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

Authors

S. Franco Ortega [1,2], J. Tomlinson [3], G. Gilardi [1], D. Spadaro [1,2 *], M.L. Gullino [1,2], A. Garibaldi [1], N. Boonham[3, 4]

[1] Centre of Competence for the Innovation in the Agro-environmental Sector - AGROINNOVA, University of Turin, via Paolo Braccini 2, I-10095 Grugliasco, TO, Italy

[2] Department of Agricultural, Forestry and Food Sciences (DiSAFA), University of Torino, via Paolo Braccini 2,I-10095 Grugliasco, TO, Italy

[3] FERA, Sand Hutton, York, UK

[4] Newcastle University, Newcastle upon Tyne, United Kingdom, NE1 7RU

*Corresponding author: davide.spadaro@unito.it

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ABSTRACT

Fusarium oxysporum f.sp. *lactucae* (FOL) is a soil and seed borne pathogen and the causal agent of Fusarium wilt on lettuce. Four races have been identified within FOL, with different worldwide distribution. Several molecular techniques have been used to detect and identify this pathogen, however, not all of them have the optimal characteristics in terms of sensitivity

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to perform FOL detection in plant and seed material. A loop-mediated isothermal amplification (LAMP) assay was developed based on the sequence characterized amplified region (SCAR) obtained in a previous Rapid Amplification of Polymorphic DNA (RAPD) study. The LAMP assay has been validated according to the EPPO standard PM7/98. The LAMP assay was tested with lettuce seeds, soil and plant material, and can be used to successfully 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.

INTRODUCTION

Fusarium oxysporum f.sp. *lactucae* (FOL) is a soil and seed borne pathogen (Garibaldi *et al.* 2004) and the causal agent of Fusarium wilt on lettuce (*Lactuca sativa* L.). 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 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 (north-western 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 it is the most widespread race, reported in the USA, Iran, Taiwan, Brazil, Portugal, Argentina, and Italy (Hubbard and Gerik, 1993; Huang & Lo, 1998; Millani *et al.* 1999; Garibaldi *et al*. 2002; Marques Ramalhete *et al.* 2006; Ventura & Costa, 2008; Malbrán *et al.*

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2014). Race 2, pathogenic on 'Patriot' and 'Costa Rica No.4' but not on 'Banchu red fire' lettuce cultivar, 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 seed borne 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 *Fusarium 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. Though these methods do not always permit accurate identification. Molecular techniques enhance the identification of *Fusarium*

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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), end-point or TaqMan real-Time PCR (Suga *et al.* 2013) based on sequence-characterized amplified region (SCAR) have all been used. Mbofung & Pryor in (2010) developed specific primers based on the intergenic spacer region to detect infected lettuce seed samples. These molecular techniques are widely used to identify a high number of *formae speciales* and races. However, these methods do not always show optimal characteristics in terms of specificity and sensitivity for seed testing. The FOL aggressiveness combined with the rapid spread amongst lettuce cultivation areas makes highly desirable a quick, easy and accurate test to detect the pathogen in seed.

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

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

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MATERIALS AND METHODS

DNA extraction from the fungal cultures

Each single-spore culture of the isolates of *Fusarium oxysporum* listed in Table 1 was grown in Potato Dextrose Broth (PDB; Sigma Aldrich, Germany) on a rotatory shaker (120 rpm) for 10 days at room temperature. Mycelium was extracted by means of filtration through Whatman No.1 filter paper. The total genomic DNA was extracted using E.Z.N.A. Fungal DNA mini kit (OMEGA Bio-Tek, Norcross, GA, USA), according to the manufacturer's instructions and was stored at -20°C. The DNA concentration of each sample was measured using NanoDrop 2000 (Thermo Fisher, Delaware, USA) and was then adjusted to 1-50 ng/ µl.

A crude extraction method was used in this study to extract plant material, including seeds, as faster as possible due to its especially utility during the field practises. It was based on the protocol of Tomlinson *et al.* (2010a) using 1 ml of PEG alkaline buffer (50g L^{-1} of PEG average Mn 4,600; 20 mM KOH; pH: 13.5) as described by Chomczynski & Rymaszewski (2006). This method includes a vigorous manual shaking of 1 ball bearing (7/16" stainless steel 316 GD Spheric Trafalgar Ltd) in a 5 ml tube during one minute using individual lettuce seeds and plant material. The same crude extraction was performed with manual shaking (3 min) using 400 lettuce seeds as a sample.

The reliability of the crude extraction method was evaluated against a commercial DNA extraction kit to validate it. The DNA were extracted from the seed samples ('Dorèe de Printemps' and 'Romabella' cultivars) using E.Z.N.A. Plant DNA kit (OMEGA Bio-Tek) according to the manufacturer's instructions after grinding with liquid nitrogen as a protocol variation.

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LAMP primer design

Six LAMP primers comprising two external primers (F3 and B3), two internal primers (FIP and BIP) and two loop primers (F-loop and B-loop) were designed for both targets according to Notomi *et al.* (2000). The primers FLA0001/FLA0001R (Shimazu *et al*. 2005) were used to perform a PCR of some FOL isolates (ATCC 76616, FOL Mya3040, FOL race 2 F9501, FOL race 3 MAFF744085, FOL race 4 04750888) and other *formae speciales* listed in Table 1 and Figure 1. The 20 µl reaction with 50 ng of the genomic DNA was performed according to the protocol of Shimazu *et al.* (2005) in a 2720 Thermal Cycler (Applied Biosystems). The amplification products were checked by means of electrophoresis in 1% agarose gel (Eppendorf). The positive PCR products were purified using a QIAquick PCR Purification Kit and sequenced in both directions using the sequencing service of BMR Genomics (Padova, Italy).

