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Optimization of a Loop-Mediated Isothermal Amplification Assay for On-Site Detection of *Fusarium fujikuroi* in Rice Seed

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Abstract: *Fusarium fujikuroi*, causing bakanae disease, is one of the most important seedborne pathogens of rice, the detection of which is paramount for seed certification and for preventing field infections. Molecular tests—qPCR and loop-mediated isothermal amplification (LAMP)—are replacing the blotter test in seed health procedures, due to higher sensitivity, specificity, fast turnaround results delivery, on-site application and the possibility of quantifying endophytic seed infections. A LAMP test, which had been previously developed with primers designed to target the elongation factor 1-α sequence of *F. fujikuroi*, was validated according to the international validation standard (EPPO, PM7/98) on thirty-four rice seed lines of different levels of susceptibility to the disease, thus comparing it to the blotter test and with two different DNA extraction procedures. The use of crude extracted DNA provided more sensitive results than the DNA extracted with the commercial kit Omega E.Z.N.A® Plant DNA kit. The results showed that the endophytic infection of *F. fujikuroi* is essential for the development of the disease in the field and that the minimum amount of the pathogen necessary for the development of the disease corresponds to 4.17×10^4 cells/µL. This study confirms the applicability of the LAMP technique on-site on rice seeds with fast and quantitative detection of the pathogen.

Keywords: bakanae; seedborne; *Oryza sativa* L.; LAMP

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1. Introduction

Rice (*Oryza sativa* L.) is a staple food consumed worldwide, with a cultivated area of 162 million ha and a production of 755 million tons. The world's largest producer is China, followed by India and Indonesia, while in Europe the largest producers are Italy and Spain [1]. In Italy, rice production is currently 1,498,133 tons, an area of 220,027 ha, concentrated in the northern regions [2] that apply technologically advanced rice cultivation systems [3].

Bakanae, caused by *F. fujikuroi*, a member of the *Gibberella fujikuroi* species complex (GFSC), is one of the most studied diseases of rice. First observed in Japan in 1828 [4] today it is widely spread throughout Asia [5] and in recent decades—California and Italy [6,7]. Yield losses worldwide can vary from 10% up to 90%, depending on the cultivar and the geographical region [8]. The most frequent symptoms of the disease are yellowing and excessive elongation of the affected seedlings [9,10] caused by the production of gibberellic acid by the pathogen [11], which has led to the Japanese name bakanae, meaning "foolish seedling". Not all infected seeds cause symptoms in seedlings [8,12]. If the level of seed borne inoculum is high, the probability of causing infection in the field increases, but several factors can influence the infection cycle, such as weather conditions, cropping practices, resistance or susceptibility of the cultivar, the virulence of the strain of the pathogen and the efficiency of the inoculum [13]. In fact, the inoculum level on the seed detected with traditional or molecular methods, and the inoculum threshold necessary to develop the

disease in the field do not always correlate [13]. *F. fujikuroi* is morphologically characterized by a white mycelium that turns purple gray with time and it produces macroconidia and microconidia [14]. The fungus can colonize seeds both internally and externally [15]. In recent years, the disease became more relevant to rice seed companies, increasing the need of specific diagnostic tools, as well as the development of effective management strategies [16].

Bakanae is a monocyclic disease, characterized by a high production of conidia on sick or dead culms in the field during the phenological stages of flowering and mature grain when the conidia can colonize the seeds [17,18]. Conidia are easily spread by wind and water [19]. Severely infected grains show red coloring due to the presence of mycelium and conidia. The fungus can also be isolated from asymptomatic seeds if they come from a highly affected field. The fungus overwinters in infected seeds or rice straw, where it can survive for at least three years in dry conditions. The pathogen can temporarily survive in soil, but it loses its pathogenicity after 180 days [17]. Low temperatures and relative humidity (30%–35%) influence the infection process and the survival of the pathogen in the soil. High levels of nitrogen in the soil increases fungal growth and host susceptibility [20].

The disease is present in all areas of rice cultivation. Bakanae causes both qualitative and quantitative losses under favorable conditions [17,21,22]. In Italy, an incidence ranging from 5% to 15% has been observed, depending on the season and the cultivar [23]. In 2008, 2009 and 2010, 146 strains of *Fusarium* spp. were isolated from infected rice plants and seeds from different cultivation areas in Piedmont [18]. Since the species belonging to the GFSC are morphologically indistinguishable, the strains have been identified at the molecular level by amplification and sequencing of the translation elongation factor gene (TEF) [24]. *F. fujikuroi* represents the most frequent *Fusarium* species present on rice causing bakanae symptoms [25,26]. Other species belonging to the GFSC, including *F. verticillioides* and *F. proliferatum*, are present in low percentages and are mainly isolated from seed [11]. Other species not belonging to the GFSC were isolated from rice, such as *F. graminearum* [27], *F. equiseti* [28] and *F. chlamydosporum* [29]. Of the 146 isolated strains, 121 were tested for pathogenicity and virulence in vivo on a susceptible cultivar, showing that only *F. fujikuroi* causes the symptoms of bakanae [18].

Different molecular techniques were used to characterize and identify the different populations of GFSC. Primer species-specifics used in PCR assays were obtained from sequences of different origin, including the elongation factor 1α. The multiple alignment of the elongation factor gene sequences (TEF) of different *Fusarium* spp. showed a deletion of six nucleotides in the *F. fujikuroi* sequence and a nucleotide polymorphism of two bases in the same region of *F. proliferatum*. These elements of variability were used to develop conventional and real-time PCR protocols [18]. The specificity of primers was confirmed by DNA analysis of the most representative species of GFSC and 298 strains of *Fusarium* spp. isolated from Italian rice plants and seeds [18]. Specific primers were used to detect fungal presence directly from infected tissues and rice seeds, providing a tool for early detection of contamination by pathogens. Based on the TEF-1α gene, 35 strains of *F. fujikuroi* and 8 strains of other species belonging to the genus *Fusarium* spp. were used to validate a real-time PCR with Taqman probe as a diagnostic method on rice seeds [30]. Specific primers and a Taqman probe were designed for the reaction. The combined action of primers and probe was tested in a quantitative PCR (qPCR), showing the amplifications of DNA fragments repeatedly and reliably from different strains of *F. fujikuroi*, but not from the other eight *Fusarium* species. The method was applied in vivo for the diagnosis of infected tissues and for the detection and quantification of the pathogen in batches of naturally contaminated rice seeds of different rice cultivars. Loop-mediated isothermal amplification (LAMP) represents a rapid, specific, sensitive and efficient molecular diagnostic technique that amplifies DNA sequences at a single temperature [31]. A LAMP protocol was validated to detect *F. fujikuroi* on rice [32]. Six primers, including two external (F3 and B3), two internal (FIP and BIP) and two loop primers (F-loop and B-loop), were designed for tef-1α target gene amplification, and the reaction was performed for 45 min at 65 °C. The fluorescence

emitted was recorded during cooling by a portable instrument, GenieII (OptiGene Ltd., Horsham, UK). This method has the potential to be used directly in the field, as a screening tool for seed batches.

