1 *Running head recto: F. oxysporum f. sp. lactucae LAMP assay*

2 *Running head verso: S. Franco Ortega et al.*

3 **Rapid detection of *Fusarium oxysporum* f. sp. *lactucae* on soil, lettuce**
4 **seeds and plants using loop-mediated isothermal amplification**

5 S. Franco Ortega^{ab}, J. Tomlinson^c, G. Gilardi^a, D. Spadaro^{ab*}, M.L. Gullino^{ab}, A. Garibaldi^a
6 and N. Boonham^{cd}

7 ^aCentre of Competence for the Innovation in the Agro-environmental Sector - AGROINNOVA,
8 University of Turin, via Paolo Braccini 2, I-10095 Grugliasco, TO; ^bDepartment of Agricultural,
9 Forestry and Food Sciences (DiSAFA), University of Torino, via Paolo Braccini 2, I-10095
10 Grugliasco, TO, Italy; ^cFera, Sand Hutton, York; and ^dNewcastle University, Newcastle upon Tyne,
11 NE1 7RU, UK

12 *E-mail: davide.spadaro@unito.it

13

14 *Fusarium oxysporum* f. sp. *lactucae* (FOL) is a soil- and seedborne pathogen and the causal agent of
15 fusarium wilt on lettuce. Four races have been identified within FOL, with different worldwide
16 distribution. Several molecular techniques have been used to detect and identify this pathogen;
17 however, not all of them have the optimal characteristics in terms of sensitivity to perform FOL
18 detection in plant and seed material. A loop-mediated isothermal amplification (LAMP) assay was
19 developed based on the sequence-characterized amplified region (SCAR) obtained in a previous
20 rapid amplification of polymorphic DNA (RAPD) study. The LAMP assay has been validated
21 according to the EPPO standard PM7/98. The LAMP assay was tested with lettuce seeds, soil and
22 plant material, and can be used successfully to amplify DNA from each of these matrices. In seed

lots artificially inoculated with FOL, the detection limit of the LAMP test was 0.004% infected seed.

Keywords: forma specialis, fusarium wilt, *Lactuca sativa*, LAMP, seedborne pathogen, soilborne pathogen

Introduction

Fusarium oxysporum f. sp. *lactucae* (FOL) is a soil- and seedborne pathogen (Garibaldi *et al.*, 2004) and the causal agent of fusarium wilt on lettuce (*Lactuca sativa*). The pathogen was initially described in Japan by Motohashi *et al.* (1960) and later identified as *Fusarium oxysporum* f. sp. *lactucae* (FOL) by Matuo & Motohashi (1967). In 1993, Hubbard & Gerik identified the causal agent of a lettuce disease in California (USA) as *Fusarium oxysporum* f. sp. *lactucum*, which was confirmed to be the same agent of the root rot in Japan. Fusarium wilt is a widespread lettuce disease (Fujinaga *et al.*, 2001; Garibaldi *et al.*, 2002; Malbrán *et al.*, 2014), which can cause the total destruction of the crop in severe outbreaks (Garibaldi *et al.*, 2002). In Italy, FOL causes important losses, especially in Lombardy (northwestern Italy), due to the intensive cultivation of lettuce in the same area, year after year (Garibaldi *et al.*, 2002).

Four races have been identified within this forma specialis. Race 1, pathogenic on Patriot and Banchu Red Fire lettuce cultivars, was described in Japan in 1967 (Matuo & Motohashi, 1967) and is the most widespread race, reported in the USA, Iran, Taiwan, Brazil, Portugal, Argentina and Italy (Hubbard & Gerik, 1993; Huang & Lo, 1998; Millani *et al.*, 1999; Garibaldi *et al.*, 2002; Marques Ramalhete *et al.*, 2006; Ventura & Costa, 2008; Malbrán *et al.*, 2014). Race 2, pathogenic on Patriot and Costa Rica No. 4 but not on Banchu Red Fire lettuce cultivars, has been reported only in Japan (Fujinaga *et al.*, 2001, Fujinaga *et al.*, 2005). Race 3, pathogenic on Patriot, Costa Rica No. 4 and Banchu Red Fire lettuce cultivars, is present in Japan and Taiwan (Fujinaga *et al.*,

2003; Lin *et al.*, 2014), while race 4 has been recently identified in the Netherlands by using pathogenicity tests and molecular analysis (Gilardi *et al.*, 2016). Vegetative compatibility groups (VCG) have been used to determine the relationship among races and formae speciales of *Fusarium oxysporum* (Pasquali *et al.*, 2005; Pintore *et al.*, 2017). Four VCGs were reported within FOL corresponding to the four races respectively (Fujinaga *et al.*, 2005; Pasquali *et al.*, 2005; Pintore *et al.*, 2017).

The easy trade of plant material between countries provides an inadvertent source of pathogen dissemination. The detection of seedborne pathogens implicated in crop yield losses is a critical point in the management of plant diseases. Garibaldi *et al.* (2004) confirmed the rapid spread of fusarium wilt on lettuce around the world due to FOL contaminated seeds.

Seed dressing with fungicides is often performed to reduce the inoculum of seedborne pathogens and to possibly obtain certified pathogen-free seed. Despite the fact that fungicides produce the most effective control against this lettuce pathogen, the current limitations in the use of chemical treatments, favoured at the European level by the adoption of the Directive 2009/128/EC, is boosting the use of prevention or alternative strategies including the use of healthy seeds.

The identification of the formae speciales within the species *F. oxysporum* has been traditionally carried out according to pathogenicity tests and morphological criteria such as size and shape of the macroconidia, the presence of microconidia or chlamydospores, and colony characteristics, such as colour or conidiophore. However, these methods do not always permit accurate identification. Molecular techniques enhance the identification of *F. oxysporum* formae speciales, and methods such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphism (AFLP), sequence-specific amplified polymorphisms (SSAP) (Baayen *et al.*, 2000; Pasquali *et al.*, 2008; Gilardi *et al.*, 2016) and end-point or TaqMan real-time PCR (Suga *et al.*, 2013) based on sequence-characterized amplified region (SCAR) have all been used. Mbofung & Pryor (2010) developed specific primers

72 based on the intergenic spacer region of the ribosomal DNA to detect infected lettuce seed samples.
73 These molecular techniques are widely used to identify a high number of formae speciales and
74 races. However, these methods do not always show optimal characteristics in terms of specificity
75 and sensitivity for seed testing. The FOL aggressiveness combined with the rapid spread amongst
76 areas of lettuce cultivation makes a quick, easy and accurate test to detect the pathogen in seed
77 highly desirable.

78 Loop-mediated isothermal amplification (LAMP) developed by Notomi *et al.* (2000)
79 consists of isothermal amplification of the target nucleic acid. The reaction is promoted by the high
80 strain displacement activity of the enzyme. This technique can be used to detect different pathogens
81 including bacteria, fungi and viruses (Tomlinson *et al.*, 2010a; Bühlmann *et al.*, 2013).
82 Fluorescence monitoring with portable and rechargeable battery machines such as the Genie II or
83 Genie III instruments (OptiGene Ltd) allows on-site testing, which is particularly useful for
84 reducing the time taken to make rapid decisions. The high sensitivity and specificity of the LAMP
85 assay makes it a strategic technology to perform seed testing (Franco Ortega *et al.*, 2018).

86 The aim of this work was to develop, test and validate a LAMP assay specific for FOL,
87 which will be used to perform quick seed and plant testing, and to evaluate its possible use in soil
88 infested with FOL.

89

90 Materials and methods

91 DNA extraction from the fungal cultures

92 Each single-spore culture of the isolates of *F. oxysporum* listed in Table 1 was grown in potato
93 dextrose broth (PDB; Sigma Aldrich) on a rotatory shaker (120 rpm) for 10 days at room
94 temperature. Mycelium was extracted by means of filtration through Whatman no. 1 filter paper.
95 The total genomic DNA was extracted using E.Z.N.A. Fungal DNA mini kit (OMEGA Bio-Tek),

96 according to the manufacturers instructions and was stored at -20°C . The DNA concentration of
97 each sample was measured using NanoDrop 2000 (Thermo Fisher) and was then adjusted to 1–50
98 $\text{ng } \mu\text{L}^{-1}$.