The sequences were merged into contigs using DNA Baser (Heracle BioSoft SRL, Romania) and an alignment was carried out using the MEGA 6.0.6. software (Tamura *et al*. 2013). Single-nucleotide polymorphisms (SNP) were targeted in the design of the LAMP primers. Potential secondary structures and hairpins were checked using OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html), while the possible interaction between primers was checked using Multiple Primer Analyzer (Thermo Scientific) (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 the NCBI web portal (http://blast.ncbi.nlm.nih.gov/Blast.cgi). HPLC purified primers were synthesized by Eurofins (The United Kingdom) and are available in kit format from OptiGene Ltd (Horsham, UK: http://www.optigene.co.uk).

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LAMP assay reaction

The 25 µl LAMP reaction included 200 nmol/l of each external primer (F3 and B3), 2 µmol/l of each internal primers (FIP and BIP), 1 µmol/l of each loop primer, 1x Isothermal Mastermix ISO-004 (OptiGene Ltd) with one microliter DNA or crude extractions. The LAMP protocol was carried out with a Genie II® instrument (OptiGene Ltd) and a StepOne (Applied Biosystem, California, USA) as follows: 45 min at 65°C, followed by the measure of annealing temperature using a reduction from 95°C to 70°C at 0.05°C/s. Seed testing was performed on the following cultivars and seed companies: 'Doréee de Printemps' (Vilmorin), 'Romabella' (Blumen), 'Costa Rica No. 4' (Rijk Zwaan', 'Ordino' (Nunhems), 'Juanita' (Syngenta), 'Boeing' (Maraldi Sementi), 'Volare' (Rijk Zwaan), and 'Ricetto' (Franchi Sementi). The test was carried out using a StepOnePlus Real Time PCR system (Applied Biosystems), setup as follows to perform the LAMP assay: quantification experiment with Standard Curve and TaqMan reagents (FAM as reporter and NFQ-MGB as quencher), by doing 40 cycles at 65°C with fluorescence measured each minute and a melting curve with the following steps: 95°C for 15 s, 70°C for 1 min and an increase of the temperature to 95°C at 0.3°C/s to record the fluorescence. A negative control and a positive control were included in each run.

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

Sensitivity of LAMP in seed test

In order to check the sensitivity of the LAMP assay, a lettuce seed sample ('Dorèe de Printemps') naturally free from FOL, checked by the agar method (Mathur & Kongsdal, 2003), was used. The seeds were prepared for inoculation with the four races of FOL after

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being treated with sodium hypochlorite (2%) for 2 minutes, washed with distilled water and air-dried.

The isolates SB1-1, F9501, FLK1001, MAFF744086, corresponding to the four races of FOL, were grown in PDB at room temperature for 1 week. The colony forming units (CFU) were counted by means of a haemocytometer, obtaining a concentration of 8.2×10^5 CFU/ml for SB1-1, 1.0×10^4 CFU/ml for F9501, 1.3×10^5 CFU/ml for FLK1001, and 8.0×10^5 CFU/ml for MAFF744086. Each sample was ten-fold serial diluted obtaining inoculum suspensions ranging from 10^5 to 10^4 CFU/ml to be used for lettuce seed infection.

Twenty-five 'Dorée de Printemps' lettuce seeds were inoculated using 5 ml of a culture of each 10-fold serial dilution suspension for each race. Inoculum was prepared in individual tubes and incubated on a rotatory shaker at 90 rpm for 45 min. All the seeds were later washed with distilled water and dried overnight before the LAMP testing.

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

Commercial seed testing

The final step of the validation using seeds, included two lettuce seed samples belonging to cultivars 'Dorée de Printemps' and 'Romabella' collected from commercial farms were used to evaluate the reliability of the LAMP assay. All the seed samples were confirmed as free from FOL with an agar test (Mathur & Kongsdal, 2003). In order to assess the sensitivity of

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the LAMP assay using commercial seed samples, both lettuce seed samples were artificially inoculated using FOL race 1 Mya 3040 isolate by 1) spraying a conidial suspension at 10^6 conidia/ml on 40% of 200 g 'Dorée de Printemps' seeds to obtain a level of contamination corresponding to 40 infected seeds in 100 total seeds; 2) mixing the talc powder of the isolate over 50% of 200 g seeds to obtain a level of contamination corresponding to 50 infected seeds in 100 total 'Romabella' seedsThe conidial suspension was prepared in PDB as previously described, while the talc formulation for chlamydospore production of FOL Mya 3040 was carried out following the protocol suggested by Locke & Colhoun (1974).

The DNA from sixteen subsamples of 25 seeds/samples obtaining a total of 400 seeds from each original 'Dorée of Printemps' and 'Romabella' lettuce samples artificially infected was extracted by both methods as described above.

In a second experiment, the two artificially infected lettuce seed samples were 10-fold diluted, by using 'Dorée de Printemps' lettuce seeds free from FOL to obtain an infection rate of 0.004% (4 infected seed out of 100,000) and 0.005% (5 infected seed out of 100,000). The DNA of 400 hundred seeds in duplicate was extracted as described above by the two methods.