The aims of this work were (1) to validate an on-site rapid molecular assay (LAMP) to detect *F. fujikuroi* on rice seed, (2) to verify the correlation between the incidence of the disease in the field and the incidence of *F. fujikuroi* detected by the LAMP method on seed, and (3) to determine the minimum threshold of *F. fujikuroi* cells on seed necessary for the development of the disease in the field.

2. Materials and Methods

2.1. Rice Seed Samples

The 34 rice seed lines (*Oryza sativa* subsp. *japonica*) used in this study (Table 1) were produced during the summer of 2019, between March and September, in Piedmont, Lombardy and Sardinia (Italy), and provided by the company SA.PI.SE. (Vercelli, Italy). The 34 lines with different degree of tolerance to the disease—from resistant (cv. Selenio) to susceptible (cv. Dorella)—were tested by the seed company and data are not shown.

Table 1. Rice seed lines (*Oryza sativa* subsp. *japonica*) used in the study.

RICE LINES Code	Cultivar	Growing Cycle (Days)	Company ID	Origin (Province)	Region
SPS.FUS.01	SOLE CL	medium (135–140 days)	ID 21	CASALBELTRAME (NO)	Piedmont
SPS.FUS.02	SOLE CL	medium (135–140 days)	ID 23	FRAZ. GIONZANA (NO)	Piedmont
SPS.FUS.03	SOLE CL	medium (135–140 days)	ID 27	ORISTANO (OR)	Sardinia
SPS.FUS.04	SOLE CL	medium (135–140 days)	ID 31	ALBANO V.SE (VC)	Piedmont
SPS.FUS.05	SOLE CL	medium (135–140 days)	ID 35	SALI V.SE (VC)	Piedmont
SPS.FUS.06	SIRIO CL	Early (125 days)	ID 36 BIS	CASALBELTRAME (NO)	Piedmont
SPS.FUS.07	SIRIO CL	Early (125 days)	ID 38	CASALBELTRAME (NO)	Piedmont
SPS.FUS.08	SIRIO CL	Early (125 days)	ID 39	GRANOZZO CON MONTICELLO (NO)	Piedmont
SPS.FUS.09	SIRIO CL	Early (125 days)	ID 43	ORISTANO (OR)	Sardinia
SPS.FUS.10	SIRIO CL	Early (125 days)	ID 48	VERCELLI (VC)	Piedmont
SPS.FUS.11	MARE CL	Medium–late (145 days)	ID 68	ORISTANO (OR)	Sardinia
SPS.FUS.12	MARE CL	Medium–late (145 days)	ID 72	ORISTANO (OR)	Sardinia
SPS.FUS.13	MARE CL	Medium–late (145 days)	ID 75	CASANOVA ELVO (VC)	Piedmont
SPS.FUS.14	MARE CL	Medium–late (145 days)	ID 79	SALI V.SE (VC)	Piedmont
SPS.FUS.15	MARE CL	Medium–late (145 days)	ID CGN-MAR	PERTENGO (VC)	Piedmont
SPS.FUS.16	LUNA CL	Medium–early (130–135 days)	ID 86	CASALINO (NO)	Piedmont
SPS.FUS.17	LUNA CL	Medium–early (130–135 days)	ID 90	SOZZAGO (NO)	Piedmont
SPS.FUS.18	LUNA CL	Medium–early (130–135 days)	ID 98	ORISTANO (OR)	Sardinia
SPS.FUS.19	LUNA CL	Medium–early (130–135 days)	ID UBZ-LUN	CERANO (NO)	Piedmont

Table 1. *Cont.*

RICE LINES Code	Cultivar	Growing Cycle (Days)	Company ID	Origin (Province)	Region
SPS.FUS.20	LUNA CL	Medium–early (130–135 days)	ID CGN-LUN	PERTENGO (VC)	Piedmont
SPS.FUS.21	BARONE CL	Medium–late (145–150 days)	ID 127	ORISTANO (OR)	Sardinia
SPS.FUS.22	BARONE CL	Medium–late (145–150 days)	ID 132	OLEVANO (PV)	Lombardy
SPS.FUS.23	BARONE CL	Medium–late (145–150 days)	ID 134	ALBANO V.SE (VC)	Piedmont
SPS.FUS.24	BARONE CL	Medium–late (145–150 days)	ID 135	TRONZANO V.SE (VC)	Piedmont
SPS.FUS.25	BARONE CL	Medium–late (145–150 days)	ID 138	VILLATA (VC)	Piedmont
SPS.FUS.26	SA1903	Early (130 days)	ID 167	BORGOVERCELLI (VC)	Piedmont
SPS.FUS.27	SA1903	Early (130 days)	ID 168	ZEME (PV)	Lombardy
SPS.FUS.28	SA1903	Early (130 days)	ID 169	S.NAZZARO SESIA (NO)	Piedmont
SPS.FUS.29	SA1903	Early (130 days)	ID SC1	BORGOVERCELLI (VC)	Piedmont
SPS.FUS.30	Vialone nano	Late (155–160 days)	ID 170 (S+N)	OLEVANO (PV)	Lombardy
SPS.FUS.31	Vialone nano	Late (155–160 days)	ID 171 (S)	OLEVANO (PV)	Lombardy
SPS.FUS.32	Vialone nano	Late (155–160 days)	ID 172 (N.T)	OLEVANO (PV)	Lombardy
SPS.FUS.33	SELENIO	Medium–late (145 days)	ID 50	ROBELLA DI TRINO (VC)	Piedmont
SPS.FUS.34	DORELLA	Medium–late (145 days)	ID LEON-TCS	OLEVANO (PV)	Lombardy

2.2. Fungal Isolates

Strain I1.3 of *F. fujikuroi*, isolated from a diseased rice plant collected in Piedmont, Italy, in 2006 and stored in the collection at AGROINNOVA, Turin, Italy, at −80 °C in 20% glycerol was used in the study.

Five isolates of different species of *Fusarium* were used in the specificity test: *F. graminearum*, isolated from maize seed in 2019; *F. oxysporum* f. sp. *lactucae*, from lettuce in 2002; *F. proliferatum*, from crown tissues of sorghum in 2020; *F. solani* from roots of sorghum in 2020; *F. verticillioides* from crown tissues of maize in 2020.

Conidial suspensions of isolate I1.3 to be used to inoculate single seed were obtained growing it in potato dextrose broth (PDB, Sigma-Aldrich, Darmstadt, Germany) on a rotary shaker (120 rpm) for 7 days at room temperature, after that the mycelium was filtrated and the suspension of conidia was centrifuged at 6000 rpm for 20 min. The supernatant was removed and the pellet was resuspended in 3 mL of sterile Ringer solution. The suspension was used for spore counts on a haemocytometer.