99 A crude extraction method was used to extract plant material, including seeds, as fast as
100 possible to replicate use during field practice. It was based on the protocol of Tomlinson *et al.*
101 (2010a) using 1 mL of PEG alkaline buffer (50 g L^{-1} PEG average Mn 4600; 20 mM KOH; pH
102 13.5) as described by Chomczynski & Rymaszewski (2006). This method included vigorous
103 manual shaking of one ball bearing (7/16" stainless steel 316 GD Spheric Trafalgar Ltd) in a 5 mL
104 tube for 1 min using individual lettuce seeds and plant material. The same crude extraction was
105 performed with manual shaking (3 min) using 400 lettuce seeds per tube as a sample.

106 The reliability of the crude extraction method was evaluated against a commercial DNA
107 extraction kit to validate it. After grinding in liquid nitrogen, the DNA of seed samples (Dorée de
108 Printemps and Romabella cultivars) was extracted using an E.Z.N.A. Plant DNA kit according to
109 the manufacturer's instructions.

110

111 **LAMP primer design**

112 Six LAMP primers comprising two external primers (F3 and B3), two internal primers (FIP and
113 BIP) and two loop primers (F-loop and B-loop) were designed for both targets according to Notomi
114 *et al.* (2000). The primers FLA0001/FLA0001R (Shimazu *et al.*, 2005) were used to perform a PCR
115 of FOL isolates (ATCC 76616, FOL Mya3040, FOL race 2 F9501, FOL race 3 MAFF744085, FOL
116 race 4 04750888) and other formae speciales listed in Table 1 and Figure 1. The 20 μL reaction
117 with 50 ng genomic DNA was performed according to the protocol of Shimazu *et al.* (2005) in a
118 2720 thermal cycler (Applied Biosystems). The amplification products were checked by means of
119 electrophoresis in a 1% agarose gel (Eppendorf). The positive PCR products were purified using a

120 QIAquick PCR Purification kit and sequenced in both directions using the sequencing service of
121 BMR Genomics (Padova, Italy).

122 The sequences were merged into contigs using DNA BASER (Heracle BioSoft SRL) and an
123 alignment was carried out using MEGA v. 6.0.6. software (Tamura *et al.*, 2013). Single-nucleotide
124 polymorphisms (SNP) were targeted in the design of the LAMP primers. Potential secondary
125 structures and hairpins were checked using OLIGO CALC
126 (<http://biotools.nubic.northwestern.edu/OligoCalc.html>), while the possible interaction between
127 primers was checked using MULTIPLE PRIMER ANALYZER (Thermo Scientific;
128 [https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-](https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html)
129 [biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-](https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html)
130 [primer-analyzer.html](https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html)). The specificity of the primers was checked by means of BLASTN analysis at
131 the NCBI web portal (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). HPLC purified primers were
132 synthesized by Eurofins and are available in kit format from OptiGene Ltd
133 (<http://www.optigene.co.uk>).

134

135 **LAMP assay reaction**

136 The 25 μ L LAMP reaction included 200 nM of each external primer (F3 and B3), 2 μ M of each
137 internal primer (FIP and BIP), 1 μ M of each loop primer, 1 $\times$ Isothermal Mastermix ISO-004
138 (OptiGene Ltd) with 1 μ L DNA or crude plant extract. The LAMP protocol was carried out with a
139 Genie II instrument and adopted to use with a StepOne (Applied Biosystem) as follows: 45 min at
140 65 $^{\circ}$ C, followed by annealing using a reduction from 95 to 70 $^{\circ}$ C at increments of 0.05 $^{\circ}$ C s $^{-1}$. The
141 StepOne Plus real-time PCR system was setup for a quantification experiment with a standard cuve
142 and for using TaqMan reagents (6-carboxyfluorescein (FAM) as reporter and non fluorescent
143 quencher – minor groove binder (NFQ-MGB) as quencher. Seed testing was performed on the

144 following cultivars (seed supplier in parentheses): Dorée de Printemps (Vilmorin), Romabella
145 (Blumen), Costa Rica No. 4 (Rijk Zwaan, Ordino (Nunhems), Juanita (Syngenta), Boeing (Maraldi
146 Sementi), Volare (Rijk Zwaan) and Ricetto (Franchi Sementi). AThe protocol was as follows: 40
147 cycles at 65 °C with fluorescence measured each minute and a melting curve with 95 °C for 15 s, 70
148 °C for 1 min and an increase of the temperature to 95 °C at increments of 0.3 °C s⁻¹ to record the
149 fluorescence. A negative control and a positive control were included in each run.

150 A cytochrome oxidase gene (*cox*) LAMP assay developed by Tomlinson *et al.* (2010b) was
151 used as a plant DNA control to confirm negative results.

152

153 **Sensitivity of LAMP in seed test**

154 In order to check the sensitivity of the LAMP assay, a lettuce seed sample (Dorée de Printemps)
155 was used that was naturally free from FOL and had been checked by the agar method (Mathur &
156 Kongsdal, 2003). The seeds were prepared for inoculation with the four races of FOL after being
157 treated with sodium hypochlorite (2%) for 2 min, washed with distilled water and air dried.

158 The isolates SB1-1, F9501, FLK1001, MAFF744086, corresponding to the four races of
159 FOL, were grown in potato dextrose broth (PDB) at room temperature for 1 week. The conidial
160 suspension were counted by means of a haemocytometer, obtaining a concentration of 8.2×10^5
161 CFU mL⁻¹ for SB1-1, 1.0×10^4 CFU mL⁻¹ for F9501, 1.3×10^5 CFU mL⁻¹ for FLK1001, and $8.0 \times$
162 10^5 CFU mL⁻¹ for MAFF744086. Each sample was 10-fold serial diluted to obtain inoculum
163 suspensions ranging from 10^5 to 10^4 CFU mL⁻¹ to be used for lettuce seed infection.

164 Twenty-five Dorée de Printemps lettuce seeds were inoculated using 5 mL of a culture of
165 each 10-fold serial dilution suspension for each race. The inoculum was added to individual tubes
166 containing the seeds and incubated at room temperature on a rotatory shaker at 90 rpm for 45 min.
167 All the seeds were then washed with distilled water and dried overnight before LAMP testing.

168 Batches of 25 seeds of lettuce seeds Dorée de Printemps were used to determine the limit of
169 detection of infection using the LAMP tests. Batches with a 4% infection rate were prepared by
170 adding 1 infected seed to 24 healthy seeds, while batches with an 8% infection rate were prepared
171 by adding 2 infected seeds to 23 healthy seeds. Three batches at 4% and 8% infection rate were
172 prepared for the four races and for each inoculum concentration. The DNA was extracted using both
173 methods described above. All the LAMP assays were performed in triplicate.

174

175 **Commercial seed testing**

176 The final step of the validation using seeds was to evaluate the reliability of the LAMP assay. Two
177 lettuce seed samples belonging to cultivars Dorée de Printemps and Romabella collected from
178 commercial farms were used. All the seed samples were confirmed as free from FOL with an agar
179 test (Mathur & Kongsdal, 2003). In order to assess the sensitivity of the LAMP assay using
180 commercial seed samples, both lettuce seed samples were artificially inoculated using FOL race 1
181 Mya 3040 isolate by (i) spraying a conidial suspension at 10^6 conidia mL^{-1} on 40% of 200 g Dorée
182 de Printemps seeds to obtain a level of contamination corresponding to 40 infected seeds per 100
183 seeds; (ii) mixing 50% of 200 g of Romabella seeds with talcum powder containing
184 chlamydospores of the isolate to obtain a level of contamination corresponding to 50 infected seeds
185 per 100 seeds. The conidial suspension was prepared in PDB as previously described, while the talc
186 formulation for chlamydospore production of FOL Mya 3040 was prepared following the protocol
187 suggested by Locke & Colhoun (1974).