Soil and plant testing

The selectivity of the assay was also evaluated using soil and plant material. Two FOL isolates (FOL1/17 (*F. oxysporum* f. sp. *lactucae* race 1) and FOL 2/17 (*F. oxysporum* f. sp. *lactucae* race 1)) were grown in PDB for ten days at room temperature on a rotatory shaker (120 rpm). The suspensions were filtered through a sterile cheese cloth and centrifuged for 15 min at 10,000 g. The conidia pellet was suspended in distilled water and counted by means of a haemocytometer to obtain a 10^6 CFU/ml suspension. The artificial inoculation of 21-25 day old lettuce seedling was performed by root immersion into the conidial suspension. Seven

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lettuce cultivars (‘Romabella’, ‘Costa Rica’, ‘Ordino’, ‘Juanita’, ‘Boeing’, ‘Volare’, ‘Ricetto’) were used to test the LAMP assay. The experiments were carried out in the greenhouse at a temperature ranging from 28°C to 30°C. Experiment 1 corresponds to the pathogenicity test using FOL1/17 (*F. oxysporum* f. sp. *lactucae* race 1), while experiment 2 corresponds to the pathogenicity test using FOL 2/17 (*F. oxysporum* f. sp. *lactucae* race 1). The number of FOL cells of both soils was determined by qPCR.

Plants were checked for typical Fusarium wilt symptoms every 10 days. Disease severity was measured 30 days after inoculation with a disease index from 0 to 4: 0= healthy plants; 1= first symptoms of leaf chlorosis, slight reduction in the development of the plant; 2= severe leaf chlorosis, evident reduction in development, sometimes asymmetric development, evident vascular browning; 3= severe leaf chlorosis, severe reduction in development and strong deformation of the plant, severe vascular browning; 4= plant totally wilted.

Crude DNA extraction using plant material was carried out as described above. After 35 days, 1 g of inoculated soil from both experiments was extracted in triplicate by using E.Z.N.A. Soil DNA kit according to manufacturer's instructions. The LAMP tests were carried out as described above using 1 µl of each sample in triplicate on a StepOne Plus Real-Time PCR system (Applied Biosystems).

qPCR and nested PCR

The primers developed by Shimazu *et al*. (2005) were used to quantify the DNA using SYBR Green,according to the protocol ofA. Cucu, Agroinnova, Turin, Italy, personal communication. The DNA quantity of soil from test 1 and test 2, and DNA from 400 seeds was measured recorded. The reaction was carried out using 1x SYBR® Green PCR Master Mix, 120 nM of each primers in 25 µl and 1 µl of soil or seed DNA. The amplification was carried out using the following protocol: 95°C for 10 minutes, 40 cycles of 15 s at 95°C, 1

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min at 60°C, and 45 s at 72°C using a OneStep Plus Real Time PCR system (Applied Biosystems). A standard curve was carried out using FOL 76616 DNA ranging from 9.4 ng/µl to 9.4 fg/µ and negative controls with water were included in the assay. The average of three technical replicates was used to calculate the DNA quantity. The Ct values generated by qPCR were compared with the standard curve to obtain the ng of DNA for the positives results. The approximate number of cells present in the 400 seeds samples was achieved by dividing the DNA quantity by the weight of the genome of *F. oxysporum* (0.0000548 ng).

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

RESULTS

LAMP primer design and validation

The primers designed by Shimazu *et al*. (2005) specific for FOL were used to design the six LAMP primers. Because, this PCR produced amplification in other non-target *formae speciales,* such as *F. oxysporum* f.sp. *conglutinans* 58385, *F. oxysporum,* f.sp. *tulipae*, *F. oxysporum* f. sp. *conglutinans* 52557 (Figure 1), single nucleotide polymorphisms between

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the PCR products of target and non-target isolates were used during the design of the six LAMP primers.

The validation of the LAMP was carried out according to EPPO standard PM7/98. The specificity was checked with an inclusivity and exclusivity panel of samples including other *formae speciales* of *Fusarium oxysporum* and other common pathogens of lettuce. Non-target amplifications were not found compared with the end-point PCR developed by Shimazu *et al.* (2005), and the average time to positive (Tp) or the time at which the fluorescence overcomes the threshold level, which is a parameter analogous to the threshold cycling time in qPCR (Tomlinson *et al.* 2013), was calculated. Tp ranged from 11 to 22 min whilst the average annealing temperature ranged from 84.93 to 85.19°C (Table 1). The sensitivity using 10-fold serial dilutions of the DNA presented differences among the different races. The lowest quantity of DNA detected corresponds to an amount of DNA ranging from10-99 pg for FOL race 1 to 100-999 pg for FOL race 2, race 3 and race 4 (Figure 2).