2.3. Seed Health Evaluation

Seed health was evaluated with the blotter method [33]. Four-hundred seeds (40 replicates of 10 seeds) from each batch were placed, without previous disinfection, in 12 × 12 cm plastic boxes over three layers of sterile filter paper soaked with a 0.05% NaClO water solution. The boxes were placed in a growth chamber at 21 ± 2 °C, under a 12 h near-ultraviolet light (NUV), 12 h dark cycle, for 5 days. At the end of the incubation time, the rice seeds were examined for the presence of *F. fujikuroi* under a stereomicroscope and, when necessary, additional observations were made with the compound microscope. Seed transmission of *F. fujikuroi* was evaluated in the greenhouse with a growing out assay where ninety seeds of each batch were sown in plastic trays filled with sterile peaty substrate

and grown at 21 ± 2 °C and irrigated twice a day. The test lasted 4 weeks, during which inspections were carried out at 7, 14, 21 and 28 days after sowing recording the percentage of seed germination and the appearance of symptoms of *F. fujikuroi*. An evaluation was carried out in the field in the experiment farm of SA.PI.SE. (Borgo Vercelli, VC) where 1200 seeds of each batch were sown in 4 m^2 plots. Percentages of germination and of sick plants were evaluated at the phenological stages of tillering, stem elongation and heading [34].

2.4. Rice Seed DNA Extraction Methods

Two sub-samples of 50 dry seeds from each batch were ground separately in liquid nitrogen with mortar and pestle to obtain a powder. One hundred mg of the powder was transferred to a 2 mL microcentrifuge tube and used for the extraction of the DNA with the Omega E.Z.N.A.® Plant DNA kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturers' instructions, after a 30-min cycle, at 25 Hz, in Tissuelyser (Qiagen®), that improves the yield of DNA extraction. To quantify the pathogen present on the surface of the seeds, two sub-samples of 50 seeds from each batch were washed twice, dipping the seeds in PEG 4600 (50 g/L) + KOH (20 mM) solution for 40 min. Seeds were then removed, and the solution was centrifuged at 6000 rpm for 20 min. The supernatant was removed, and the pellets resuspended in 2 mL of sterile Ringer solution. The sample was transferred in a 2 mL microcentrifuge tube and centrifuged at the maximum speed (25,000 rpm) for 5 min. At the end, the supernatant was removed, and the pellet was used for the DNA extraction with Omega E.Z.N.A.® Fungal DNA mini kit (Omega Bio-Tek) according to the manufacturers' instructions, after a 15-min cycle, at 25 Hz, in Tissuelyser (QIAGEN®). The extracted DNA was quantified with the spectrophotometer Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Two sub-samples of 25 seeds from each line were subjected to a DNA crude extraction where the seeds and 2 mL of a PEG 4600 (50 g/L) + KOH (20 mM), an alkaline buffer (pH 13.5), were put in a 5 mL tube and then vigorously shaken for 3 min [35]. The use of this alkaline buffer allows the testing in molecular assays of a wide variety of biological samples, such as rice seeds and their fungal pathogens, directly without previously neutralization or DNA isolation steps [35].

2.5. Real-Time PCR

Real-time PCR assays were carried out with specific primers TqF2/TqR and probe FfujiPq [29] and GoTaq® Probe qPCR Master Mix mix (Promega, Madison, WI, USA) to quantify *F. fujikuroi*. All real-time PCR tests were performed, including two biological replications for each seed batch and three technical replications for each biological replication. Each analysis included three technical replications of a positive and a negative control. The real-time PCR amplification was carried out using a StepOne Plus™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) thermal cycler, under the following conditions: 1 µL of genomic DNA as template, 5 µL of GoTaq® Probe qPCR Master Mix, 0.5 µL of each primer (5 µM), 0.25 µL of probe (0.25 µM) in a final volume of 10 µL. The reaction was carried out with an initial incubation at 50 °C for 2 min and at 95 °C for 10 min and followed by 40 cycles at 95 °C for 15 sec and at 62 °C for 1 min [30]. The results were displayed through the StepOne software, connected to the thermal cycler. A standard curve was obtained by the correlation of the Ct and the concentrations of ten-fold serial dilutions of the DNA of one strain of *F. fujikuroi*, ranging from 10 ng/µL to 100 fg/µL.

2.6. LAMP

The LAMP reaction was set up using six primers, two external (F3 and B3), two internal (FIP and BIP) and two loop primers (F-loop and B-loop), designed based on the EF-1α sequence [32]. The assays were carried out on a GenieII instrument (OptiGene Ltd., Horsham, UK) in a final volume of 10 µL containing 1 µL of genomic DNA as template, 0.5 µL of each external primer (10 µM), 0.5 µL of each internal primer (100 µM), 0.25 of each

loop primer (100 µM) and 6 µL of Isothermal Mastermix ISO-004 (1×) (OptiGene Ltd.). The amplification protocol was carried out at 65 °C for 45 min, followed by an annealing step where the fluorescence was measured while cooling from 95 °C to 70 °C at 0.05 °C/s. The same protocol can be adopted on a StepOne Plus™ Real-Time PCR System thermal cycler. The protocol was verified by the evaluation of sensitivity, specificity, repeatability and reproducibility according to the EPPO PM7/98 standard (2019) [36].

The limit of analytical sensitivity was obtained with a standard curve by the correlation of time to positive (Tp) and the concentrations of ten-fold serial dilutions of the DNA extracted from mycelium of *F. fujikuroi* isolate I1.3. The limit was also tested with single rice seeds (cv. Dorella) artificially inoculated with serial dilutions of isolate I1.3 (0.48 and 4.8 ng/mL).

To verify the analytical specificity of the protocol to detect *F. fujikuroi*, an assay was set up with DNA samples extracted from *F. proliferatum* and *F. verticillioides* (GFSC) *F. graminearum*, *F. oxysporum* and *F. solani.*

DNA extracted from rice seed (cv. Dorella) artificially inoculated with a conidial suspension of *F. fujikuroi* isolate I1.3 (0.48 and 4.8 ng/mL) was used to verify repeatability and reproducibility. The repeatability of the method was tested through the degree of concordance between three independent tests carried out under unchanged analysis and instrument conditions, in the same laboratory and by the same operator, considering the results of biological and technical replication. The reproducibility of the method was tested by different operators on different days.

The validated protocol was adopted to test the 34 rice lines to compare two DNA extraction methods: extraction with the commercial kit Omega E.Z.N.A.® Plant DNA kit and crude extraction.