188 The DNA was extracted by each of the methods described above from sixteen subsamples of
189 25 seeds per sample, obtaining a total of 400 seeds from each artificially infected sample of Dorée
190 de Printemps and Romabella lettuce seeds.

191 In a second experiment, the two artificially infected lettuce seed samples were repeatedly
192 diluted by 10 using lettuce seeds free from FOL to obtain a range of infection rates from 40% to
193 0.004% (4 infected seed out of 100 000) or from 50% to 0.005% (5 infected seed out of 100 000).
194 The DNA of 400 hundred seeds from each sample was extracted by each of the two methods
195 described above.

196

197 **Soil and plant testing**

198 The selectivity of the assay was also evaluated using soil and plant material. Two FOL isolates
199 (FOL1/17 (*F. oxysporum* f. sp. *lactucae* race 1) and FOL 2/17 (*F. oxysporum* f. sp. *lactucae* race 1))
200 were grown in PDB for 10 days at room temperature on a rotatory shaker (120 rpm). The
201 suspensions were filtered through a sterile cheese cloth and centrifuged for 15 min at 10 000 *g*. The
202 conidial pellet was suspended in distilled water and counted by means of a haemocytometer to
203 obtain a 10⁶ CFU mL⁻¹ suspension. Lettuce seedlings, 21–25-day-old, were artificially inoculated
204 by root immersion in the conidial suspension. Seven lettuce cultivars (Romabella, Costa Rica,
205 Ordino, Juanita, Boeing, Volare, Ricetto) were used to test the LAMP assay. The experiments were
206 carried out in the greenhouse at a temperature ranging from 28 to 30 °C. Experiment 1
207 corresponded to the pathogenicity test using FOL1/17 (*F. oxysporum* f. sp. *lactucae* race 1), while
208 experiment 2 corresponded to the pathogenicity test using FOL 2/17 (*F. oxysporum* f. sp. *lactucae*
209 race 1).

210 Plants were checked for typical fusarium wilt symptoms every 10 days. Disease severity was
211 measured 30 days after inoculation using a disease index from 0 to 4: 0 = healthy plants; 1 = first
212 symptoms of leaf chlorosis, slight reduction in the development of the plant; 2 = severe leaf
213 chlorosis, evident reduction in development, sometimes asymmetric development, evident vascular

214 browning; 3 = severe leaf chlorosis, severe reduction in development and strong deformation of the
215 plant, severe vascular browning; 4 = plant totally wilted.

216 After assessing the disease severity, a crude DNA extraction was performed on the plant material as
217 described above. Additionally, 1 g of soil in which the infected plants had grown from both
218 experiments was extracted in triplicate using an E.Z.N.A. Soil DNA kit according to the
219 manufacturer's instructions. The LAMP tests were carried out as described above using 1 μ L of
220 each sample in triplicate on a StepOne Plus Real-Time PCR system. The number of FOL cells of
221 both soils was determined by qPCR.

222

223 **qPCR and nested PCR**

224 The primers developed by Shimazu *et al.* (2005) were used to quantify the DNA using SYBR
225 Green, according to the protocol of A. Cucu, Agroinnova, Turin, Italy, personal communication.
226 The quantity of FOL DNA in the soil from tests 1 and 2, and DNA from 400 seeds was measured
227 and recorded. The reaction was carried out using 1 $\times$ SYBR Green PCR Master Mix, 120 nM of each
228 primer in 25 μ L and 1 μ L of soil or seed DNA. The amplification was carried out using the
229 following protocol: 95 $^{\circ}$ C for 10 min, 40 cycles of 15 s at 95 $^{\circ}$ C, 1 min at 60 $^{\circ}$ C, and 45 s at 72 $^{\circ}$ C
230 using a OneStep Plus Real Time PCR system. A standard curve was compiled using FOL 76616
231 DNA ranging from 9.4 ng μ L⁻¹ to 9.4 fg μ L⁻¹ and negative controls with water were included in the
232 assay. The average of three technical replicates was used to calculate the DNA quantity. The C_t
233 values generated by qPCR for each sample were compared with the standard curve to obtain the
234 amount of DNA in the positive samples. The approximate number of cells present in the 400 seed
235 samples was calculated by dividing the DNA quantity by the weight of the genome of *F. oxysporum*
236 (0.0000548 ng).

237 For the specificity test, FLA001F/FLA0001R SCAR primers for FOL, designed on a RAPD
238 by Shimazu *et al.*, (2005) were used for specific PCR of FOL DNA from a pure culture. A nested
239 PCR using the primers designed by Mbofung & Pryor (2010), GYCF1 and GYCR4C in the first
240 amplification and R943 as the reverse primer for the second amplification, was carried out
241 according to the following protocol: initial denaturation at 94 °C for 1 min, and 25 cycles at 94 °C
242 for 30 s, 65 °C for 2 min 24 s, and 72 °C for 1 min. The second run of the nested PCR was carried
243 out with the following protocol: initial denaturation at 94 °C for 1 min; 25 cycles at 94 °C for 30 s,
244 63 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. This assay was
245 performed using 1 µL of DNA from the 25-seed batches, extracted with a kit, to verify the
246 reliability of the primers with seeds.

247

248 Results

249 LAMP primer design and validation

250 The primers designed by Shimazu *et al.* (2005) specific for FOL were used to design the six LAMP
251 primers. Because, the PCR using the original primers produced amplification in other non-target
252 formae speciales, such as *F. oxysporum* f. sp. *conglutinans* 58385, *F. oxysporum* f. sp. *tulipae*, *F.*
253 *oxysporum* f. sp. *conglutinans* 52557 (Fig. 1), SNPs between the PCR products of target and non-
254 target isolates were used during the design of the six LAMP primers.

255 The validation of the LAMP was carried out according to EPPO standard PM7/98. The
256 specificity was checked with an inclusivity and exclusivity panel of samples including other formae
257 speciales of *Fusarium oxysporum* and other common pathogens of lettuce. Non-target
258 amplifications were not found compared with the end-point PCR developed by Shimazu *et al.*
259 (2005), and the average time to positive (Tp), i.e. the time at which the fluorescence overcomes the
260 threshold level, which is a parameter analogous to the threshold cycling time in qPCR (Franco

261 Ortega *et al.*, 2018), was calculated. T_p ranged from 11 to 22 min whilst the average annealing
262 temperature ranged from 84.93 to 85.19 °C (Table 1). The sensitivity using 10-fold serial dilutions
263 of the DNA presented differences among the different races. The lowest quantity of DNA detected
264 corresponds to an amount of DNA ranging from 10–99 pg for FOL race 1 to 100–999 pg for FOL
265 race 2, race 3 and race 4 (Fig. 2).

266 The sensitivity of the assay was also evaluated with individually infected lettuce seeds and
267 25-seed batches at 4% (1 infected seed out of 25) and 8% (2 infected seeds out of 25) infection rate
268 using the crude extraction method. Average T_p of the five FOL infected seed replicates for each
269 race are listed in Figure 3.

270 For individually infected seeds extracted with crude extraction method, the FOL races 1 and
271 2 produced the lowest T_p (14 min 24 s and 17 min for the two inoculum concentrations of race 1,
272 and 16 min 47 s for race 2 at concentration 1×10^4 CFU mL⁻¹) in comparison with the seeds
273 infected with the other FOL races in both dilutions (T_p ranging from 17 min 22 s to 28 min 59 s;
274 Fig. 3). The best results for individually infected seeds were obtained with the seeds inoculated with
275 8.2×10^5 CFU mL⁻¹ (FOL race 1), 1×10^4 CFU mL⁻¹ (FOL race 2), 1.3×10^5 CFU mL⁻¹ (FOL race
276 3), and 8×10^5 CFU mL⁻¹ (FOL race 4), respectively. The time to positive of race 4 infected seeds
277 was higher than for race 1 and race 2 infected seeds, reaching 28 min and 59 s and 21 min 40 s for
278 each dilution. The LAMP assay did not produce positive amplification for all the replicates of seeds
279 infected with FOL race 3 and race 4. Only some of the replicates produced a positive amplification
280 using the *cox* LAMP; however, the purpose of this assay was only to interpret negative results of the
281 pathogen LAMP (Table S1).

