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Development of loop-mediated isothermal amplification assays for the detection of seedborne fungal pathogens, *Fusarium fujikuroi* and *Magnaporthe oryzae*, in rice seeds

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ABSTRACT

Bakanae disease (caused by *Fusarium fujikuroi*) and rice blast (caused by *Magnaporthe oryzae*) are two of the most important seedborne pathogens of rice. The detection of both pathogens in rice seed is necessary to maintain high quality standards and avoid production losses. Currently, blotter tests are used followed by morphological identification of the developing pathogens to provide an incidence of infection in seed lots. Two loop-mediated isothermal amplification (LAMP) assays were developed with primers designed to target the elongation factor 1-alpha sequence of *F. fujikuroi* and the calmodulin sequence of *M. oryzae*. The specificity, sensitivity, selectivity, repeatability and reproducibility for each assay was assessed in line with the international validation standard published by EPPO (PM7/98). The results showed a limit of detection of 100-999 fg DNA of *F. fujikuroi* and 10-99 pg *M. oryzae* DNA. When combined with a commercial DNA extraction kit, the assays were

demonstrated to be effective for use in detection of the pathogens in commercial batches of infected rice seed of different cultivars, giving results equivalent to the blotter method, thus demonstrating the reliability of the method for the surveillance of *F. fujikuroi* and *M. oryzae* in seed testing laboratories.

INTRODUCTION

Cereals represent 40% of the global crop yield (tons) (Oerke and Dehne 2004), and rice is second only to wheat in the amount produced. Rice feeds over half the world's population and supplies 70% of their daily calories, especially in Asia (Delseny et al. 2001), where it is a staple crop for over 3 billion people.

The number of organisms that threaten rice production is extremely high, and the maintenance of high-quality rice seed is therefore necessary to secure food availability and reach high quality standards (Teng 1994). Until now, the treatments against seed-borne pathogens have been carried out using fungicides that allow pathogens to be eradicated or reduced, especially on commercial seeds where healthy certified seeds, free from pathogens, are required.

Seedborne plant pathogens can cause important yield and quality losses as well as being an unnoticed source of pathogen spread and dissemination (Du Toit, 2004).

Magnaporthe oryzae (T.T. Hebert) M.E. Barr *(Pyricularia oryzae* Cavara, *anamorph)* is a hemibiotrophic pathogen and the causal agent of rice blast and leaf spot in over 50 grass species (Ou 1985). Rice blast is the most destructive rice disease, and it is endemic in all rice growing areas (Manandhar et al. 1998). The high adaptability of rice to different weather conditions makes it ideal for cultivation in different parts of the world including Asia, Africa and America as well as Mediterranean countries such as Italy, Spain, and Portugal (World Rice Production 2017/2018). European countries are characterized by unfavorable weather conditions for the development of blast disease in the dry season, but at the end of the season, long periods of wet weather can favour panicle infection, blast development and related yield losses (Chataigner, 1996). *M. oryzae* is widespread in

rice seed, as indicated by recent analyses performed on seeds of different rice varieties (Sun *et al.*, 2015).

Fusarium fujikuroi Nirenberg [teleomorph *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura] is both a seed and a soil-borne pathogen, and it is the causal agent of bakanae disease. *F. fujikuroi* was initially described as *Lisea fujikuroi* Sawada, in 1919, and later renamed *Gibberella fujikuroi* by Sun and Snyder (1981). *Fusarium fujikuroi* is part of the *Gibberella fujikuroi* species complex (GFSC), which is formed by monophyletic lineages with anamorphs in *Fusarium* spp. (O'Donnell and Cigelnik 1997). Many of the species within this complex are pathogenic and important producers of secondary metabolites, including phytohormones and mycotoxins (Siciliano *et al.*, 2015). Bakanae has become more widespread over the years, threatening rice producing countries such as Japan, Korea, Thailand, Taiwan, India, the Philippines, Italy, Portugal, Spain, and the USA (Bashyal et al. 2014). Under favorable conditions for the disease, bakanae can cause yield losses up to 10-20 %. Severe infections, due to secondary infection through conidia dissemination, can reach 70% of rice plants (Ito and Kimura 1931; Ou 1985). Chemical seed dressing has been the most common way of controlling fungal diseases in rice (Zhou et al. 1994), however, with the reduction in fungicide usage in the EU, the incidence of some rice diseases has increased and it can now be difficult to source pathogen free certified seed (Matic et al. 2017).

In order to achieve faster pathogen identification, nucleic acid-based methods have become widespread. Typically molecular methods have high specificity and sensitivity and are much quicker to perform than morphological identification using microscopy (Boonham et al. 2008). Specific primers and probes for end-point and real-time PCR (TaqMan) assays have been developed by Amatulli *i*(2012) and Amaral Carneiro et al. (2017), designed to target the 1-alpha elongation factor (EF-1α) region to detect *F. fujikuroi*. while a real-time PCR (TaqMan) assay for *M. oryzae,* designed to amplify a unigene scytolone dehydratese has been developed by Su'udi et al. (2013).