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

Individually infected seeds extracted with crude extraction method of the FOL races 1 and 2 produced the lowest Tp (14 min 24 s and 17 min for the two inoculum concentration of race 1, and 16 min 47 s for race 2 at concentration 1 x 10^4 CFU/ml) in comparison with the other FOL races infected seeds in both dilutions (Tp ranging from 17 min 22 s to 28 min 59 s) (Figure 3). The best results for individually infected seeds were obtained with the seeds inoculated with 8.2 x 10^5 CFU/ml (FOL race 1), 1 x 10^4 CFU/ml (FOL race 2), 1.3 x 10^5 CFU/ml (FOL race 3), and 8 x 10^5 CFU/ml (FOL race 4), respectively. The time to positive of race 4 infected seeds was higher compared with the results of race 1 and race 2 infected

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seeds, reaching 28 min and 59 s and 21 min 40 s for each dilution. The LAMP assay did not produce positive amplification for all the replicates of FOL race 3 and race 4 seeds. Only some of the replicates produced a positive amplification using the COX LAMP however the purpose of this assay it is only to interpretate negative results of the pathogen LAMP (Table S1).

The crude extraction DNA of the 25 seed batches was not diluted to increase the sensitivity of the technique. The 8% infection rate seed batch (2 infected out of 25 seeds) produced the most reliable and repeatable result, for all the races and concentrations The best results were obtained using the FOL race 1 infected seeds at 8% infection rate and at the highest concentration (8.2×10^5 CFU/ml) followed by race 3, race 4 and race 2 at the highest concentration: 1.3×10^5 CFU/ml for FOL race 3, 8×10^5 CFU/ml for FOL race 4, and 1×10^4 CFU/ml for FOL race 2. The reliability of a crude DNA extraction was compared with DNA extracted using a commercial kit on 25-seeds batches extracted with both methods.In all cases, the Tp and the annealing temperature were slightly lower with the DNA prepared using the crude DNA extraction method compared with the DNA extraction kit (Figure 4 and Table S2). The same extractions were simultaneously tested using the nested-PCR developed by Mbofung & Pryor (2010) and no amplification was detected in any of the samples.

The LAMP assay was validated according to EPPO standard PM7/98. The specificity was validated using three technical replicates of the fungi listed in Table 1, using different *formae speciales* of *F. oxysporum*, and different lettuce pathogens. The sensitivity was validated in triplicate using 10-fold serial dilutions of the 4 races within FOL. FOL infected seed samples tested individually and in batches, as explained above were used to check the LAMP sensitivity. Seed testing was performed using individual and batches seeds (three biological replicates in each test) and the LAMP assay was performed in triplicate. The reproducibility was verified in different machines, used by different researchers in different days. The

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selectivity of the LAMP assay was checked using three matrices: artificially infested soil from a pathogenicity test, two batches of lettuce seeds ('Dorée de Printemps' and 'Romabella'), and plant material of seven lettuce cultivars ('Romabella', 'Costa Rica', 'Ordino', 'Juanita', 'Boeing', 'Volare', 'Ricetto'). Commercial lettuce seeds were used to evaluate the reliability of the assay, by testing 400 seed samples with different infection rates.

Commercial lettuce seeds

Two commercial lettuce seed batches ('Dorèe de Printemps' and 'Romabella') were used to check the reliability of the LAMP assay. The two seed samples were extracted using commercial DNA extraction kit and crude extraction using four hundred 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 repetitive results using both types of DNA extraction with the 25-seeds subsamples, obtaining a Tp 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 S3). The Tp 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 Tp 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 which 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 (Figure 5).

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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%. The 'Dorée de Printemps' seed samples at 40% of infection rate also gave positive results using the crude extraction method. Using the LAMP assay for testing 400 seeds at 10-fold dilution 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 (Table 2, Table S4). Not all the biological replicates, such as the 0.4% one, produced positive amplification. Cell number in both samples was measured using qPCR, however, when testing the seed of 'Dorée de Printemps' not all the replicates gave a positive result. None of the 10-fold diluted batches (from 4-5% to 0.004-0.005%) was detected by qPCR. Only the cells of the 40% conidia infected lettuce seeds sample (1.1 cells/ µl) and the 50% chlamydospores-infected lettuce seeds (sample 5.05-10.42 cells/µl) were quantified by qPCR.

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

Soil and plant testing

Plant samples of seven lettuce cultivars ('Romabella', 'Costa Rica', 'Ordino', 'Juanita', 'Boeing', 'Volare' and 'Ricetto') with Fusarium wilt symptoms produced positive amplification using the LAMP assay after a crude DNA extraction in both experiments (Figure 6). The infested soil from both pathogenicity tests produced positive amplification after DNA was extracted using a commercial DNA extraction kit (E.Z.N.A Soil DNA Kit).

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The results of the qPCR with SYBR Green showed that the number of cells was 12.7 and 14.37 cells/µl respectively (Table 3).

DISCUSSION

Different molecular markers, such as the intergenic spacer region, elongation factor 1-alpha, mitochondrial small subunit, or polygalacturonase genes (Hirano & Arie, 2009, Mbofung *et al.* 2007), have been used to distinguish *formae speciales* of *F. oxysporum.* The difficulty to find differences among *formae speciales* with molecular markers was overcome by the high level of polymorphisms (Lievens *et al.* 2008) found in the genome using random amplified polymorphic DNA (RAPD) markers (Shimazu *et al.* 2005). Markers previously identified using RAPD were used in this work to design a LAMP assay with optimal characteristics able to detect FOL in three different matrices including plant material, soil and seeds.