282 The DNA from the crudely extracted 25-seed batches was not diluted in order to increase
283 the sensitivity of the technique. The 8% infection rate seed batch (2 infected out of 25 seeds)
284 produced the most reliable and repeatable result for all the races and concentrations. The best results
285 were obtained using the FOL race 1 infected seeds at 8% infection rate and at the highest

286 concentration (8.2×10^5 CFU mL⁻¹) followed by race 3, race 4 and race 2 at the highest
287 concentrations: 1.3×10^5 CFU mL⁻¹ for FOL race 3, 8×10^5 CFU mL⁻¹ for FOL race 4, and 1×10^4
288 CFU mL⁻¹ for FOL race 2. The reliability of a crude DNA extraction was compared with DNA
289 extracted using a commercial kit on 25-seed batches extracted with both methods. In all cases, the
290 T_p and the annealing temperature were slightly lower with the DNA prepared using the crude DNA
291 extraction method compared with the DNA extraction kit (Fig. 4; Table S2). The same extractions
292 were simultaneously tested using the nested-PCR developed by Mbofung & Pryor (2010) and no
293 amplification was detected in any of the samples.

294 The LAMP assay was validated according to EPPO standard PM7/98. The specificity was
295 validated using three technical replicates of the fungi listed in Table 1, using different formae
296 speciales of *F. oxysporum*, and different lettuce pathogens. The sensitivity was validated in
297 triplicate using 10-fold serial dilutions of the four races within FOL. FOL infected seed samples
298 tested individually and in batches, as explained above, were used to check the LAMP sensitivity.
299 Seed testing was performed using individual and batches seeds (three biological replicates in each
300 test) and the LAMP assay was performed in triplicate. The reproducibility was verified in different
301 machines, used by different researchers in different days. The selectivity of the LAMP assay was
302 checked using three matrices: artificially infested soil from a pathogenicity test, two batches of
303 lettuce seeds (Dorée de Printemps and Romabella), and plant material of seven lettuce cultivars
304 (Romabella, Costa Rica, Ordino, Juanita, Boeing, Volare, Ricetto). Commercial lettuce seeds were
305 used to evaluate the reliability of the assay, by testing 400 seed samples with different infection
306 rates.

307

308 **Commercial lettuce seeds**

Two commercial lettuce seed batches (Dorée de Printemps and Romabella) were used to check the reliability of the LAMP assay. The two seed samples were extracted using a commercial DNA extraction kit and crude extraction using 400 seeds in a single reaction and dividing the same number of seeds into sixteen 25-seed subsamples. The Dorée de Printemps and Romabella lettuce seed samples produced reliable and repeatable results using both types of DNA extraction with the 25-seeds subsamples, obtaining a T_p with the commercial DNA extraction kit of 22 min 57 s and 14 min 57 s for Dorée de Printemps and Romabella samples respectively (Table S4). The T_p using a crude extraction method was 17 min 01 s and 16 min 48 s on average for the Dorée de Printemps and Romabella seed samples, respectively. The annealing temperature ranged from 85.37 to 85.47 °C for the Dorée de Printemps sample and from 85.49 to 85.14 °C for Romabella seed sample. Despite the small differences in the T_p and annealing temperatures, the DNA extracted with the commercial kit produced a higher number of positive results than the DNA extracted using the crude method; DNA extracted using a kit gave 33/48 and 48/48 positives for Dorée de Printemps and Romabella respectively in comparison with 1/48 and 2/48 positives using the crude extraction methods for each cultivar (Fig. 5).

All the replicates of 400-seeds of Dorée de Printemps and Romabella produced positive amplification with the commercial DNA extraction kit at infection rates of 40% and 50%, respectively. The Dorée de Printemps seed samples at 40% infection rate also gave positive results using the crude extraction method. Using the LAMP assay for testing 400 seeds with a 10-fold dilution series of infection rates produced positive amplification until infection rates were as low as 0.004% (corresponding to 4 seeds out of 100 000) of the seeds infected with conidia and 0.05% (5 infected seeds out of 10 000) of the seeds infected with chlamydospores (Tables 2 & S4). Not all the biological replicates, such as the replicates of seeds with 0.4% infection, produced positive amplification. Cell number in seed samples was measured by qPCR. However, when testing the seed of Dorée de Printemps, not all the replicates gave a positive result; FOL was not detected by qPCR in any of the 10-fold diluted batches (from 4–5% to 0.004–0.005%). It was only possible to

335 quantify the number of cells of FOL in the 40% conidia-infected lettuce seed sample (1.1 cells
336 μL^{-1}) and the 50% chlamydospore-infected lettuce seed sample (5.05–10.42 cells μL^{-1}).

337 The 400-seed batches of Dorée de Printemps lettuce infected at 40% gave positive results
338 using crude extraction. In contrast, the PCR carried out with the primers of Mbofung & Pryor
339 (2010) did not produce any amplification using the DNA extracted from the 25-seed batches (data
340 not shown).

341

342 **Soil and plant testing**

343 Plant samples of seven lettuce cultivars (Romabella, Costa Rica, Ordino, Juanita, Boeing, Volare
344 and Ricetto) with fusarium wilt symptoms produced positive amplification using the LAMP assay
345 after a crude DNA extraction in both experiments using FOL1/17 or FOL2/17 (Fig. 6). The infested
346 soil from both pathogenicity tests also produced positive amplification using the LAMP assay after
347 DNA was extracted using a commercial DNA extraction kit (E.Z.N.A Soil DNA kit). The results of
348 the qPCR with SYBR Green showed that the number of FOL cells in the soil for FOL1/17 and
349 FOL2/17 was 12.7 and 14.37 cells μL^{-1} , respectively (Table 3).

350

351 **Discussion**

352 Different molecular markers, such as the intergenic spacer region of the ribosomal DNA, elongation
353 factor 1-alpha, mitochondrial small subunit, or polygalacturonase genes (Mbofung *et al.*, 2007;
354 Hirano & Arie, 2009), have been used to distinguish formae speciales of *F. oxysporum*. The
355 difficulty in finding differences among formae speciales with molecular markers was overcome by
356 the high level of polymorphisms (Lievens *et al.*, 2008) found in the genome using RAPD markers
357 (Shimazu *et al.*, 2005). Markers previously identified using RAPD were used in this work to design

358 a LAMP assay with optimal characteristics able to detect FOL in three different matrices including
359 plant material, soil and seeds.

360 Crop protection against FOL on lettuce requires an effective, reliable and economically
361 acceptable detection technique to help growers and breeders in the lettuce-growing areas. The
362 LAMP assay developed in this study has been validated according to the international EPPO
363 standard 7/98 to detect FOL in plant material, soil and seeds. Although there is no specific
364 phytosanitary legislation within the EU for the detection of FOL in lettuce seeds in trade or lettuce
365 plant material, the increased spread of the pathogen and the recent identification of a new race
366 within FOL (Gilardi *et al.*, 2016, 2017) makes the development and availability of new techniques
367 for the diagnosis of FOL essential.

368 Garibaldi *et al.*, (2004) demonstrated the presence of FOL in lettuce seeds, assessing 27
369 samples of different cultivars of commercial lettuce seeds from Lombardy (north Italy). They
370 reported infection rates as low as 0.15% in some of the samples tested, suggesting the necessity of
371 using healthy seeds and plant material to avoid the spread of the pathogen within the trade.
372 However, until now, no assay was able to detect the pathogen in seed samples contaminated at low
373 levels. A quick and easy test to identify the presence of FOL is an essential requirement in routine
374 seed diagnostic laboratory practices to avoid a long turnaround time between the reception of the
375 samples and the results. Use of a LAMP assay to identify contaminated batches of seeds and plant
376 material can reduce the time for identification and disposal of infected seed batches from days to
377 hours, avoiding subsequent yield losses.