Loop-mediated isothermal amplification (LAMP) is a DNA amplification method that can be used to amplify nucleic acid in a target specific way without the need for thermal cycling (Notomi et al. 2000;

78 Nagamine et al. 2001). LAMP uses enzymes that are less affected by compounds that inhibit PCR
79 reactions and as a result can be easier and quicker to perform than PCR based methods as complex
80 DNA extraction is not required. The enzymes tend to copy faster than PCR and the lack of thermal-
81 cycling means reactions can be run on hand-held, battery-powered platforms such at the Genie III
82 (Optigene, Horsham, UK). Taken together these characteristics identify LAMP as being well suited
83 to diagnostic use and also facilitates the potential use of LAMP in remote locations such as seed
84 stores, pack houses or directly in the field as an alternative to sending samples to a centralized testing
85 laboratory. The LAMP method has been demonstrated for the detection of bacteria (Hodgetts et al.
86 2015), fungi (Tomlinson et al. 2010a), phytoplasma (Hodgetts et al. 2011) and viruses (Tomlinson et
87 al. 2013). The LAMP product can be visualized by means of gel electrophoresis, by means of
88 magnesium pyrophosphate precipitation, turbidimetric (Mori et al. 2004), and colorimetric reactions
89 using color-changing reagents, such as hydroxy naphthol blue (Goto et al. 2009). However due the
90 large amount of target DNA amplified during a LAMP reaction closed tube methods for resolving
91 results are preferable and real-time methods based on turbidity (Mori et al. 2004) or fluorescence
92 (Tomlinson et al. 2010b; Tomlinson et al. 2010c) are better suited for routine diagnostics.
93 The efficient detection of *F. fujikuroi* and *M. oryzae* is essential during seed certification and to allow
94 early containment and control measures to be implemented. Two LAMP assays have been developed
95 in this study for the detection of *F. fujikuroi* and *M. oryzae* on rice. We have explored the use of the
96 assays for more rapid seed testing and have validated the tests to the international validation standard
97 published by EPPO (PM7/98).
98
99 **MATERIALS AND METHODS**
100
101 Fungal isolates
102 All the samples used for DNA extraction are listed in Table 1. The isolates, belonging to the
103 *Magnaporthe* genus, were kindly provided by Dr. Didier Tharreau (UMR BGPI, Unité Mixte de

104 Recherche, Biologie et Génétique des Interactions Plante-Parasite, Montpellier, Franc). The
105 *Gibberella fujikuroi* species complex-isolates and the fungi commonly found in rice paddies used in
106 this study were obtained from Agroinnova (University of Turin, Italy) and FERA (York, UK).
107 *Fusarium* isolates had previously been identified by sequencing using elongation factor-1-alpha
108 analysis, using EF1/EF2 primers that provide species-level discrimination (Geiser et al. 2004). The
109 fungal isolates were stored at -80°C in 20% glycerol.
110
111 DNA extraction from the fungal cultures
112 Each single-spore culture of the isolates listed in Table 1 was grown in potato dextrose broth (PDB;
113 Sigma Aldrich, Germany) on a rotary shaker (120rpm) for 10 days at room temperature. Mycelium
114 was collected, by means of filtration through Whatman No.1 filter paper, and was then stored at -
115 20°C. The total genomic DNA was obtained using an E.Z.N.A Fungal DNA mini kit (OMEGA Bio-
116 Tek, Norcross, GA, USA), according to the manufacturer's instructions. The DNA concentration of
117 each isolate was measured by Nanodrop 2000 (ThermoFisher, Delaware, USA) and was adjusted to
118 1-50 ng/µl.
119
120 Loop-mediated isothermal amplification (LAMP) reactions
121 Six LAMP primers (external primers F3 and B3, internal primers (FIP and BIP) and loop primers (F-
122 loop and B-loop) were designed for both targets, according to the methods reported by Notomi et al.
123 (2000).
124 The calmodulin sequence was used to design specific LAMP primers for *M. oryzae.* Sequence
125 alignment using MEGA 6.0.6 was carried out using the sequence of several isolates of *M. oryzae* and
126 its closest specie *Magnaporthe grisea* (Choi *et al.*, 2013), as well as other *Pyricularia* spp. (GenBank
127 accession numbers AF396017, AF396019, AF396013, AF396027, AF396022, AF396020,
128 AF306910, AF396011, AY063738, KC167645, AY063739, KC167646, AF396012, KC167624,
129 KC167601, AF396008, AF396005, KC167639, KC167643).

130 The LAMP primers for *F. fujikuroi* were designed on the basis of EF-1α sequence. This gene was
131 characterized by a 6 bp deletion in the sequence of all the strains of *F. fujikuroi* in the 688 position in
132 the second intron of the EF-1α gene, as reported by Amatulli et al. (2012). The sequence alignment
133 carried out using MEGA 6.0.6. included the following strains of the *Gibberella fujikuroi* species
134 complex: *Fusarium commune, F. andiyazi, F. graminearum, F. proliferatum, F. verticillioides*, *F.*
135 *oxysporum*, and *F. equiseti*, as well as some *F. fujikuroi* isolates (GenBank accession number:
136 KR071742, KR071743, KR071745, KR071744, AF160279, KR071746, JN092344, HM804942,
137 KC954401, JX118988, KR071740, KR071703, KT794174, KF499577).
138 The possibility of secondary structures and hairpins was checked by using OligoCalc
139 (http://biotools.nubic.northwestern.edu/OligoCalc.html). A BLASTn analysis of the target sequence
140 was carried out to evaluate the specificity of the primers. The primers were synthesized and HPLC
141 purified by Eurofins (UK) and can be obtained with positive controls material as a kit from OptiGene
142 Ltd (Horsham, UK).
143 The LAMP reactions were carried out with 1µl of DNA of the different isolates, with the
144 concentration adjusted to 1-30ng/ µl or 1 µl of the crude extractions. The 25 µl reaction included 0.2
145 µmol /l of the external primers (F3 and B3), 2 µmol/l of each internal primer (FIP and BIP), 1 µmol/l
146 of each loop primer and 1x Isothermal Mastermix ISO-004® (OptiGene Ltd). Amplification was
147 performed for 45 min at 65°C, after which the annealing temperature was measured by recording
148 fluorescence whilst cooling from 95°C to 70°C at 0.05°C/s using a Genie_ II® instrument (OptiGene
149 Ltd). Negative controls with water were included in each run. The same amplification protocol was
150 adopted on a StepOne instrument (Applied Biosystem, California, USA) to test the rice. The Real
151 Time machine was setup using a Quantification experiment type with Standard Curve with TaqMan
152 reagents (FAM as reporter and NFQ-MGB as quencher). The programme was setup to do 40 cycles
153 at 65°C with fluorescence measured each minute and a melting curve with the following steps: 95°C
154 for 15s, 70°C for 1 min and an increase of the temperature to 95°C at 0.3°C/s to record the
155 fluorescence At the same time, a COX (cytochrome oxidase gene) LAMP assay, developed by