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

Garibaldi *et al.* (2004) demonstrated the presence of FOL in lettuce seeds assessing 27 samples of different cultivars of commercial lettuce seeds from Lombardy (north Italy) and reported infection rates as low as 0.15% in some of the samples tested, suggesting the necessity of using healthy seeds and plant material to avoid the pathogen spread in trade. However, until now, no assay is able to detect the pathogen in seed samples contaminated at

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low levels. A quick and easy test to identify the presence of FOL is an essential requirement in routine seed diagnostic laboratory practices to avoid a long turnaround time between the reception of the samples and the results. The use of the LAMP assay to identify contaminated batches of seeds and plant material can reduce the reaction time from days to hours to discard an infected seed batch and avoid subsequent yield losses.

The international standard rules for seed testing (ISTA Rules) are based on time-consuming procedures including incubation times of one week and later identification by morphological features of the fungi. These type of practices require screening seed under a stereoscopic microscope and identification of the conidia of the pathogens by trained diagnosticians. Detection by isolation of the pathogen in culture is labor intensive and requires specialized mycological skills, whereas the use of molecular methods can mitigate the latter problem and can, therefore, be utilized more widely for efficient pathogen detection.

A nested PCR was previously developed to detect the presence of FOL by Mbofung & Pryor (2010) however, it includes long incubation times and it does not present optimal characteristics in terms of sensitivity with a detection limit of 0.1% infection rate for FOL. The LAMP assay developed in this study can overcome these disadvantages due to an easy interpretation of the results by the end-users and an improved sensitivity in comparison with the nested PCR. The sensitivity of the LAMP assay was evaluated using DNA, individual seeds and batches inoculated with the four races before testing commercial seeds batches. The results were repeatable, reproducible and reliable to detect the presence of the four races in lettuce seeds with Tp inferior to 30 min for all *F. oxysporum* f. sp. *lactucae* races. In comparison with the negative results using DNA from 25-seeds batches at an infection rate of 4% (1 infected seed out of 25) and 8% (2 infected seed out of 25) for the four races obtained

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using the nested PCR developed by Mbofung & Pryor (2010), the LAMP assay was able to detect the pathogen even using a crude extraction method. On the other hand, the crude extracted DNA from the 400 commercial seeds samples was not detected using the LAMP assay. However, the sensitivity of the LAMP assay was improved by incorporating a commercial DNA extraction kit in the protocol, ensuring the detection of the pathogen to an infection rate of 0.004% conidia FOL artificially inoculated lettuce seeds (4 infected seed out of 100,000 lettuce seeds) and at 0.05% with FOL chlamydospores (5 infected seed out of 10,000 lettuce seeds), which is significantly more sensitive than the test developed by Mbofung & Pryor (2010). Furthermore, the LAMP assay was demonstrated to be specific using a panel of target and non target species, which is a significant advantage over the traditional methods where discriminating the *formae speciales* of *Fusarium oxysporum* is difficult.

The LAMP assay was also reliable for the detection of FOL on the plant material from the different cultivars tested, becoming a potential tool for the diagnosis of FOL in plants to avoid the pathogen spread.

The detection of soilborne pathogens such as *Fusarium* also has potential for breeders who grow the same crop in the same soil each year. Routine testing using the LAMP assays in DNA extracted from soils can provide advanced warning about the need of effective crop protection strategies, due to the presence of chlamydospores of FOL which can survive between subsequent cropping seasons.

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In the present study, the application of a new LAMP assay was demonstrated to detect FOL in soil, plant material and infected seeds at rates as low as 0.004%. The seed and plant tests with LAMP may avoid the diffusion of FOL to lettuce growing areas in other countries, avoiding new outbreaks of lettuce Fusarium wilt, while soil testing may help growers to take actions to control the disease spread in the field and to disinfest contaminated soils.

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FIGURES

Figure 1. Amplification using the primers FLA0001F/FLA0001R based on sequence tagged site (STS) markers designed by Shimazu *et al. 2004,* using target isolates of FOL and non-target *formae speciales.* The size of the products were estimated by comparsion to the GelPilot 1-kb DNA plus Ladder (Qiagen).

Figure 2. A. Sensitivity testing of the FOL LAMP assay reporting the average of four replicates and standard deviation of 4 isolates corresponding to the four races of FOL. **B**. The table indicates the number of positive results detected for each range of DNA for each race and the total number for the four races.

Figure 3. LAMP assay results for sensitivity using individual lettuce seeds extracted using the crude extraction method at different concentration of inoculum. The four FOL races were used in the test. The concentration of 10^5 CFU/ml of race 2 was not tested.

Figure 4. Comparison of the LAMP assay using 25 lettuce seeds batches among the FOL races, where the DNA was extracted using a crude extraction method or a commercial extraction kit. The table shows the average and standard deviation of the Tp (Time to positive in hour: minutes: seconds), Anneal (Annealing temperature in °C). **A**. FOL race 1 results. **B**. FOL race 2 results. **C**. FOL race 3 results. **D**. FOL race 4 results.

Figure 5. Seed testing using 400 seeds of two different lettuce seed batches (‘Dorée de Printemps’ seeds inoculated spraying a conidial suspension at 10^6 conidia/ml on 40% of 200 g seeds and ‘Romabella’ seeds inoculated mixing the talc powder of the isolate over 50% of 200 g seeds)and testing 16 subsamples of 25 seeds each. The two bar charts show the results

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comparing the commercial kit and crude DNA extraction method. **A**. The bar graph represents the number of positives subsamples from the total subsamples tested, **B.** Time to positive

Figure 6. Results of the LAMP assay using plant material. The experiment 1 corresponds with the pathogenicity test performed with the isolate FOL 1/17. While the experiment 2 corresponds with the pathogenicity test performed with the isolates FOL 2/17.**A**.The bar graph represents the Tp of the different lettuce cultivars **.B**. Representation of the disease severity index in function of the symptoms .**C**. Results of the LAMP assay after a crude extraction of the plant material. The disease index is reported in the Table ranging from 0 to 4, being 0 disease absence and 4 total destruction of the plant.