378 The international standard rules for seed testing (ISTA Rules) are based on time-consuming
379 procedures including incubation times of one week followed by identification of the fungi by their
380 morphological features. These practices require screening of seed under a stereoscopic microscope
381 and identification of the pathogen conidia by trained diagnosticians. Detection by isolation of the
382 pathogen in culture is labour-intensive and requires specialized mycological skills, whereas the use

383 of molecular methods can mitigate the latter problem and can, therefore, be used more widely for
384 efficient pathogen detection.

385 A nested PCR was previously developed to detect the presence of FOL by Mbofung & Pryor
386 (2010). However, it includes long incubation times and does not present optimal characteristics in
387 terms of sensitivity, with a detection limit of 0.1% infection rate for FOL. The LAMP assay
388 developed in the present study can overcome these disadvantages, as the results are easily
389 interpreted by end-users and the sensitivity is improved in comparison with the nested PCR. The
390 sensitivity of the LAMP assay was evaluated using FOL DNA, individual infected seeds and
391 batches of commercial seeds inoculated with the four races of FOL before testing. The results were
392 repeatable, reproducible and reliable for detection of the four races in lettuce seeds with a Tp of less
393 than 30 min for all FOL races. When DNA from 25-seed batches with FOL infection rates of 4% or
394 8 were tested by the nested PCR developed by Mbofung & Pryor (2010), results for all four races
395 were negative. In contrast, the LAMP assay was able to detect the pathogen even using a crude
396 extraction method. On the other hand, in crude extracted DNA from samples of 400 commercial
397 seeds, FOL was not detected using the LAMP assay. However, the sensitivity of the LAMP assay
398 was improved by incorporating DNA extraction using a commercial kit, leading to the detection of
399 the pathogen to an infection rate of 0.004% in lettuce seeds artificially inoculated with FOL conidia
400 and 0.05% if inoculated with FOL chlamydospores; this is significantly more sensitive than the test
401 developed by Mbofung & Pryor (2010). Furthermore, the LAMP assay was demonstrated to be
402 specific using a panel of target and non-target species, which is a significant advantage over the
403 traditional methods where discriminating the formae speciales of *Fusarium oxysporum* is difficult.

404 The LAMP assay was also reliable for the detection of FOL on the plant material from the
405 different cultivars tested, becoming a potential tool for the diagnosis of FOL in plants to avoid the
406 pathogen spread. In addition, the assay successfully detected FOL in the soil. The detection of
407 soilborne pathogens such as *Fusarium* also has potential for breeders who grow the same crop in the

408 same soil each year. Routine testing using LAMP assays in DNA extracted from soils can provide
409 advanced warning of the need for effective crop protection strategies, due to the presence of
410 chlamydospores of FOL which can survive between cropping seasons.

411 In conclusion, a new LAMP assay has been developed for the detection of FOL and its
412 application has been demonstrated for detection of FOL in soil, plant material and infected seeds at
413 rates as low as 0.004%. LAMP testing of seeds and plants may help prevent the spread of FOL to
414 lettuce growing areas in other countries, avoiding new outbreaks of lettuce fusarium wilt; in
415 addition, soil testing may help warn growers to take action to control the disease spread in the field
416 and to disinfest contaminated soils.

417

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423

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511

512 Supporting Information

513 Additional Supporting Information may be found in the online version of this article at the
514 publisher's web-site.

515 **Table S1** Sensitivity of the LAMP assay for detection of *Fusarium oxysporum* f. sp. *lactucae*
516 (FOL), tested using DNA crudely extracted from single lettuce seeds inoculated with the four races
517 of FOL at different inoculum concentrations. The table represents the average of three replicates

518 with the standard deviation of the time to positive (Tp), and the annealing temperature. The results
519 of the *cox* assay were included as a plant DNA control.

520 **Table S2** Comparison of LAMP tests for detection of *Fusarium oxysporum* f. sp. *lactucae* (FOL),
521 performed using DNA extracted by different methods. The table shows the average of three
522 replicates in terms of time to positive (Tp) and annealing temperature of the assay using DNA
523 obtained by a crude extraction method or a commercial DNA extraction kit for the four FOL races
524 at both concentration and infection rates.

525 **Table S3** Sensitivity of the LAMP assay for detection of *Fusarium oxysporum* f. sp. *lactucae*
526 (FOL), tested using DNA from a crude extraction of batches of 25 lettuce seeds with different
527 infection rates and different inoculum concentrations. The four FOL races were used in the test. The
528 table represents the average and standard deviation of three replicates of the time to positive, and
529 the annealing temperature. The results of the *cox* assay were included as a plant DNA control. An
530 infection rate of 4% corresponds to 1 infected seed and 24 healthy lettuce seeds, while 8%
531 corresponds to 2 infected seeds and 23 healthy lettuce seeds.

532 **Table S4** Results of the LAMP assay for detection of *Fusarium oxysporum* f. sp. *lactucae* (FOL)
533 using commercial lettuce seeds of Dorée de Printemps inoculated artificially by conidial spraying or
534 Romabella seeds inoculated with talc power from chlamydospore. The table shows the time to
535 positive and the annealing temperature of the assay of DNA obtained by two DNA extraction
536 methods (crude extraction and commercial kit). The number of cells was calculated by qPCR. The
537 LAMP assay was carried out in triplicate on DNA from two different samples of 400 lettuce seeds.
538 The number of positives show the positive amplification taking into account the six replicates.

539

540

541 **Table 1.** Specificity results of the LAMP assay following testing of a range of *F.oxysporum formae specialis* and other lettuce pathogens showing
542 the average of time to postive and annealing temperature of three technical replicates. The Table also shows the results obtained using the specific
543 primers for FOL (Shimazu *et al.*, 2005)

Unique identifier	Species	Year of isolation	Origin	LAMP assay for FO f.sp. <i>lactucae</i>		FLA0001F/ FLA0001R primers- PCR results
				Tp(min:s)	Annel (°C)	
ATCC 76616	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 1	2002	USA	16:49	84.94	Positive
Fus lat 5.14	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 1	2002	Italy	13:26	85.04	Positive
Fus lat 6.14	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 1	2002	Italy	13:04	85.06	Positive
Fus lat 7.14	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 1	2002	Italy	13:15	84.92	Positive
Fus lat 11.10	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 1	2002	Italy	13:49	84.98	Positive
Fus lat 7.16	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 1	2016	France	11:37	84.94	Positive
Fus lat 8.16	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 1	2016	France	14:30	84.90	Positive
Fus lat 9.16	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 1	2016	France	11:41	85.04	Positive
Fus lat 10.16	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 1	2016	France	13:40	85.10	Positive
ATCCMya30-40	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 1	2002	Italy	22:40	84.97	Positive
SB1-1(MAFF244120)	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 1	1997	Japan	08:00	84.93	Positive
9501	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 2	1995	Japan	16:00	85.19	Positive
F9501(MAFF244121)	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 2	unknown	Japan	10:19	85.11	Positive
MAFF744085	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 3	unkonwn	Japan	12:37	84.75	Positive
MAFF744086	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 3	unkonwn	Japan	15:19	84.83	Positive
FLK1001(MAFF244122)	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 3	2010	Japan	13:40	85.04	Positive
FL 015/04750896	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 4	2015	Nederlands	12:19	84.72	Positive
FL 015/04750888	<i>Fusarium oxyporum</i> f.sp. <i>lactucae</i> race 4	2015	Nederlands	21:22	84.86	Positive
Polizzi	<i>Fusarium oxysporum</i> f. sp. <i>opuntiarum</i>	unknown	Italy	Undetermined	Undetermined	Positive
297	<i>Fusarium oxysporum</i> f. sp. <i>asparagae</i>	unknown	unknown	Undetermined	Undetermined	Negative
2745	<i>Fusarium oxysporum</i> f. sp. <i>lini</i>	unknown	UK	Undetermined	Undetermined	Negative
ATTC 52422	<i>Fusarium oxysporum</i> . f. sp. <i>chrysantemi</i>	unknown	unknown	Undetermined	Undetermined	Negative
ATTC 52557	<i>Fusarium oxysporum</i> . f. sp. <i>conglutinans</i>	1987	USA	Undetermined	Undetermined	Negative
ATCC 58385	<i>Fusarium oxysporum</i> . f. sp. <i>conglutinans</i>	unknown	unknown	Undetermined	Undetermined	Positive