156 Tomlinson *et al.* (2010c) was used as internal control to detect the plant DNA and to confirm the
157 DNA presence in case of negative results in the LAMP assays made just after crude extraction.
158
159 Rice seed batches
160 Pathogen-free seeds of rice cv. Dorella confirmed by PDA test (Mathur and Kongsdal 2003) were
161 artificially infected to check the lowest infection rate which could be detected using the LAMP assays.
162 In order to verify the absence of natural infection from the two target pathogens, 400 seeds were
163 placed onto potato dextrose agar (PDA, Sigma Aldrich, Germany) (25 seeds/Petri dish) and left for 7
164 days at 22 °C in alternating 12 h light-darkness cycles. After this check, rice seeds were treated with
165 sodium hypochlorite (2%) for 2 minutes, rinsed with distilled water and then air-dried for 30 minutes.
166 Single-spore cultures of *F. fujikuroi* CsC8 and *M. oryzae* Guy11 grown on potato dextrose broth
167 (PDB, Sigma Aldrich) for 7 days at room temperature, were filtered through sterilized cotton cheese-
168 cloth and brought to a concentration of 2×10^5 CFU/ml for CsC8 and 3.3×10^3 CFU/ml for *M. oryzae*
169 Guy11, by counting with a haemocytometer. Both concentrations were 10-fold serial diluted to 20
170 CFU/ml for *F. fujikuroi* CsC8 and 33 CFU/ml for *M. oryzae* Guy11, respectively. Three hundred rice
171 seeds were inoculated with 7-days conidial suspensions, incubated on a rotary shaker at 90 rpm for
172 45 min, and air-dried overnight. The effective concentration in number of conidia on the rice seeds
173 was determined using real time PCR (TaqMan) (Table S2 and S3). Infected rice seed batches were
174 prepared at 4.0%, 6.6%, 8.0%, 13.3% and 33.3% by mixing infected seeds with healthy seeds at
175 different ratios: 1 infected seed in 24 healthy seeds for 4.0% infected rate, 1 infected seed in 14 healthy
176 seeds for 6.6%infected rate, 2 infected seeds in 23 healthy seeds for 8%infected rate, 2 infected seeds
177 in 13 healthy seeds for 13.3% infected rate and 5 infected seeds in 10 healthy seeds for 33.3% infected
178 rate. Four seed batches representing biological replicates were prepared: three replicates for crude
179 DNA extraction method (explained below) and one for the E.Z.N.A Plant DNA kit extraction
180 (OMEGA Bio-Tek).
181

182 Rice seed DNA extraction

183 Alkaline DNA extraction (Chomczynski and Rymaszewski, 2006), used as crude extraction method,
184 was modified for rice seed batches. One 7/16" stainless steel 316 GD ball (Spheric Trafalgar Ltd.)
185 and 2 ml of pH 13 PEG buffer (50g l^{-1} PEG average Mn 4,600; 20 mmol/l KOH; pH 13.5) were
186 placed in a 5 ml tube, and homogenized by vigorous manual shaking for three minutes (Figure 3).
187 Three individual rice seeds at different inoculum concentrations and one seed batch for each inoculum
188 concentration and infection ratio were ground with liquid nitrogen. All the plant material was
189 extracted with E.Z.N.A Plant DNA kit (OMEGA Bio-Tek) according to manufacturer's instructions,
190 to compare the reliability of the crude extraction method and to check whether the LAMP reaction
191 would be affected by inhibitors.

192

193 Real Time PCR

194 Ten-fold diluted DNA (1 µl) from each DNA extraction method was used to measure the target
195 concentrations by quantitative real-time PCR on an ABI Prism 7900HT instrument (Applied
196 Biosystems) and to conduct a comparative analysis with the LAMP assay both for *M. oryzae* and *F.*
197 *fujikuroi*. To quantify *M. oryzae*, a real-time PCR, designed on the MHP1 gene, was carried out in
198 triplicate under the following conditions described by Su'udi et al. (2013): an initial denaturation at
199 95°C for 4.5 min, 40 cycles of 15 s at 95°C and 15 s at 60°C. A standard curve was obtained using
200 10-fold dilutions of the previously measured DNA samples of *M. oryzae*, ranging from 1.37ng/µl to
201 0.137 fg/µl of DNA tested in triplicate. The real-time PCR primers and probe designed by Amaral
202 Carneiro et al. (2017) were used to quantify *F. fujikuroi* in the rice seeds. The reaction was carried
203 out in triplicate with an initial incubation at 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C
204 for 15 s and at 62°C for 1 min. A standard curve was obtained by making serial dilutions of *F. fujikuroi*
205 DNA ranging from 6.18ng/µl to 0.618 fg/µl. Positive (fungal DNA) and negative (water) controls
206 were included in both experiments. The average of the three technical replicates of the real-time PCR
207 assay was used to calculate the DNA quantity of each individual seed and seed batch. The number of