2.7. Relationship between Seed Contamination and Disease Development

Four samples of 150 seeds of line SPS.FUS.14, selected for the low percentage of contamination found both in the field and because of molecular analyses, were used to determine the minimum concentration of cells of *F. fujikuroi* necessary to observe the development of the disease in the seedlings. The seeds were artificially inoculated with serial dilutions of a conidial suspension of *F. fujikuroi* (10^2, 10^3, 10^4 and 10^5 conidia/mL), while a sample of 150 seeds was used as a healthy control. Sixty seeds of each group were used for the DNA extraction from the whole seed with Omega E.Z.N.A.® Plant DNA kit with a preliminary lysis with liquid nitrogen, while other 60 seeds of each group were used for the DNA extraction from the surface of the seeds with serial washes in PEG 4600 (50 g/L) + KOH (20 mM), followed by using the commercial kit Omega E.Z.N.A.® Fungal DNA mini kit. In addition, 30 seeds of each group were sown in plastic trays filled with sterile soil and incubated in the greenhouse at 21 ± 2 °C. The test lasted 4 weeks, during which inspections were carried out at 7, 14, 21 and 28 days after sowing to record symptoms of *F. fujikuroi*. Real-time PCR was carried out to quantify the pathogen [30]. The results obtained were used to calculate the number of *F. fujikuroi* cells per sample, using the parameter of interest "Quantity mean", which is the average amount of DNA of *F. fujikuroi* of the three technical replications of the same sample analyzed. From this parameter, we calculated the approximate number of fungal cells in each sample by dividing the DNA quantity by the weight of the genome of the pathogen (0.000048 ng) [37].

2.8. Data Analyses

Baseline range, threshold cycle (CT) values and real-time PCR standard curves were automatically generated using StepOne software, while time to positive (Tp) and temperature of annealing of the LAMP products were visualized using Genie explorer software. Statistical analyses of the data were performed with the Statistical Package for Social Science (SPSS, IBM, Chicago, IL, USA) version 27.0.

3. Results

3.1. Seed Health Evaluation

The incubation tests with blotter method showed a percentage of seeds infected with *F. fujikuroi* always lower than 2.5%. These results, which are not statistically significant, may be due to a low sensitivity of the detection method or a low incidence of the pathogen in the seeds. The growing out assays carried out in the greenhouse showed a high variability among the different seed lines as percentage of plants with bakanae symptoms. Statistical analyses showed the highest percentage of plants showing symptoms of *F. fujikuroi* in lines SPS.FUS.01 and SPS.FUS.20, with values of up to 89% (Figure 1). The evaluation of the percentage of plants infected in the field did not show significant differences among the lines, with a percentage of symptomatic plants always lower than 14% (Table 2).

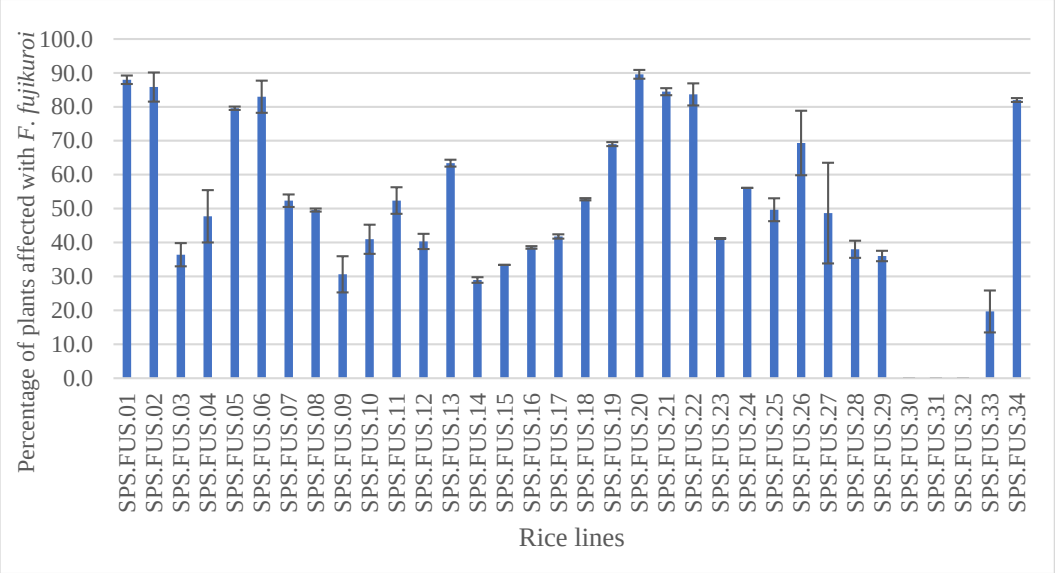

Figure 1. Percentage of plants grown in the greenhouse, affected with *F. fujikuroi*. The values are the means ± standard deviations (ANOVA, Tukey test, $p \leq 0.05$).

3.2. Real-Time PCR

Analytical sensitivity was assessed setting up a standard curve with ten-fold serial dilutions of DNA of *F. fujikuroi*. The cycle threshold (Ct) was interpolated with the initial concentration of the pathogen in the standard curve and the regression line was obtained with the determination coefficient (R^2) value of 0.9992. The minimum Ct recorded corresponds to a concentration of *F. fujikuroi* of 10 ng/µL while the highest Ct to a concentration of the pathogen was 100 fg/µL. The average reaction efficiency, calculated from the slope of the line, was 97% (Figure 2A,B).

Table 2. Percentage of seeds and plants infected with *F. fujikuroi* observed with blotter method (A), in the greenhouse (B) and in the field (C), in the 34 seed lines used in this study. Quantitation (pg/g) of *F. fujikuroi* in the seeds (D) and on their surface (E) with RealTime PCR with TaqMan probe and amplification with LAMP assay (Tp and T° annealing) of *F. fujikuroi* extracted by the seeds with commercial kit (F) and with crude extraction (G). * ND = not detected.