SUPPLEMENTARY MATERIAL

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.

Table S2. Comparison of the LAMP tests performed using different DNA extraction methods. The Table shows the average of three replicates in terms of time to positive and annealing temperature using a crude extraction method and commercial DNA extraction kit for the four FOL races for both concentration and infection rates.

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

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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.

Table S4. Results of the LAMP assay using commercial lettuce seeds of 'Dorée de Printemps' inoculated artificially by conidial spraying or talc power from chlamydopospore. 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.

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

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

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2745	*Fusarium oxysporum* f. sp. *lini*	*unknown*	*UK*	Undetermined	Undetermined	Negative
ATTC 52422	*Fusarium oxysporum.* f. sp. *chrysantemi*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
ATTC 52557	*Fusarium oxysporum.* f. sp. *conglutinans*	*1987*	*USA*	Undetermined	Undetermined	Negative
ATCC 58385	*Fusarium oxysporum.* f. sp. *conglutinans*	*unknown*	*unknown*	Undetermined	Undetermined	**Positive**
ATCC 744009	*Fusarium oxysporum.* f. sp. *fragariae*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
ATTC 58110	*Fusarium oxysporum.* f. sp. *raphani*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
ATCC 66274	*Fusarium oxysporum.* f. sp. *chrysantemi*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
ATCC 16603	*Fusarium oxysporum.* f. sp. *matthioli*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
ATTC 16608	*Fusarium oxysporum.* f. sp. *tracheiphilum*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
GR15	*Fusarium oxysporum* f. sp. *Raphani*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
Lav DIC03	*Fusarium oxysporum* f. sp. *lavand*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
FOtu	*Fusarium oxysporum* f. sp. *tulipae*	*unknown*	*Italy*	Undetermined	Undetermined	**Positive**
Baudino Racconigi	*Fusarium oxysporum* f. sp. *radicis lycopersici*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
F.O.R.C. - AFU-68A -	*Fusarium oxysporum* f. sp. *radicis cucumerinum*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
IRF 658/11 -	*Fusarium oxysporum* f. sp. *papaveris*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
SIS - La Lota 5	*Fusarium oxysporum* f. sp. *melonis*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
Panero	*Fusarium oxysporum* f. sp. *lycopersici*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
FOlilii1	*Fusarium oxysporum* f. sp. *lilii*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
78.65	*Fusarium oxysporum* f. sp. *gladioli*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
RAG 2	*Fusarium oxysporum* f. sp. *anemoni*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
710	*Fusarium poae*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
820	*Fusarium avenaeum*	*unknown*	*UK*	Undetermined	Undetermined	Negative
822	*Fusarium sporotrichioides*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
1100	*Fusarium proliferatum*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
Race 4 - DSM 62390	*Fusarium redolens*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
Novaro	*Fusarium tabacinum*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
reis prova 1	*Fusarium verticillioides 2*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
CER 47	*Fusarium culmorum*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
L8FEQ	*Fusarium equiseti*	*unknown*	*Italy*	Undetermined	Undetermined	Negative

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HPV04	*Fusarium graminearum*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
31369L10	*Alternaria alternata*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
Py 20.11	*Pythium ultimum*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
Colldem	*Colletotrichum dematicum*	*unknown*	*unknown*	Undetermined	Undetermined	Negative
Cercos1	*Cercospora* sp.	*unknown*	*unknown*	Undetermined	Undetermined	Negative
RS1	*Rhizoctonia solani*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
294	*Sclerotinia sclerotiorum*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
2581	*Sclerotinia minor*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
2662	*Sclerotium trifoii*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
PE1	*Phoma exigua*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
BC1	*Botrytis cinerea*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
VD1	*Verticillium dahliae*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
398	*Verticillium longisporum*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
Phynic1	*Phytophthora nicotianae*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
268?	*Trichoderma viridae*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
Phyinf1	*Phytophthora infestans*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
Phycap1	*Phytophthora.capsici*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
Phoma latt t152	*Phoma tropica*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
404	*Pythium ultimum*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
N4 reis	*Myrothecium roridum*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
N5lat	*Myrothecium roridum*	*unknown*	*Italy*	Undetermined	Undetermined	Negative
Peron1	*Peronospora* sp.	*unknown*	*Italy*	Undetermined	Undetermined	Negative
Pho_macro	*Phoma macrostoma*	*unknown*	*Italy*	Undetermined	Undetermined	**Positive**
405	*Cladosporium cladosporioides*	*unknown*	*Unknown*	Undetermined	Undetermined	Negative
Plasmo	*Plasmopora* sp.	*unknown*	*Italy*	Undetermined	Undetermined	Negative
N17	*Phoma betae*	*unknown*	*Italy*	Undetermined	Undetermined	Negative