208 detected cells was then calculated by dividing the DNA quantity by the weight of the genome of each
209 pathogen: 0.00004756 ng for *F. fujikuroi* (Jeong et al. 2013) and 0.0000378 ng for *M. oryzae* (Kumar
210 et al. 2017). The Ct values generated by the real-time assay were compared with the standard curve
211 to obtain the amount (ng) of each positive result.
212
213 Validation of the LAMP assays
214 Both tests were validated according to EPPO PM7/98 standard, by evaluating the selectivity,
215 specificity, sensitivity, repeatability and reproducibility. The specificity panel was composed of nine
216 *F. fujikuroi* isolates, 30 isolates of phylogenetically closely related *Fusarium* species, six *M. oryzae*
217 isolates, five isolates of phylogenetically closely related *Magnaporthe* species, together with other
218 rice pathogens. All the samples were used in three independent assays for each test. The sensitivity
219 was checked by using 10 fold serial dilutions of DNA extracted from pure culture of two different
220 isolates of *M. oryzae* and from four isolates of *F. fujikuroi,* and DNA from seeds. The seed testing
221 included individual rice seeds inoculated at different concentrations (from 2 x 10^5 cells/ml to 20
222 cells/ml for *F. fujikuroi* CsC8 isolate, and from 3.3 x 10^3 cells/ml to 33 cells/ml for *M. oryzae* Guy11)
223 and seed batches with different infection ratios, previously described. The selectivity of the LAMP
224 assays was evaluated using seeds from different rice cultivars ('Carnise', 'Deneb', 'Selenio',
225 'Galileo' and 'Dorella') naturally infected with *M. oryzae* or *F. fujikuroi.* Different artificially
226 inoculated seed batches were used to determine the infection rate, which was established by using the
227 LAMP assay, and to evaluate the *in vivo* sensitivity.
228 For repeatability and reproducibility, the DNA was extracted with both methods. Five individual
229 seeds (biological replicates), inoculated at different concentrations (2 x 10^5 CFU/ml and 2 x 10^4
230 CFU/ml for CsC8 and 3.3 x 10^3 CFU/ml for *M. oryzae* Guy11), were extracted with the crude
231 extraction method, while three seeds were extracted with the kit extraction method. Regarding the
232 four seed batches, three of them were extracted with the crude extraction method, while the fourth
233 was extracted with the kit extraction method. Individual seeds and seed batches were quantified in

triplicate (technical replicates) with the real-time PCR and the LAMP assay. Repeatability was checked by running three independent assays for each pathogen (taking into account the results of the biological and technical replicates), while reproducibility was tested by different operators on different days with different machines.

Identity of LAMP products for *F. fujikuroi*

For the *F. fujikuroi* assay, to confirm the identity of the crude extracted samples, which gave an annealing temperature lower than the pure DNA, the LAMP products (1µl) were digested either with MspI or with PvuI, according to the manufacturer's instructions (Promega, Wisconsin, USA). The reaction mixtures were incubated at 37°C for 1 h and the digestion products were visualized in 3% agarose gels. The profiles were compared with a GeneRuler, 100 bp DNA ladder (Thermo Scientific, Massachusetts USA).

To explore the impact of crude extraction on the recorded annealing temperature, a *F. fujikuroi* CsC8 LAMP product, with an annealing temperature of 88.2 °C, was used. The LAMP product was 2-fold serially diluted, and 1 µl of each dilution was combined with 24 µl of Mastermix ISO-004® and primers, at the same conditions described above to replicate the LAMP assay. At the same time, the DNA of each dilution (1 µl) was combined with 23 µl of Mastermix ISO-004® and primers and 1 µl of the crude seed extract. The annealing temperature of both experiments was obtained with a Genie II® instrument.

Field samples

Rice seeds from the five rice cultivars described above were used to check the reliability of the LAMP assay. The DNA of four hundred rice seeds, divided into 25-seed subsamples per cultivar were extracted using the crude extraction method and the E.Z.N.A Plant DNA kit (OMEGA Bio-Tek). LAMP assays were carried out as described above with a StepOne Plus Real-Time PCR system (Applied Biosystems), using 1 µl of the extraction and recording the Ct values as the Time to positive

(Tp), a parameter analogous to the threshold cycling time in polymerase chain reaction (PCR) (Tomlinson *et al.*, 2013).

The same rice cultivars samples were plated on PDA to check for the presence of *F. fujikuroi* and *M. oryzae* as decribed by Mathur and Kongsdal (2003) and were morphologically identified after 7 days at room temperature and 12 h light/darkness.

Genomic DNA was extracted from some single spore colonies of the *Fusarium* isolated from rice seeds as described above to determine the species using DNA barcoding as follows. The elongation factor 1-alpha (EF-1α) gene was amplified using the EF1 and EF2 primers under the following conditions: 94 °C for 5 minutes followed by 40 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 1 min, with a final extension of 10 min at 72 °C. The PCR assay was carried out in a 20 µl reaction volume with 50 ng of genomic DNA and 0.25 mmol/l of deoxynucleotide triphosphates, 1.0 mmol/l of $MgCl_2$, 0.5 µmol/l for each primer, 1:10 diluted Qiagen PCR buffer and 1U of Taq DNA polymerase (Qiagen, Chatsworth, CA, USA) in a T-100 thermal cycler (Bio-Rad, California,USA). Amplification products were separated by electrophoresis in 1% agarose gel (Eppendorf, Hamburg, Germany) and purified using QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA, USA) before being sequenced by BMR-Genomics (Padova, Italy). BLASTn analysis of the sequences was carried out to identify each isolate.