		Blotter method	Greenhouse	Field	RealTime PCR with TaqMan Probe		LAMP			
		A	B	C	D	E	DNA Extraction with Commercial Kit F		Crude extraction G	
Sample	Cultivar	Percentage of Seeds with *F. fujikuroi* (%)	Percentage of Plants affected by *F. fujikuroi* (%)	Percentage of Plants Affected by *F. fujikuroi* (%)	Average Quantity of *F. fujikuroi* (pg/g)	Average Quantity of *F. fujikuroi* on the Surface (pg/g)	Tp (min:sec)	T° Annealing (°C)	Tp (min:sec)	T° Annealing (°C)
SPS.FUS.01	SOLE CL	0.25	88.0	9.0	175.7	0.2	33:05	89.52	28:12	88.2
SPS.FUS.02	SOLE CL	0.0	85.8	14.0	200.2	ND	28:53	89.22	28:31	88.2
SPS.FUS.03	SOLE CL	0.0	36.4	7.0	ND *	ND	23:27	89.37	ND	75.32
SPS.FUS.04	SOLE CL	0.0	47.7	9.0	ND	ND	ND	74.88	ND	75.32
SPS.FUS.05	SOLE CL	0.75	79.6	11.0	44.4	1.1	29:31	88.33	28:39	88.48
SPS.FUS.06	SIRIO CL	0.75	83.0	6.0	375.6	0.2	26:09	88.47	27:53	88.18
SPS.FUS.07	SIRIO CL	0.0	52.3	5.0	5.5	ND	ND	75.46	35:11	88.34
SPS.FUS.08	SIRIO CL	0.25	49.6	6.0	1.7	ND	36:01	88.3	31:14	88.34
SPS.FUS.09	SIRIO CL	0.25	30.6	4.0	ND	ND	21:26	89.96	ND	75.45
SPS.FUS.10	SIRIO CL	0.25	41.0	4.0	ND	ND	ND	75.16	ND	75.3
SPS.FUS.11	MARE CL	1.0	52.4	4.0	ND	ND	ND	74.88	ND	74.99
SPS.FUS.12	MARE CL	0.25	40.3	4.0	ND	ND	ND	75.47	32:13	88.88
SPS.FUS.13	MARE CL	0.25	63.4	3.0	25.4	0.4	ND	75.47	ND	75.31
SPS.FUS.14	MARE CL	0.0	28.9	2.0	ND	ND	ND	74.57	ND	75.31
SPS.FUS.15	MARE CL	0.25	33.4	3.0	1.4	ND	29:15	88.92	24:38	89.07
SPS.FUS.16	LUNA CL	0.0	38.5	3.0	ND	ND	33:50	89.07	21:14	89.07
SPS.FUS.17	LUNA CL	2.5	41.8	4.0	9.3	ND	ND	74.72	21:56	89.07
SPS.FUS.18	LUNA CL	0.75	52.7	3.0	9.9	ND	ND	74.87	ND	75.45
SPS.FUS.19	LUNA CL	0.75	69.0	3.0	6.3	2.6	25:41	89.07	24:31	89.08
SPS.FUS.20	LUNA CL	1.0	89.6	6.0	8656.5	3.8	21:14	89.07	26:24	88.93
SPS.FUS.21	BARONE CL	1.5	84.5	2.0	146.9	3.4	30:00	88.78	27:29	88.92
SPS.FUS.22	BARONE CL	0.5	83.7	4.0	216.2	ND	ND	74.87	25:10	88.63
SPS.FUS.23	BARONE CL	0.0	41.2	3.0	5.9	0.8	ND	74.72	27:06	88.64
SPS.FUS.24	BARONE CL	0.75	56.1	6.0	5.1	ND	ND	75.01	29:13	88.33

Table 2. *Cont.*

		Blotter method	Greenhouse	Field	RealTime PCR with TaqMan Probe		LAMP			
		A	B	C	D	E	DNA Extraction with Commercial Kit F		Crude extraction G	
Sample	**Cultivar**	**Percentage of Seeds with *F. fujikuroi* (%)**	**Percentage of Plants affected by *F. fujikuroi* (%)**	**Percentage of Plants Affected by *F. fujikuroi* (%)**	**Average Quantity of *F. fujikuroi* (pg/g)**	**Average Quantity of *F. fujikuroi* on the Surface (pg/g)**	**Tp (min:sec)**	**T° Annealing (°C)**	**Tp (min:sec)**	**T° Annealing (°C)**
SPS.FUS.25	BARONE CL	0.75	50.0	3.0	22.8	1.5	ND	74.57	ND	75.31
SPS.FUS.26	SA1903	0.0	70.0	3.0	11.7	1.1	ND	74.72	ND	75.31
SPS.FUS.27	SA1903	0.0	49.0	3.0	ND	ND	ND	74.87	ND	75.47
SPS.FUS.28	SA1903	0.0	38.0	2.0	8.3	ND	ND	75.16	37:07	88.19
SPS.FUS.29	SA1903	0.0	36.0	3.0	ND	ND	ND	75.02	33:37	89.07
SPS.FUS.30	VIALONE NANO	0.75	.	.	2.3	0.7	ND	75.16	ND	75.02
SPS.FUS.31	VIALONE NANO	0.25	.	.	2.9	ND	ND	74.57	ND	75.17
SPS.FUS.32	VIALONE NANO	0.0	.	.	1.5	ND	ND	75.16	ND	75.61
SPS.FUS.33	SELENIO	0.25	20.0	4.0	12.8	ND	28:10	88.42	32:12	88.8
SPS.FUS.34	DORELLA	0.25	82.0	6.0	20.3	ND	23:40	88.12	21:05	89.07

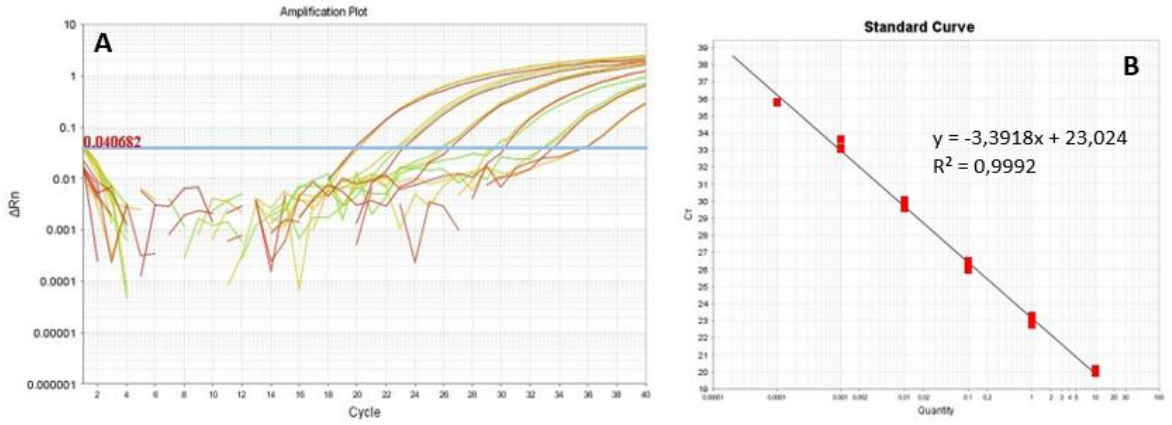

Figure 2. (**A**) Amplification with real-time PCR assay of serial dilutions of DNA of *F. fujikuroi* I1.3. The threshold cycle values (Ct) of the fungal DNA ten-fold serial dilutions are represented in red and green. (**B**) Regression line obtained. The equation of the line and the coefficient of determination are shown in the graph.

Tests carried out on the rice samples showed the sensitivity of the method in quantifying the DNA of *F. fujikuroi* both when extracted from the whole seed and from the seed surface only. A high quantity of *F. fujikuroi* was detected in sample SPS.FUS.20, with an average concentration of 8.66 ng/g in the whole seed (Figure 3) and an average concentration of 3.8 pg/g on the seed surface that did not show statistically significant differences from the other rice lines. Ten out of 34 rice seed lots tested produced a negative result, indicating absence or a low concentration not detectable by the probe of *F. fujikuroi* (Table 2).