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Table 2. Results of the LAMP assay using commercial 'Dorée de Printemps' lettuce seeds inoculated artificially by conidial spraying or talc powder from chlamydopospore 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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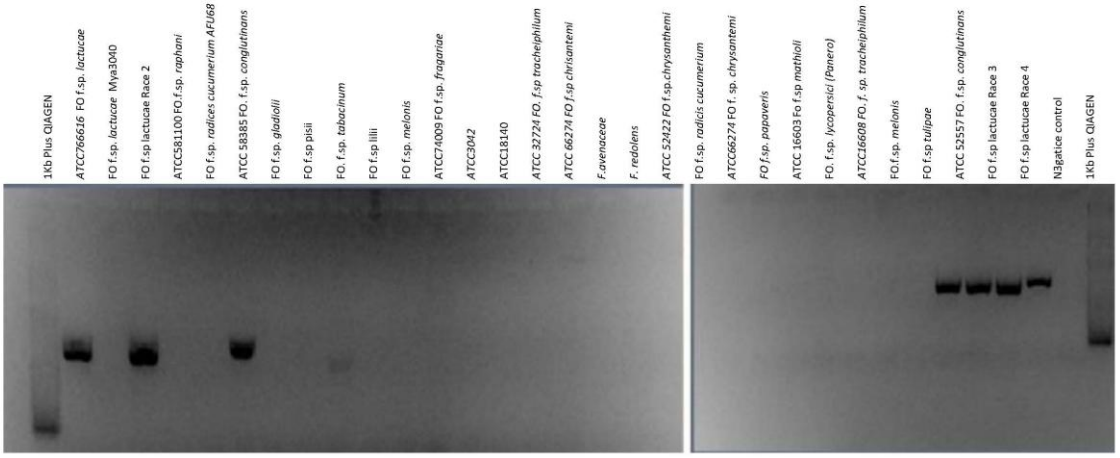
Table 3. LAMP assay results using soil from the test 1 and test 2. The experiment 1 with corresponds with the soil inoculated with FOL 1/17 (*F. oxysporum* f.sp. *lactucae* race 1) while the experiment 2 corresponds with the soil inoculated with FOL 2/17 (*F. oxysporum* f.sp. *lactucae* race 1). The 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/µl**
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



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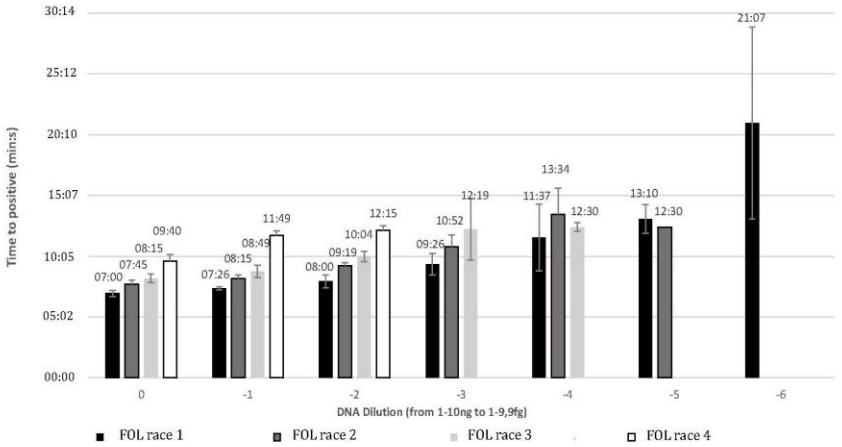
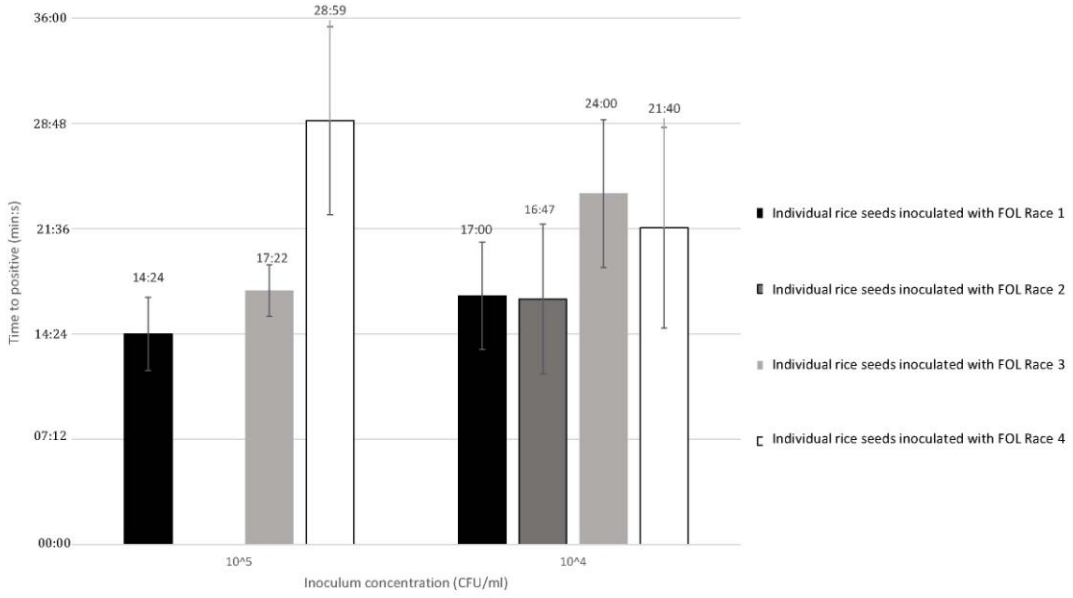