RESULTS

Validation of the LAMP assays

The specificity of the *F. fujikuroi* and *M. oryzae* amplicons was checked against the NCBI Nucleotide database using BLASTn which showed a 100% identity with the GenBank sequence accessions KT257540.1 and KM485261.1, respectively. The LAMP assays developed for *F. fujikuroi* and *M. oryzae* were validated according to EPPO standard PM7/98. When tested with an inclusivity and exclusivity panel of fungal isolates, amplification was not detected in any DNA sample from non-

target species (Table S1). The average Tp for the *F. fujikuroi* LAMP assay, using DNA concentration of 1-30 ng/µl ranged from 18 to 22 min with an annealing temperature ranging from 87.97 to 88.38 °C, while the Tp for the *M. oryzae* LAMP assay ranged from 13 to 16 min with an annealing temperature ranging from 88.41 to 89 °C.

The specificity of the assays was compared with the published real-time PCR (TaqMan) for each assay. None of the non-targets amplified with the *M. oryzae* real-time PCR assay, however non-specific amplifications were recorded for several *Fusarium* spp. (Amaral Carneiro et al. 2017; Su'udi et al. 2013) using the *F. fujikuroi* real-time PCR assay.

The sensitivity of both assays was tested using different several approaches (Figures 1 and 2). The lowest quantity of DNA amplified using the *F. fujikuroi* LAMP assay corresponds to an amount of DNA ranging between 100-999 fg (270 fg for strain C1, 23.1 fg for strain CsSP1, 3.06 pg for strain CsAg3 and 61.8 pg for strain VPG2), while the *M. oryzae* LAMP assay amplified DNA ranging between 10-99 pg (13.7 pg for strain Guy11 and 183 pg for strain AG0043). The same 10-fold serial dilutions of DNA were simultaneously tested by means of qPCR, for *F. fujikuroi* the qPCR assay amplified DNA amounts ranging between 100-999 fg and the *M. oryzae* assay amplified DNA ranging between 1-9.9 pg.

The sensitivity was also checked by using individual rice seeds inoculated with 10-fold serial dilutions of both pathogens, and then extracting the DNA with the crude extraction method and a commercial DNA extraction kit. The results of these tests are reported in Figure 3. The LAMP assay for *F. fujikuroi* repeatedly (in all replicates) detected the two first serial dilutions ($2x10^5$ CFU/ml and $2x10^4$ CFU/ml), while the other dilutions were detected with time to positive results in excess of 35 min, which was considered the time threshold of the LAMP test (data not shown). The *M. oryzae* LAMP assay could be used to repeatedly (in all replicates) detect the first serial dilution ($3.3 x10^3$ cells/ml), while only some replicates of the dilution $3.3 x10^2$ cells/ml were detected.

For both assays, DNA from the individual seeds was extracted using crude and commercial kit extraction and amplified with qPCR, confirming the inoculum. The qPCR quantification showed that

312 the individual seeds inoculated with 3.3 x10^3 *M. oryzae* CFU/ml had an average of 3900 cells, while
313 the individual seeds inoculated with 2x10^5 CFU/ml *F.fujikuroi* CFU/ml had 102 cells. However, both
314 qPCR were not repeatable: only one or two biological replicates out of the five tested amplified with
315 the commercial kit, while with the crude extraction the qPCR did not produce any amplification.
316 On the contrary, positive amplification was obtained for the assays with both kit and crude extraction
317 methods using LAMP, although a slight variation of the positive amplification was reported, and a
318 faster detection was obtained with the kit extraction method in the lowest dilution for the *F. fujkuroi*
319 assay (from 34 min 10 s to 29 min 37 s respectively) (Figures 3 and 4) and in the two serial dilutions
320 of *M. oryzae* (from 15 min 50 s to 13 min 35 s for the highest dilution and 36 min 43 s to 33 min 21
321 s for the lowest dilution).
322 Based on the positive results obtained for the individual seed testing, batches of 15 and 25 rice seeds,
323 including different concentrations of infected seeds inoculated at 2x10^5 and 2x10^4 CFU of *F.*
324 *fujikuroi/*ml and 3.3x10^3 CFU of *M. oryzae/*ml, were used to determinate the detectable infection rate
325 of a batch. The most repeatable and reliable results obtained using infected seed inoculated with 2x10^5
326 CFU of *F. fujikuroi*/ml corresponded to a 33.3% infection rate, while an 8 % infection rate was
327 detected in all batches and replicates, with variations in the positive time amplification among
328 replicates. The best result obtained from the batches with the seeds inoculated at 2x10^4 CFU of *F.*
329 *fujikuroi*/ml corresponds to a 33.3% infection rate, but the results were not repeated. These results
330 indicate that the batches with a 33.3% infected rate can be detected regardless of the concentration of
331 the pathogen in the seed. The best results of the *M. oryzae* infected seeds were obtained for the 8%
332 and 13.3% infection rate batches. The batches used for the kit extraction produced more repeatable
333 results, obtaining positive amplification in all the LAMP replicates. (Figure 6).
334
335 Identity of LAMP products for *F. fujikuroi*
336 The specificity of the *F. fujikuroi* LAMP assay was checked by means of enzyme DNA restriction,
337 using the MpsI and PvuI enzymes, and the same pattern was obtained for all the samples (pure DNA

and crude samples), even with products that gave different annealing temperatures, as reported in Figure 7. Further exploration demonstrated that the crude seed extracts influenced the annealing temperature of more than 1°C for each dilution (Table 2).

Testing naturally infected rice

Five seed batches were tested to compare the traditional culture method of 400 seeds with the LAMP assay implemented using two different DNA extraction techniques.