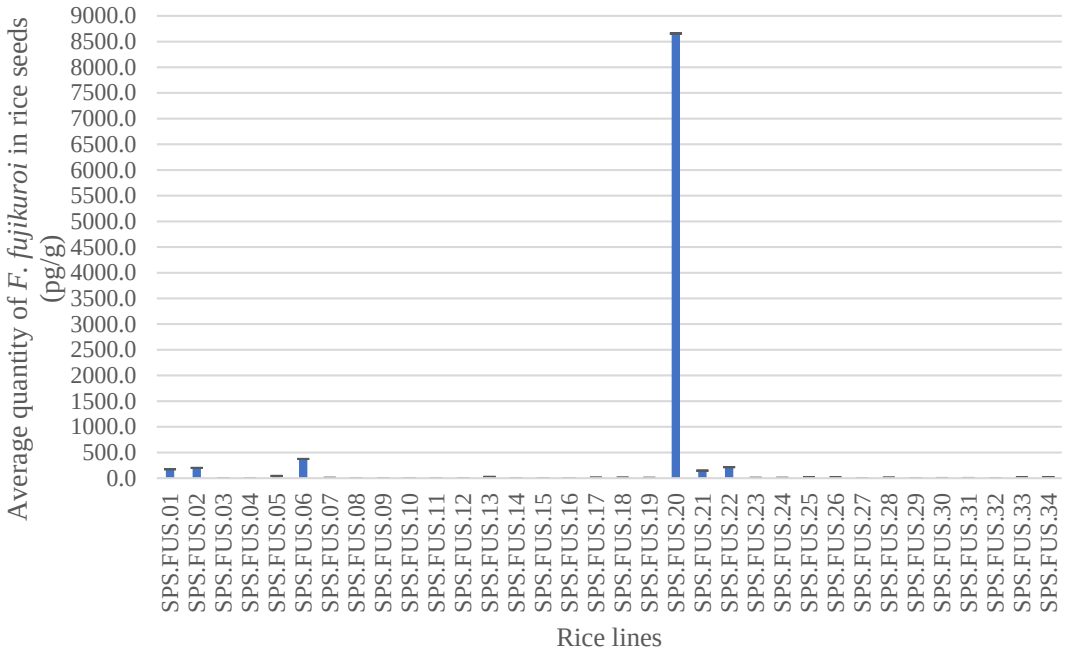

Figure 3. Average quantity of *F. fujikuroi* in the whole seed obtained by the detection of 34 rice lines with real-time PCR with TaqMan probe in triplicate. The values are the means ± standard deviations (ANOVA, Tukey test, $p \leq 0.05$).

3.3. LAMP

Analytical sensitivity of the LAMP reaction was determined with a standard curve obtained by correlating Tp and ten-fold serial DNA concentrations extracted from fresh

mycelium of *F. fujikuroi* (I1.3). The curve set up by testing seven serial DNA dilutions of I1.3 showed amplification for the first six points (from 10 ng/µL to 100 fg/µL), determining the amount of 100 fg/µL as the sensitivity limit of the reaction (Figure 4). The analytical sensitivity limit was also tested on artificially inoculated single rice seeds (cv. Dorella) with serial dilutions (0.48 and 4.8 ng/mL) of the pathogen *F. fujikuroi*. The results of the amplifications obtained are shown in Table 3, confirming the sensitivity of the method to detect the pathogen in seeds.

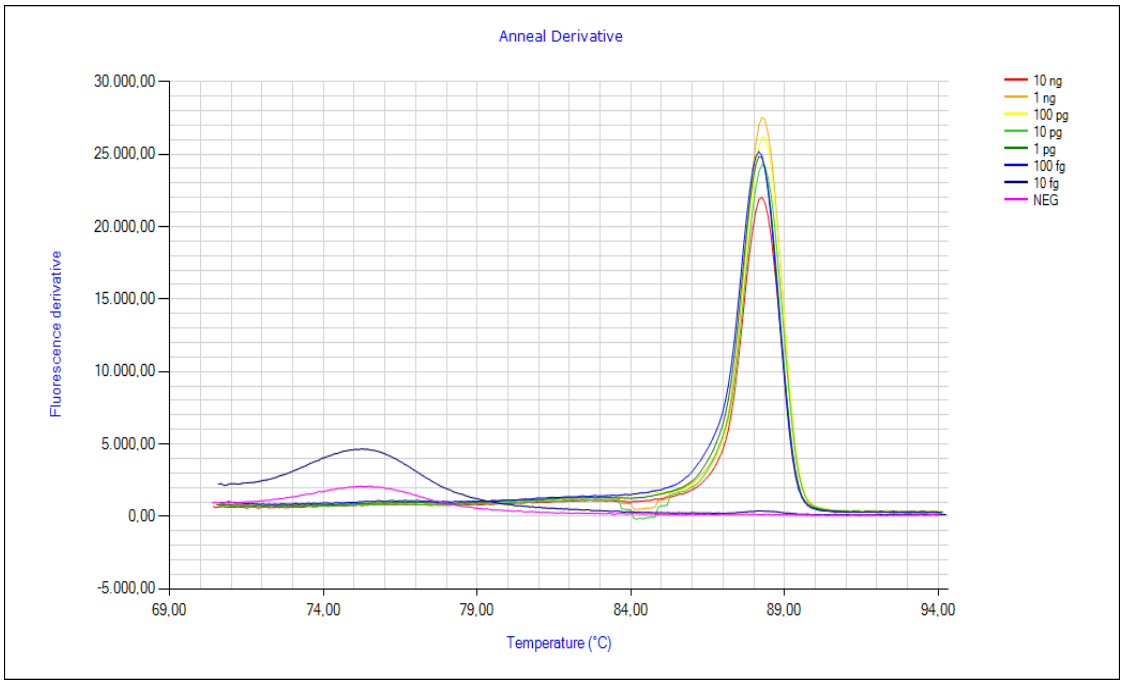

Figure 4. Analytical sensitivity of the LAMP assay obtained by the amplification of ten-fold serial dilutions of DNA of *F. fujikuroi* (I1.3) from a concentration of 10 ng/µL to 100 fg/µL.

Table 3. Results of the analytical sensitivity limit of the LAMP assay for *F. fujikuroi* on artificially inoculated rice seeds cv. Dorella. The table shows Time to positive (Tp) and average annealing temperature of the three technical replicates tested.

Sample	Concentration of the Inoculum (ng/mL)	Tp (min:sec)	T° Annealing
Seed 1	0.48	28:15	87.7
Seed 2	0.48	30:20	87.9
Seed 3	0.48	29:45	87.8
Seed 1	4.8	27:50	88.2
Seed 2	4.8	28:10	88.2
Seed 3	4.8	27:45	88.2

The LAMP assay was set up testing *F. graminearum*, *F. solani* and *F. oxysporum* f. sp. *lactucae* and three isolates belonging to the GFSC, *F. fujikuroi*, *F. verticillioides* and *F. proliferatum* to evaluate the analytical specificity of the reaction, that was confirmed by the amplification of *F. fujikuroi* only (Figure 5). The repeatability and reproducibility of LAMP assays for *F. fujikuroi* were confirmed by producing 100% reliable amplifications among replications, both biological and technical, when tested under unchanged analysis conditions or with different operators.