B

	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	10/116
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



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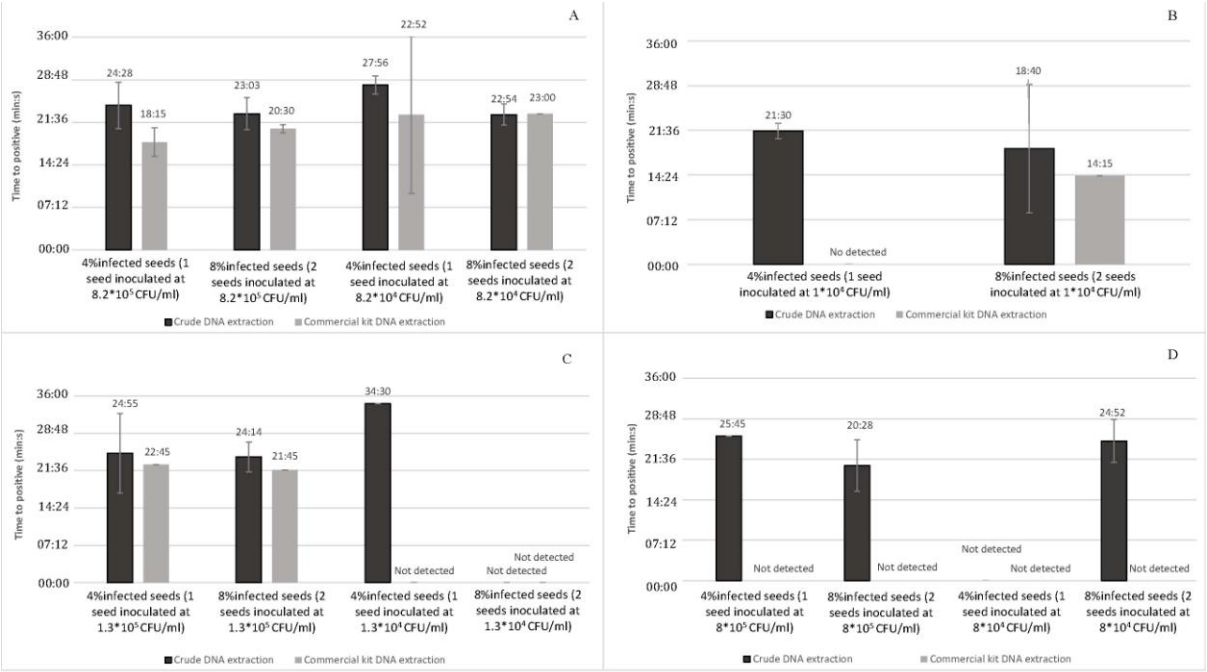

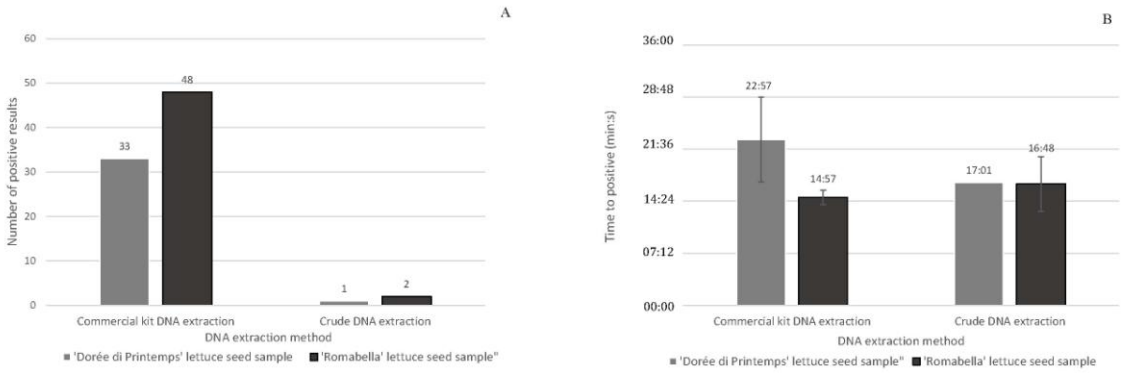

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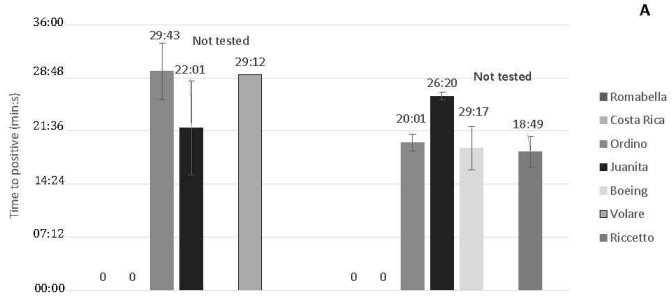
A



B

C

Lettuce cultivar	Experiment	Disease severity index	LAMP result after crude extraction
'Romabella'	1	0	-
	2	0	-
'Costa Rica'	1	0	-
	2	0	-
'Ordino'	1	2	+
	2	1.2	+
'Juanita'	1	4	+
	2	4	+
'Boeing'	1	Not tested	Not tested
	2	2.8	+
'Volare'	1	4	+
	2	Not tested	Not tested
'Riccetto'	1	Not tested	Not tested
	2	3.6	+

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