The results of the cultivation test showed that rice cultivars 'Deneb', 'Selenio' and 'Galileo' were naturally infected with *M. oryzae* at 0.01%, 0.01% and 0.005% respectively, whilst 'Carnise' and 'Dorella' were free of this pathogen (data not shown). On the other hand, the PDA test showed that 'Deneb', 'Carnise' and 'Selenio' were naturally infected with *F.fujikuroi* at 0.075%, 0.025% and 0.025% infection rates, respectively; whilst 'Dorella' and 'Galileo' were not infected with *F. fujikuroi*. Seed from the cv 'Deneb' were the most infected cultivar with *F. fujikuroi* producing a large number of colonies of this pathogen.

A number of colonies (n = 8) were tested using DNA barcoding and the results of the BLASTn analysis confirmed the *Fusarium* species as *F. fujikuroi.* An example sequence was deposited in GenBank with the accession number: MF677976, MF677977, MF677978, MF677979, MF677980, MF677981, MF677982, MF677983.

The results of testing batches of seed using the LAMP assays confirmed the blotter results. When testing for *M. oryzae,* seeds from the cv 'Deneb', 'Selenio' and 'Galileo' gave positive results. The number of positive results varied depending on the cultivar tested using both types of DNA extraction method. More positive results were found for the cv 'Deneb' followed by 'Selenio' and 'Galileo'. The commercial DNA extraction kit gave more positive results (28/48, 15/48 and 9/48 for cv 'Deneb', 'Selenio' and 'Galileo' respectively) than the crude DNA extraction method (7/48, 2/48 and 3/48 for cv 'Deneb', 'Selenio' and 'Galileo' respectively (Table 3).

363 The LAMP for *F. fujikuroi* also confirmed the blotter test, confirming the presence of the fungi in the
364 cultivars 'Deneb', 'Carnise' and 'Selenio'. However for fusarium only the DNA from the commercial
365 DNA extraction kit produced positive amplification, whilst the crude extracted DNA was not detected
366 in any of the rice cultivars tested (Table 4).
367
368 **DISCUSSION**
369 Two LAMP assays have been developed in this work to obtain a simple, fast and cheap diagnostic
370 method for screening rice seed for the presence of the pathogens *F. fujikuroi* and *M. oryzae*. Currently,
371 the method recommended by the International Rules for Seed Testing 2017 (ISTA;
372 http://www.bibme.org/citation-guide/apa/website/) consists of blotting on a 90 mm filter paper
373 soaked with distilled water a working sample of 400 seeds divided into 25 seeds subsamples,
374 recording the percentage of infected seeds for 7 days at 22°C in alternating cycles of 12 h
375 light/darkness, confirmed by stereoscopic examination of each seed. Despite the ease of the blotting
376 method and the extensive use in diagnostic laboratories, a correct screening requires trained
377 diagnosticians who are able to identify the conidia of the resulting fungal growth under a stereoscopic
378 microscope. One of the aspects that should be taken into account during this activity is the presence
379 of other conidia with similar morphological features, which may be misidentified as rice blast, such
380 as the saprophytic fungi *Cladosporium* spp. (ISTA 2017). A misidentification can lead to economic
381 losses for any company that is attempting to certify the absence of pathogens in rice seeds. The other
382 disadvantage of the blotting method is the long incubation of 7 days. This also requires sizable and
383 expensive controlled environment chambers to perform the tests.
384 PCR-based methods are often used in well-equipped laboratories, to carry out routine identification
385 tests. However, the complexity of time-consuming PCR-based methods reduces the possibility of on-
386 site sampling and detection, thus increasing the delay between sampling (process) and results. One
387 of the drawbacks of the PCR-based method is its sensitivity to inhibitors, which can modify the
388 specificity and even produce false negative results. To avoid this problem, the PCR is combined with

389 long extraction methods to obtain high-quality nucleic acids from the sampled material (Boonham et
390 al. 2008). These inhibitors are a heterogeneous group of poorly characterized compounds including
391 phenols, polysaccharides, melanin, humic/tannic acids, and proteins present in rice seeds (Tian et al.
392 2004; Schrader et al. 2012). The LAMP assay shows optimal characteristics, as it allows rapid,
393 sensitive, specific and easy field-detection and is potentially less sensitive to inhibitors (Kaneko et al.
394 2007).
395 A LAMP assay for *M. oryzae* has been recently developed by Villari et al. (2017) for the detection of
396 airborne inoculum in turfgrass. However this LAMP assay was not tested on *M. oryzae* isolated from
397 *O. sativa* unlike the test presented here, which was able to amplify the DNA of *M. oryzae* in less than
398 20 minutes. The *F. fujikuroi* LAMP assay developed in this study was found to be more specific than
399 the previously published real-time PCR assay (Amaral Carneiro et al. 2017). The sensitivity of the
400 LAMP assays was validated using both DNA samples and *in vivo* samples obtaining consistent and
401 reproducible results using batches of 25 rice seeds with different infection rates. Despite both
402 published real-time PCRs were reported to be more sensitive than the so-far developed LAMP assays,
403 none of them produced reproducible results using rice seeds.
404 During the development, it was noted that the annealing temperature of the *F. fujikuroi* LAMP assay
405 was affected by the addition of crude extracts of rice seed. Whilst the annealing temperature was
406 impacted, the amplification itself was not adversely affected. Restriction digests demonstrated the
407 specificity of the amplified products in the presence of seed extracts despite the variation in the
408 annealing temperature observed.
409 The reliability of both LAMP assays using commercial rice seed samples was assessed by comparing
410 them to the traditional blotting test recommended by ISTA (ISTA 2017) with five different rice
411 cultivars. Other LAMP assays have been developed to test cereal seeds, but in these methods the
412 DNA is extracted with an electric grinder (Abd-Elsalam et al. 2011), thus making on-site detection
413 difficult. We explored the potential of the crude extraction method developed by Tomlinson et al.
414 (2010c), which is based on alkaline lysis combined with manual shaking, for testing rice seeds in the

present study. This method has the potential for achieving results directly on-site as a screening tool for seed batches, or could become a quick and easy detection tool in the laboratory without expensive equipment and time-consuming DNA extraction.