ATCC 744009	<i>Fusarium oxysporum</i> f. sp. <i>fragariae</i>	unknown	unknown	Undetermined	Undetermined	Negative
ATTC 58110	<i>Fusarium oxysporum</i> f. sp. <i>raphani</i>	unknown	unknown	Undetermined	Undetermined	Negative
ATCC 66274	<i>Fusarium oxysporum</i> f. sp. <i>chrysantemi</i>	unknown	unknown	Undetermined	Undetermined	Negative
ATCC 16603	<i>Fusarium oxysporum</i> f. sp. <i>matthioli</i>	unknown	unknown	Undetermined	Undetermined	Negative
ATTC 16608	<i>Fusarium oxysporum</i> f. sp. <i>tracheiphilum</i>	unknown	unknown	Undetermined	Undetermined	Negative
GR15	<i>Fusarium oxysporum</i> f. sp. <i>Raphani</i>	unknown	unknown	Undetermined	Undetermined	Negative
Lav DIC03	<i>Fusarium oxysporum</i> f. sp. <i>lavand</i>	unknown	Italy	Undetermined	Undetermined	Negative
FOtu	<i>Fusarium oxysporum</i> f. sp. <i>tulipae</i>	unknown	Italy	Undetermined	Undetermined	Positive
Baudino Racconigi	<i>Fusarium oxysporum</i> f. sp. <i>radicis lycopersici</i>	unknown	Italy	Undetermined	Undetermined	Negative
F.O.R.C. - AFU-68A -	<i>Fusarium oxysporum</i> f. sp. <i>radicis cucumerinum</i>	unknown	Italy	Undetermined	Undetermined	Negative
IRF 658/11 -	<i>Fusarium oxysporum</i> f. sp. <i>papaveris</i>	unknown	Italy	Undetermined	Undetermined	Negative
SIS - La Lota 5	<i>Fusarium oxysporum</i> f. sp. <i>melonis</i>	unknown	Italy	Undetermined	Undetermined	Negative
Panero	<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i>	unknown	Italy	Undetermined	Undetermined	Negative
FOlilii1	<i>Fusarium oxysporum</i> f. sp. <i>lilii</i>	unknown	Italy	Undetermined	Undetermined	Negative
78.65	<i>Fusarium oxysporum</i> f. sp. <i>gladioli</i>	unknown	Italy	Undetermined	Undetermined	Negative
RAG 2	<i>Fusarium oxysporum</i> f. sp. <i>anemoni</i>	unknown	unknown	Undetermined	Undetermined	Negative
710	<i>Fusarium poae</i>	unknown	Italy	Undetermined	Undetermined	Negative
820	<i>Fusarium avenaeum</i>	unknown	UK	Undetermined	Undetermined	Negative
822	<i>Fusarium sporotrichioides</i>	unknown	unknown	Undetermined	Undetermined	Negative
1100	<i>Fusarium proliferatum</i>	unknown	unknown	Undetermined	Undetermined	Negative
Race 4 - DSM 62390	<i>Fusarium redolens</i>	unknown	unknown	Undetermined	Undetermined	Negative
Novaro	<i>Fusarium tabacinum</i>	unknown	unknown	Undetermined	Undetermined	Negative
reis prova 1	<i>Fusarium verticillioides</i> 2	unknown	unknown	Undetermined	Undetermined	Negative
CER 47	<i>Fusarium culmorum</i>	unknown	unknown	Undetermined	Undetermined	Negative
L8FEQ	<i>Fusarium equiseti</i>	unknown	Italy	Undetermined	Undetermined	Negative
HPV04	<i>Fusarium graminearum</i>	unknown	Italy	Undetermined	Undetermined	Negative
31369L10	<i>Alternaria alternata</i>	unknown	Italy	Undetermined	Undetermined	Negative
Py 20.11	<i>Pythium ultimum</i>	unknown	Italy	Undetermined	Undetermined	Negative
Colldem	<i>Colletotrichum dematicum</i>	unknown	unknown	Undetermined	Undetermined	Negative
Cercos1	<i>Cercospora</i> sp.	unknown	unknown	Undetermined	Undetermined	Negative
RS1	<i>Rhizoctonia solani</i>	unknown	Italy	Undetermined	Undetermined	Negative

294	<i>Sclerotinia sclerotiorum</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
2581	<i>Sclerotinia minor</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
2662	<i>Sclerotium trifolii</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
PE1	<i>Phoma exigua</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
BC1	<i>Botrytis cinerea</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
VD1	<i>Verticillium dahliae</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
398	<i>Verticillium longisporum</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
Phynic1	<i>Phytophthora nicotianae</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
268?	<i>Trichoderma viridae</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
Phyinf1	<i>Phytophthora infestans</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
Phycap1	<i>Phytophthora.capsici</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
<i>Phoma latt t152</i>	<i>Phoma tropica</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
404	<i>Pythium ultimum</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
N4 reis	<i>Myrothecium roridum</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
N5lat	<i>Myrothecium roridum</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
Peron1	<i>Peronospora</i> sp.	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
Pho_macro	<i>Phoma macrostoma</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Positive
405	<i>Cladosporium cladosporioides</i>	<i>unknown</i>	<i>Unknown</i>	Undetermined	Undetermined	Negative
Plasmo	<i>Plasmopora</i> sp.	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative
N17	<i>Phoma betae</i>	<i>unknown</i>	<i>Italy</i>	Undetermined	Undetermined	Negative

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550 Table 2. Results of the LAMP assay using commercial ‘Dorée de Printemps’ lettuce seeds inoculated artificially by conidial spraying or talc powder
 551 from chlamydospore at different concentrations. The table shows the results of both DNA extraction.

			Crude DNA extraction	Commercial DNA extraction kit
Conidia -infested lettuce seeds	40%	40 infected seeds in 100 lettuce seeds	+	+
	4%	4 infected seeds in 100 lettuce seeds	-	+
	0.40%	4 infected seeds in 1000 lettuce seeds	-	-
	0.04%	4 infected seeds in 10000 lettuce seeds	-	+
	0.004%	4 infected seeds in 100000 lettuce seeds	-	+
Chlamydospore -infested lettuce seeds	50%	50 infected seeds in 100 lettuce seeds	+	+
	5%	5 infected seeds in 100 lettuce seeds	-	+
	0.50%	5 infected seeds in 1000 lettuce seeds	-	+
	0.05%	5 infected seeds in 10000 lettuce seeds	-	+
	0.005%	5 infected seeds in 100000 lettuce seeds	-	-

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555 Table 3. LAMP assay results using soil from the test 1 and test 2. The experiment 1 with corresponds with the soil inoculated with FOL 1/17 (*F.*
 556 *oxysporum* f.sp. *lactucae* race 1) while the experiment 2 corresponds with the soil inoculated with FOL 2/17 (*F. oxysporum* f.sp. *lactucae* race 1).
 557 The tables represents the Tp annealing temperature and the number of cells calculated according to the standard curve.