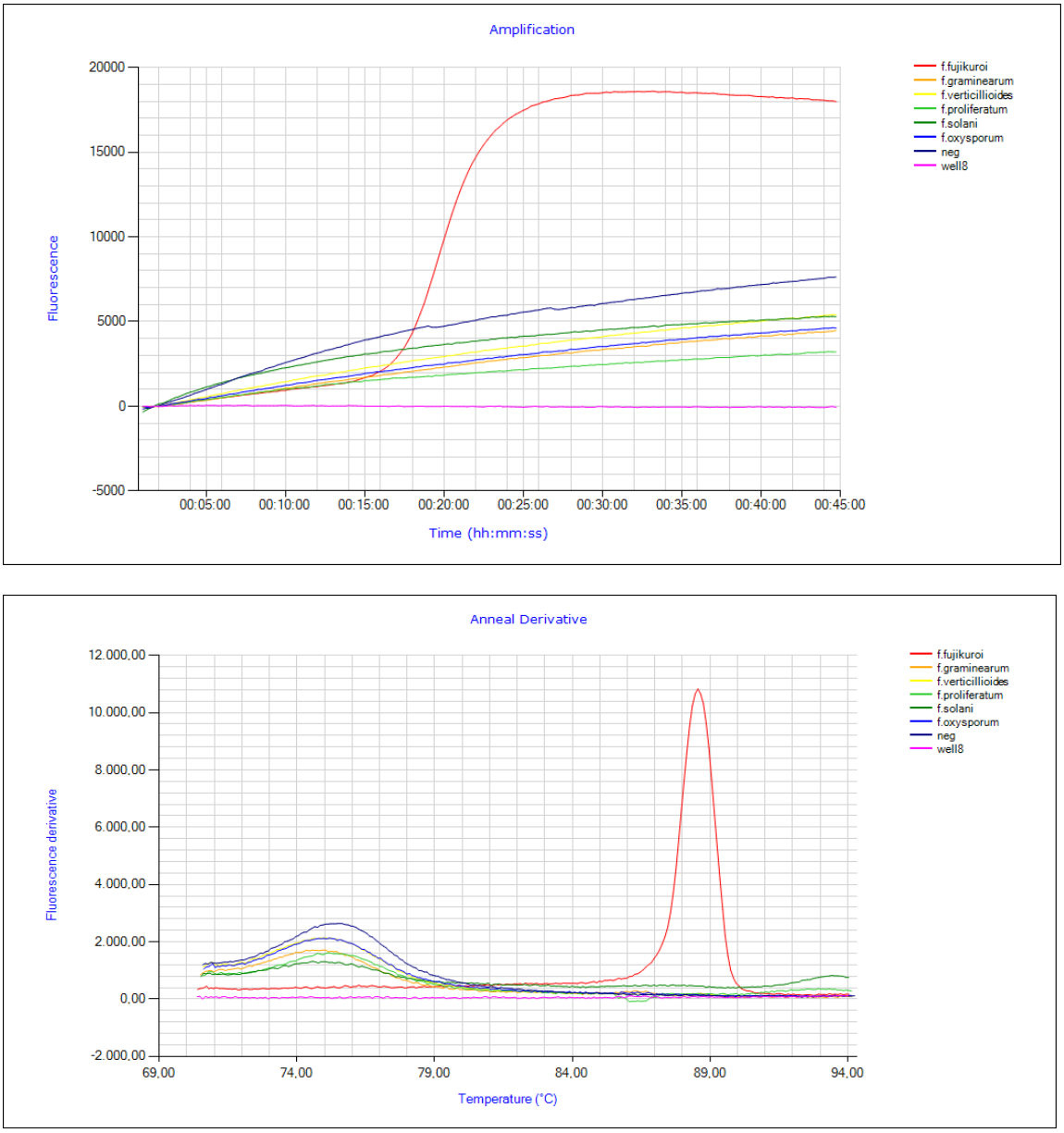

Figure 5. Validation of the analytical specificity of the LAMP reaction obtained with amplification of *F. fujikuroi* DNA (I1.3) compared to five other isolates of *Fusarium* belonging to the GFSC, *F. proliferatum* and *F. verticillioides*, and *F. graminearum*, *F. oxysporum* and *F. solani*.

The results of the analyses carried out on the 34 rice lines tested are listed in Table 2. Positive amplifications were obtained with LAMP assays both with DNA extracted with the commercial kit E.Z.N.A. and with DNA obtained with crude extraction. Higher analytical sensitivity in the detection of *F. fujikuroi* was observed in six seed lines over a total of 34 tested with the use of DNA from crude extraction (Figure 6).

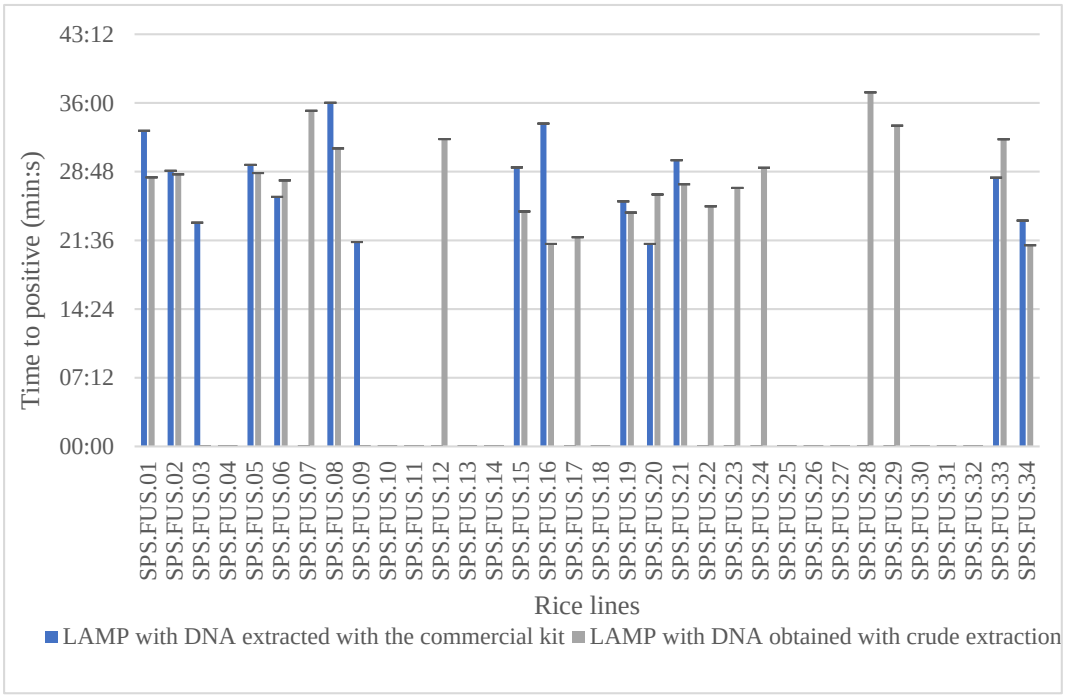

Figure 6. Results obtained with LAMP in which 34 rice lines were used and the DNA was extracted using a kit extraction method and a crude extraction method in triplicate. The graph shows the time to positive (Tp) obtained using both types of DNA extraction method among the different rice lines. The values are the means ± standard deviations (ANOVA, Tukey test, $p \leq 0.05$).