The reliability of both assays was improved when a commercial DNA extraction kit was used, presumably due to an improvement in the sensitivity of the tests. In conjunction with the commercial extraction kit, both LAMP assays may become a potential routine test for the detection of *F. fujikuroi* and *M. oryzae* in rice seeds especially due to the improved turnaround time to perform the experiment (some hours) against the 7 days of a blotting method. The *M. oryzae* LAMP assay gave the same results as the blotter test when using the crude extraction which would ensure a quicker result, perhaps as a screening test performed in the field. However, a laboratory confirmation test using a commercial DNA extraction kit may be needed for negative results.

In order to ensure the DNA extraction from rice seeds and interpret the negative results from false negatives, the use of a LAMP assay, based on cytochrome oxidase, may be a useful indicator, as it is able to distinguish between a failed acid nucleic extraction and the negative presence of the pathogen in samples (Tomlinson et al. 2010a).

In summary, two LAMP assays have here been designed for *F. fujikuroi* and *M. oryzae* and validated according to international validation standard published by EPPO (PM7/98). They have been found to be sufficiently sensitive and specific to provide a viable and rapid alternative to the current morphological identification methods. Furthermore, when the LAMP assays were used on Genie II or Genie III platforms (OptiGene) they are a rapid (less than 1 hour) way of testing the pathogen levels in batches of rice seed in order to facilitate correct preventive and control measures before pathogen dispersal.

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443

444 **LITERATURE**

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556 **Tables**

557 **Table 1.** Isolates used in this study

Isolate code	*Species*	Geographical origin	Year of isolation
l1.3	*Fusarium fujikuroi*	Italy	2006
VPG2	*Fusarium fujikuroi*	Italy	2008
G3S	*Fusarium fujikuroi*	Italy	2008
VPG4	*Fusarium fujikuroi*	Italy	2008
CsSP1	*Fusarium fujikuroi*	Italy	2009
CsAg3	*Fusarium fujikuroi*	Italy	2009
CS.C8	*Fusarium fujikuroi*	Italy	2009
GCe2	*Fusarium fujikuroi*	Italy	2009
M1149	*Fusarium fujikuroi*	Taiwan	unknown
19-113	*Fusarium proliferatum*	Italy	2006
11-47	*Fusarium proliferatum*	Italy	2006
FP1	*Fusarium proliferatum*	Italy	unknown
FP2	*Fusarium proliferatum*	Italy	unknown
FP3	*Fusarium proliferatum*	Italy	unknown
1100	*Fusarium proliferatum*	unknown	unknown
2-130	*Fusarium napiforme*	Italy	2006
FA1	*Fusarium andiyazi*	Italy	unknown
FA2	*Fusarium andiyazi*	Italy	unknown
FC1	*Fusarium commune*	Italy	unknown
FC2	*Fusarium commune*	Italy	unknown
FC3	*Fusarium commune*	Italy	unknown
19-9	*Fusarium verticillioides*	Italy	2006
FV3	*Fusarium verticillioides*	Italy	unknown
FV2	*Fusarium verticillioides*	Italy	unknown
Mya3040	*Fusarium oxysporum* f.sp. *lactucae*	Italy	2002
Gr15	*Fusarium oxysporum* f.sp. *raphani*	Italy	unknown
ATCC52557	*Fusarium oxysporum* f.sp. *conglutinans*	unknown	unknown
ATCC58385	*Fusarium oxysporum* f.sp. *conglutinans*	unknown	unknown
ATCC52422	*Fusarium oxysporum* f.sp. *chrysantemi*	unknown	unknown
ATCC744009	*Fusarium oxysporum* f.sp. *fragariae*	Unknown	unknown
HPV04	*Fusarium graminearum*	unknown	unknown
1498	*Fusarium graminearum*	unknown	unknown
822	*Fusarium sporotrichioides*	unknown	unknown
575	*Fusarium sulphureum*	unknown	1991
710	*Fusarium poae*	UK	1993
L8FEQ	*Fusarium equiseti*	unknown	unknown
832	*Fusarium culmorum*	unknown	unknown
1098	*Fusarium trienetrum*	unknown	unknown
54	*Fusarium avenaceae*	unknown	1991
FP	*Fusarium panidoroserum*	unknown	unknown

RS1	*Rhizoctonia solani*	unknown	unknown
E.S. 34015	*Alternaria alternata*	unknown	unknown
Py5.10	*Pythium ultimum*	unknown	unknown
Scscletìro1	*Sclerotinia sclerotiorum*	unknown	unknown
Scmin1	*Sclerotinia minor*	unknown	unknown
Vertsp1	*Verticillium*	unknown	unknown
CV1	*Curvularia sp.*	unknown	unknown
1095	*Cochliobolus*	unknown	2000
1238	*Cochliobolus*	unknown	1998
PE1	*Phoma exigua*	unknown	unknown
TC1	*Trichoderma*	unknown	unknown
CO1	*Colletotrichum sp.*	unknown	unknown
PF1	*Penicillium expansum*	unknown	unknown
br0011-Guy11	*Magnaporthe oryzae*	South America	1978
br0156	*Magnaporthe oryzae*	Africa	1989
br0071-GrF2	*Magnaporthe oryzae*	The USA	1998
MO1	*Magnaporthe oryzae*	unknown	unknown
AG0041	*Magnaporthe oryzae*	unknown	unknown
AG0043	*Magnaporthe oryzae*	unknown	unknown
br0029	*Magnaporthe grisea*	Brazil	1989
br0067	*Pyricularia pennisetigena*	Brazil	1990
br0017	*Pyricularia penniseticola*	Africa	1990
br0001-RG1	*Pyricularia zingibericola*	Indian Ocean	unknown
br0001-CT4	*Pyricularia ctenantheicola*	Greece	1998
BO3	*Bipolaris oryzae*	unknown	unknown
BO5	*Bipolaris oryzae*	unknown	unknown

558

559 **Table 2.** Annealing temperature of the LAMP product of *F. fujikuroi* strain CsC8 2-fold serial diluted with

560 and without seed extracts.