	Tp (min:s)	Annel (°C)	Number of cells
Soil experiment 1	28:17±03:34	85.09±0.24	(3.7-21.66)12.7±12.7
Soil experiment 2	21:37±07:34	85.08±0.08	(4.02-24.7)14.37±14.6

Figure 1 Amplification using the primers FLA0001F/FLA0001R based on sequence tagged site (STS) markers designed by Shimazu et al. (2005), using target isolates of *Fusarium oxysporum* (FO) f. sp. *lactucae* and non-target formae speciales. The size of the products were estimated by comparison to the GelPilot 1-kb DNA plus ladder (QIAGEN).

B

	FOL Race 1	FOL Race 2	FOL Race 3	FOL Race 4	TOTAL
1-10ng	4//4	4//4	4//4	4//4	16//16
100-999pg	4//4	4//4	4//4	4//4	16//16
10-99pg	4//4	4//4	4//4	2//4	14//16
1-9,9pg	4//4	4//4	4//4	0//4	12//16
100-999fg	4//4	4//4	2//4	0//4	10//16
10-99fg	3//4	1//4	0//4	0//4	4//16
1-9,9fg	2//4	0//4	0//4	0//4	2//16

Figure 2 (a) Sensitivity testing of the LAMP assay for detection of four isolates corresponding to the four races of *Fusarium oxysporum* f. sp. *lactucae* (FOL). The time taken to a positive result with 10-fold dilutions of DNA extracted from each isolate is shown. Each bar represents the average and standard deviation of four replicates. (b) The table indicates the number of positive results detected for each range of DNA quantity for each race and the total number for the four races.

Figure 3 Sensitivity of LAMP assay for detection of *Fusarium oxysporum* f. sp. *lactucae* (FOL) in individual lettuce seeds inoculated with different inoculum concentrations of the four races. DNA was extracted using a crude extraction method. The concentration of 105 conidia mL⁻¹ of race 2 was not tested.

Figure 4: Comparison of results of the LAMP assay for detection of *Fusarium oxysporum* f. sp. *lactucae* (FOL) using two methods of DNA extraction from batches of 25 lettuce seeds inoculated with different concentrations of FOL. The DNA was extracted using a crude extraction method or a commercial extraction kit. Infected seeds were inoculated with two concentrations of inoculum and batches of seed had either 4% or 8% infected seeds (1 or 2 seeds per batch, respectively). The graph shows the average and standard deviation of the time to a positive result (Tp) in hour: minutes: seconds. (a) Results for FOL race 1. (b) Results for FOL race 2. (c) Results for FOL race 3. (d) Results for FOL race 4.

Figure 5 Detection by LAMP assay of *Fusarium oxysporum* f. sp. *lactucae* (FOL) race 1 in batches of 400 seeds of two different lettuce cultivars. Dorée de Printemps seeds were inoculated by spraying 40% of 200 g seeds with a conidial suspension at 106 conidia mL⁻¹. Romabella seeds were inoculated by mixing 50% of 200 g seeds with talcum powder containing chlamydospores. Testing was carried out on 16 subsamples of 25 seeds for each lettuce cultivar. The DNA was extracted from seeds using either a commercial kit or by a crude DNA extraction method. (a) Number of positive subsamples from the total subsamples tested. (b) Time to positive results (Tp).

Figure 6 Results of the LAMP assay for detection of *Fusarium oxysporum* f. sp. *lactucae* (FOL) in plant material. Experiment 1 corresponds to the pathogenicity test performed with isolate FOL 1/17. Experiment 2 corresponds to the pathogenicity test performed with the isolate FOL 2/17. (a) Bar graph representing the time to a positive result (Tp) with different lettuce cultivars. (b) Representative samples showing the symptoms for the disease severity index ranging from 0 to 4, 0 being disease absence and 4 total destruction of the plant. (c) The disease severity index and results of the LAMP assay after a crude extraction of plant material for each lettuce cultivar.

Table S1. LAMP assay results of the sensitivity testing using single inoculated lettuce seeds after a crude DNA extraction at different inoculum concentration. The four FOL races were used in the test. The table represents the average of three replicates with the standard deviation of the time to positive, and the annealing temperature. The results of the COX assay were included as plant DNA control.

		LAMP assay		COX LAMP (Control of Plant DNA)		
		AVERAGE of three replicates		AVERAGE of three replicates		
		Tp (min:s)	Annel °C	Tp (min:s)	Annel °C	
FOL race 1	8,2x10 ⁵ CFU/ml	1st seed	11:45±21	82.64±0.21	Undetermined	Undetermined
		2nd seed	14:00±01:53	82.67±1.38	19:30±01:46	81.83±2.16
		3rd seed	13:10±00:31	83.64±0.98	Undetermined	Undetermined
		4th seed	17:40±03:53	83.31±0.33	Undetermined	Undetermined
		5th seed	15:25±02:16	83.06±0.50	Undetermined	Undetermined
		AVERAGE	14:24±02:31	83.06±0.42	19:30:00±01:46	81.83
	8,2x10 ⁴ CFU/ml	1st seed	22:35±05:59	82.85±0.41	14:52±00:32	82.74±0.18
		2nd seed	13:10±01:23	82.88±0.73	Undetermined	Undetermined
		3rd seed	17:55±04:58	82.7±1.35	Undetermined	Undetermined
		4th seed	14:20±01:17	83.04±0.3	12:52±11:29	81.15±3.05
		5th seed	17:00±01:19	80.95±0.65	14:22±01:14	82.6±0.99
		AVERAGE	17:00±03:40	82.49±0.87	14:03±01:02	82.16±0.88
FOL race 2	1x10 ⁴ CFU/ml	1st seed	10:55±00:38	83.45±0.49	17:22±03:43	81.86±0.88
		2nd seed	17:30±01:19	82.95±0.62	16:37±02:18	82.35±0.33
		3rd seed	12:10±00:38	83.58±0.75	21:30±07:47	80.26±1.17
		4th seed	22:15±00:45	81.4±1.66	Undetermined	Undetermined
		5th seed	21:05±06:14	81.24±1.18	Undetermined	Undetermined
		AVERAGE	16:47±05:07	82.52±1.12	18:30±02:38	81.49±1.09
FOL Race 3	1,3x10 ⁵ CFU/ml	1st seed	18:25±04:48	82.46±0.25	28:52±10:47	81.865±0.11
		2nd seed	16:40±04:51	81.93±0.92	Undetermined	Undetermined
		3rd seed	14:55±02:15	82.7±0.54	Undetermined	Undetermined
		4th seed	17:15±03:11	82.9±0.02	Undetermined	Undetermined
		5th seed	19:35±01:23	82.58±1.23	16:22±32	81.92±0.4
		AVERAGE	17:22±01:46	82.52±0.36	22:37±08:51	81.83±0.04
	1,3x10 ⁴ CFU/ml	1st seed	24:15±06:04	82.2±1.03	15:15	82.715±0.2
		2nd seed	31:00	83.64	18:15	80.76
		3rd seed	17:30	81.9	23:45	77.45
		4th seed	21:15±03:53	81.29±2.92	19:45±01:46	81.155±3.05
		5th seed	26:00	81.5	15:15	81.9
		AVERAGE	24:00±05:04	82.105±0.93	18:27±3:33	80.796±2.01
FOL Race 4	8x10 ⁵ CFU/ml	1st seed	23:45	81.66	Undetermined	Undetermined
		2nd seed	35:20±06:17	82.06±1.57	20:30	83.17
		3rd seed	20:15±08:08	76.45±0.11	Undetermined	Undetermined
		4th seed	Undetermined	Undetermined	Undetermined	Undetermined

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8x10 ⁴ CFU/ml	5th seed	23:45	81.68	Undetermined	Undetermined
	AVERAGE	28:59±06:26	81.8±0.22	20:30	83.17
	1st seed	18:55±02:06	81.94±1.28	Undetermined	Undetermined
	2nd seed	33:55±12:10	81.57±1.30	Undetermined	Undetermined
	3rd seed	18:55±06:32	82±2.32	Undetermined	Undetermined
	4th seed	17:40±06:00	82.86±0.96	16:15	80.33
	5th seed	18:52±32	82.525±0.98	17:15	79.78
	AVERAGE	0:21:40±06:52	82.18±0.51	16:45±42	80.055±0.39

