse, C. B., Albermann, S., Wagner, D., Bergner, S. V., Connolly, L. R., Fischer, A., Reuter, G., Kleigrewe, K. Bald, T., Wingfield, B. D., Ophir, R., Freeman, S., Hippler, M., Smith, K. M., Brown, D. W., Proctor, R. H., Münsterkötter, M., Freitag, M., Humpf, H. U., Güldener, U., and Tudzynski, B. 2013. Deciphering the Cryptic Genome: Genome-wide Analyses of the Rice Pathogen *Fusarium fujikuroi* Reveal Complex Regulation of Secondary Metabolism and Novel Metabolites. PLOS Pathog. 9:e1003475.

Wulff, E. G., Sørensen, J. L., Lübeck, M., Nielsen, K. F., Thrane, U., and Torp, J. 2010. *Fusarium* spp. associated with rice Bakanae: ecology, genetic diversity, pathogenicity and toxigenicity. Environ. Microbiol. 12:649–657.

TABLES

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)

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]-TCACGTGTCAAACTAAA-[NF-MGB]
TqF1	93	CGAGTGATGGGCGCGTTTTG
TqR		AGCGGCTTCCTATTGTCGAA
TaqFuji		[FAM]-CGCATTGTCACGTGTCAAAC-[NF-MGB]
FfujiFq	116	CACGTGTCAAACTAAACATTCG
FfujiRq		GATGGTGATACCACGCTCAC
BakanPq		[FAM] – GCCGCTGAGCTCGGTAAGGG -[NF-MGB]

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

[a] Forty seeds per variety were tested in three replicates per treatment. 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.