3.4. Relationship between Seed Contamination and Disease Development

The DNA extracted from whole seeds and from seed surface from artificially inoculated rice samples with ten-fold serial cell suspensions of *F. fujikuroi* was tested with a real-time PCR assay to quantify the pathogen and the results were used to quantify the number of *F. fujikuroi* cells per sample. From the quantity mean of each sample, the number of cells/µL was calculated. At the same time, artificially inoculated seeds were sown in the greenhouse and the percentage of symptomatic plants for each concentration of inoculum was recorded. Seed lines obtained from seeds inoculated with a conidial suspension of 10^2 conidia/mL did not show symptoms of the disease, while symptomatic seedlings were observed when seed was inoculated at 10^3, 10^4 and 10^5 conidia/mL. The minimum concentration of the fungus necessary to observe the development of the disease corresponds to a concentration of 4.17×10^4 cells/µL of *F. fujikuroi* (Table 4).

Table 4. Number of cells/µL of *F. fujikuroi* detected in seed samples artificially inoculated with ten-fold serial dilution of the pathogen.

Sample	Inoculum Concentration (Conidia/mL)	Number of Cells/µL	Number of Cells/µL on the Surface
Seed sample 1	1×10^2	4.58×10^3	ND
Seed sample 2	1×10^3	4.17×10^4	ND
Seed sample 3	1×10^4	9.13×10^4	ND
Seed sample 4	1×10^5	1.90×10^5	ND
Seed sample 5	None	ND	ND

4. Discussion

The aim of the study was to optimize and validate a rapid, sensitive and efficient molecular diagnostic technique to replace the blotter test in seed health procedures, due to

its higher sensitivity, specificity, fast turnaround results delivery, on-site application and the possibility of quantifying endophytic seed infections. Such a tool could represent an advantage for seed companies that may detect *F. fujikuroi* in rice seeds before sowing and decide to adopt seed dressing or other seed treatments.

The evaluation of seed health shows different percentages of infection of each seed lot when detected with different methods as a function of the sensitivity of the method and of the external conditions that can influence the development of the disease such as temperature, humidity and fertilization [20]. Blotter tests did not provide statistically significant results for seed health assessment in this study. Significant results were obtained in the greenhouse growing out tests that were carried out under controlled conditions of temperature and relative humidity. These parameters, like temperatures from 27 °C to 30 °C [18], create favorable conditions for the development of the disease and the appearance of the symptoms on plants affected by *F. fujikuroi*.

In the field, percentages of symptomatic plants were lower than in the experiments carried out in the greenhouse. These data could be influenced by environmental and crop management conditions. In fact, the rice field where the trial was carried out was often dry, influencing and the expression of symptomatic plants.

Moreover, the different degrees of virulence among different strains of *F. fujikuroi* present on or in rice seeds and plants in the same field and in the same cultivar can influence the level of infection in the field [18].

When the level of infection on or in whole rice seeds was assessed with real-time PCR it was observed that the endophytic infection was related to greenhouse expression of symptoms, although not statistically significant.

The quantitation of *F. fujikuroi* with real-time PCR showed different values among the different seed lines, as a function of the level of susceptibility of the cultivar, their origin and the environment and the soil conditions during their production. The survival of the fungus in the grains was strictly correlated to the soil temperature that is optimal at 35 °C [17] and the seeds infection can increase at high temperature during the flowering stage [38]. The disease incidence is also related to the genetic characteristics of the cultivar. Several studies have shown that aromatic cultivars were more sensitive that the non-aromatic ones [39], coarse varieties were more resistant [40] and the susceptibility may change throughout the crop stages [15]. The study highlighted that the microbiological component present on the seed surface did not affect the incidence of the disease, while the endophytic component of *F. fujikuroi* was relevant. Sunani et al. [41] hypothesized that the fungus can infect seeds and reach the embryo in three ways: by systemic infection of the vascular system, by stylar canal, or through the growth of hyphae from the husk to the ovary. Furthermore, the microconidia of *F. verticillioides* can represent a source of infection of the embryo [42] and it is possible that the conidia of *F. fujikuroi* also follow this inoculation mechanism and systemically infect the seeds [41]. This information is important because it supports the choice of the most effective chemical treatment for disease control [5].

A LAMP assay, based on the protocol described by Franco-Ortega et al. [32], was validated verifying the efficiency of the reaction by setting up a curve of standards obtained from ten-fold serial dilutions of DNA of *F. fujikuroi*. The original protocol was optimized reducing the final volume of the reaction to 10 µL. The method was validated following the EPPO (PM7/98) guidelines to determine the analytical sensitivity, specificity, repeatability and reproducibility. The analytical sensitivity of the LAMP assay was determined by testing ten-fold serial dilutions of *F. fujikuroi* and corresponds to a concentration of 100 fg/µL. The specificity was confirmed by testing the DNA of *Fusarium* species belonging or not to the same species complex and highlighting the amplification of only *F. fujikuroi*. Repeatability and reproducibility were confirmed by replication of LAMP tests under unchanged and modified test conditions. The validated protocol was used to quantify *F. fujikuroi* in the commercial rice seed lines testing both the DNA extracted with the commercial kit "E.Z.N.A. Plant DNA kit" of Omega Bio-Tek and crude extraction. The use of crude extraction produces more sensitive results in the detection of the pathogen, and it simplifies

the diagnostics assays, reducing time and enabling the processing of many samples with lower costs [35].

Results obtained with artificial single seed inoculation with serial DNA dilutions and detection with real-time PCR showed that the minimum amount necessary for the development of the disease in the greenhouse corresponds to a concentration of 4.17×10^4 cells/µL of *F. fujikuroi* in the seeds. Therefore, this result can be considered as a warning threshold for seed phytosanitary quality assessment. A similar evaluation was made on the quantity of *F. fujikuroi* detected with real-time PCR carried out with DNA extracted from artificially inoculated single rice seeds by Franco-Ortega et al. [32].

5. Conclusions

This study confirms the on-site applicability of the LAMP method as a fast, effective and sensitive diagnostic technique, such as published previously by Rong et al. [43], who developed a similar technique based on the IGS region. The advantage of this diagnostic tool is represented by the easy application of the protocol by field operators without prior molecular experience or the support of a laboratory structure [44]. In addition, the use of the crude extraction allows obtaining a sensitive screening of the level of infection with *F. fujikuroi* in rice seed batches. Finally, the evaluation of the minimum concentration of the pathogen necessary for the development of the disease represents a way to select rice batches and exclude those with high levels of contamination, which would lead to consequent yield and economic losses for the seed company.

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