566 **Table S2.** Comparison of the LAMP tests performed using different DNA extraction methods. The Table shows the average of three replicates in
 567 terms of time to positive and annealing temperature using a crude extraction method and commercial DNA extraction kit for the four FOL races for
 568 both concentration and infection rates.
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		Crude DNA extraction		Commercial DNA extraction kit	
		Tp (min:s)	Anneal (°C)	Tp (min:s)	Anneal (°C)
FOL race 1	1 infected seed inoculated at 8.2x10 ⁵ ml in 24 healthy seeds (4% infected seeds)	24:28±03:56	84.23±0.27	18:15±02:25	83.20±0.03
	2 infected seeds inoculated at 8.2x10 ⁵ ml in 23 healthy seeds (8% infected seeds)	23:03±02:43	82.29±0.75	20:30±42	82.7±0.36
	1 infected seed inoculated at 8.2x10 ⁴ ml in 24 healthy seeds (4% infected seeds)	27:56±01:30	81.87±0.17	22:52±13:15	84.35±1.06
	2 infected seeds inoculated at 8.2x10 ⁴ ml in 23 healthy seeds (8% infected seeds)	22:54±01:48	82.43±0.86	23:00	82.73
FOL race 2	1 infected seed inoculated at 1x10 ⁴ ml in 24 healthy seeds (4% infected seeds)	21:30±01:14	84.23±0.48	Undetermined	Undetermined
	2 infected seeds inoculated at 1x10 ⁴ ml in 23 healthy seeds (8% infected seeds)	18:40±10:22	82.92±2.82	14:15:00	84.43
FOL race 3	1 infected seed inoculated at 1.3x10 ⁵ ml in 24 healthy seeds (4% infected seeds)	24:55±07:39	81.29±1.58	22:45	83.21
	2 infected seeds inoculated at 1.3x10 ⁵ ml in 23 healthy seeds (8% infected seeds)	24:14±02:51	82.18±0.69	21:45	83.30
	1 infected seed inoculated at 1.3x10 ⁴ ml in 24 healthy seeds (4% infected seeds)	34:30	84.23	Undetermined	Undetermined
	2 infected seeds inoculated at 1.3x10 ⁴ ml in 23 healthy seeds (8% infected seeds)	Undetermined	Undetermined	Undetermined	Undetermined
FOL race 4	1 infected seed inoculated at 8x10 ⁵ ml in 24 healthy seeds (4% infected seeds)	25:45	84.23	Undetermined	Undetermined
	2 infected seeds inoculated at 8x10 ⁵ ml in 23 healthy seeds (8% infected seeds)	20:28±0:04:35	80.98±1.55	Undetermined	Undetermined
	1 infected seed inoculated at 8x10 ⁴ ml in 24 healthy seeds (4% infected seeds)	Undetermined	Undetermined	Undetermined	Undetermined
	2 infected seeds inoculated at 8x10 ⁴ ml in 23 healthy seeds (8% infected seeds)	24:52±0:03:51	81.32±0.91	Undetermined	Undetermined

Table S3. LAMP assay results demonstrating the sensitivity using 25-lettuce seeds batches after a crude DNA extraction with different infection rates and different inoculum concentration. The four FOL races were used in the test. The table represents the average of three replicates with the standard deviation of the time to positive, and the annealing temperature. The results of the COX assay were included as a plant DNA control. An infection rate of 4% corresponds to 1 infected seed in 24 healthy lettuce seeds, while 8% infected rate corresponds with 2 infected seeds in 23 healthy lettuce seeds.

		LAMP assay		COX LAMP (Control of Plant DNA)		Number of positive results (considering positive amplification under 35 min of Tp)
		AVERAGE of three replicates		AVERAGE of three replicates		
		Tp (min:s)	Anneal °C	Tp (min:s)	Anneal °C	
FOL race 1	4% infected seeds at 8.2*10 ⁵ CFU/ml	24:28±03:56	84.23±0.27	14:58±02:27	82.19±0.53	5 out of 9-->55.5%
	8% infected seeds at 8.2*10 ⁵ CFU/ml	23:03±02:43	82.29±0.75	15:28±01:15	81.87±0.63	9 out of 9-->100%
	4% infected seeds at 8.2*10 ⁴ CFU/ml	27:56±01:30	81.87±0.17	17:03±01:15	80.91±0.40	3 out of 9-->33.3%
	8% infected seeds at 8.2*10 ⁴ CFU/ml	22:54±01:48	82.43±0.86	15:05±01:42	81.53±1.04	6 out of 9-->66.6%
FOL race 2	4% infected seeds at 1*10 ⁴ CFU/ml	0:21:30±01:14	84.23±0.48	15:45±01:59	81.31±0.34	3 out of 9-->33.3%
	8% infected seeds at 1*10 ⁴ CFU/ml	18:40±10:22	82.92±2.82	13:05±00:11	81.44±0.71	6 out of 9-->66.6%
FOL race 3s	4% infected seeds at 1.3*10 ⁵ CFU/ml	24:55±24:55	81.29±1.58	12:40±00:37	82.197±1.21	5 out of 9-->55.5%
	8% infected seeds at 1.3*10 ⁵ CFU/ml	24:14±02:51	82.18±0.69	13:13±00:35	80.93±0.63	6 out of 9-->66.6%
	4% infected seeds at 1.3*10 ⁴ CFU/ml	34:30	81.82	16:10±06:41	81.1±1.18	1 out of 9-->11.1%

	8% infected seeds at 1.3×10^4 CFU/ml	23:15±01:35	80.87±0.12	17:27±01:15	81.23±0.83	4 out of 9-->44.4%
FOL race 4	4% infected seeds at 8×10^5 CFU/ml	25:45	84.23	17:41±:006:06	80.71±1.62	5 out of 9-->55.5%

Table S4. Results of the LAMP assay using commercial lettuce seeds of Dorée de Printemps inoculated artificially by conidial spraying or Romabella lettuce seed inoculated with chlamydospores. The table shows the results of both DNA extraction of the time to positive and the annealing temperature. The number of cells was calculated according to the qPCR. The LAMP assay was carried out in triplicate in two different 400 lettuce seeds samples. The number of positive show the positive amplification taking into account the six replicates.

		Crude DNA extraction		Commercial DNA extraction kit		Number of positive results	Number of cells/μl
		Tp(min:s)	Annel($^{\circ}$ C)	Tp(min:s)	Annel($^{\circ}$ C)		
Conidia - infected lettuce seeds	40%	15:32 $\pm$ 07:11	85.49 $\pm$ 0.63	28:19 $\pm$ 03:34	85.1 $\pm$ 0.24	6	1.11577
	4%	Undetermined	Undetermined	29:12: $\pm$ 04:35	84.99 $\pm$ 0.38	4	Undetermined
	0.40%	Undetermined	Undetermined	Undetermined	Undetermined	0	Undetermined
	0.04%	Undetermined	Undetermined	29:23	85.173	2	Undetermined
	0.004%	Undetermined	Undetermined	23:00	85.62	2	Undetermined
Chlamydospore -infected lettuce seeds	50%	Undetermined	Undetermined	21:37: $\pm$ 07:34	85.08 $\pm$ 0.08	6	(5.05-10.42) 6,85 $\pm$ 3,02
	5%	Undetermined	Undetermined	25:58 $\pm$ 03:34	85.12 $\pm$ 0.32	5	Undetermined
	0.50%	Undetermined	Undetermined	20:4 $\pm$ 2.78	85.26 $\pm$ 0.17	6	Undetermined
	0.05%	Undetermined	Undetermined	25:14 $\pm$ 02:43	85.02 $\pm$ 0.39	3	Undetermined
	0.005%	Undetermined	Undetermined	Undetermined	Undetermined	0	Undetermined