561

CsC8 DNA - LAMP product	Annealing temperature (°C) from LAMP product without seed extracts	Annealing temperature (°C) from LAMP product with 1 µl of seed extract
	87.36	86.05
Dilution -2	87.00	85.50
Dilution -4	87.00	86.14
Dilution -6	87.10	86.34
Dilution -8	87.19	85.94
Dilution -10	75.11	73.97
Dilution -12	75.41	73.42
Dilution -14	75.31	74.22

562

563

Table 3. Results of testing 400 seed batches of 5 cultivars in 25 seed-subsample batches using the *M. oryzae* LAMP assay representing average Ct values for the positive seed batches extracted using each of the two DNA extraction methods.

	Commercial DNA extraction kit		Crude DNA extraction	
Cultivar	Tp (h:min:s)	Anneal(°C)	Tp (min:s)	Anneal(°C)
Deneb	Tp (min:s)	89.18±1.64	17:30±07:23	89.2±0.19
Selenio	15:72±04:14	89.27±0.11	19:21±07:34	89.21±0.22
Galileo	15:38±02:22	89.04±0.17	19:49±01:50	88.85±0.61
Dorella	0	0	0	0
Carnise	0	0	0	0

575 **Table 4.** Results of testing 400 seed batches of 5 cultivars in 25 seed-subsample batches using the
576 *F.fujikuroi* LAMP assay representing average Ct values for the positive seed batches extracted
577 using each of the two DNA extraction methods.

578

	Commercial DNA extraction kit		Crude DNA extraction	
Cultivar	Tp (min:s)	Anneal(°C)	Tp (min:s)	Anneal(°C)
Galileo	0	0	0	0
Dorella	0	0	0	0
Deneb	25:91±04:04	88.11±0.46	0	0
Carnise	29:91±02:87	88.14±0.6	0	0
Selenio	33:76±04:06	88.24±0.38	0	0

579
580

Figures

Figure 1. Sensitivity test using 10-fold serial dilutions of the DNA of two *M. oryzae* isolates, where the average Tp time (Time to positive) of two isolates in three independent assays are reported for each isolate. Negative control (with water) and positive control (with DNA from the fungi of the Table 1) were included in each run.

Figure 2. Sensitivity test using a 10-fold serial dilution of the DNA of four *F .fujikuroi* isolates, where the average of three replicates of Tp time (Time to positive) of four isolates tested in three independent assays are reported for each isolate. Negative control (with water) and positive control (with DNA from the fungi of the Table 1) were included in each run.

Figure 3. **A** .Results of the sensitivity test of the *F. fujikuroi* LAMP assay in which individual rice seeds were used and the DNA was extracted using a crude extraction method and a kit extraction method in triplicate. Cytochrome oxidase (COX) LAMP assay was included as an internal control of the DNA extraction. The table listed the time to positive (Tp) and the annealing temperature (Anneal) on average considering only positive amplification results with a Tp of less than 35 min. **B**. The bar graph represents the differences between the time to positive (Tp) obtained using both types of DNA extraction method and the different inoculum concentrations. Negative control (with water) and positive control (with DNA from the fungi of the Table 1) were included in each run.

Figure 4. A. Sensitivity of the *M. oryzae* LAMP assay with individual rice seeds, where DNA was extracted using crude and kit extraction methods in triplicate. Cytochrome oxidase (COX) LAMP assay was included as internal control of the DNA extraction. The table listed the time to positive (Tp) and the annealing temperature (Anneal) on average considering only positive amplification results with a Tp of less than 35 min. **B**. The bar graph represents the differences between the time to positive (Tp) obtained using both types of DNA extraction method and the different inoculum concentrations. Negative control (with water) and positive control (with DNA from the fungi of the Table 1) were included in each run.

Figure 5. Sensitivity of the *F. fujikuroi* LAMP assay with rice seed batches with different percentages of infected seeds, where DNA was extracted using crude and kit extraction methods in triplicate. Average and standard deviation of the time to positive of three replicates are reported, according to the DNA extraction method used. Negative control (with water) and positive control (with DNA from the fungi of the Table 1) were included in each run. **A**. The bar graph represents the inoculum concentration $2x10^5$ CFU/ml.**B**. The bar graph on the right the inoculum concentration $2x10^4$ CFU/ml.

Figure 6. Sensitivity of the *M. oryzae* LAMP assay with rice seed batches with different percentages of infected seeds, where DNA was extracted using crude and kit extraction methods in triplicate. Average and standard deviation of the time to positive of three replicates are reported, according to the DNA extraction method used. Negative control (with water) and positive control (with DNA from the fungi of the Table 1) were included in each run.

Figure 7. **A**. MspI and **B.** PvuI digestions of the different LAMP products. Undigested LAMP assay product of *F. fujikuroi* CsC8 was included as control. The table reports the lane and the annealing temperature of each sample tested.