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ORIGINAL ARTICLE

A fast approach to discard false negative susceptible lettuce genotypes to *Fusarium oxysporum* f.sp. *lactucae* race 1

Martina Sanna[1] | Giovanna Gilardi[1] | Maria Lodovica Gullino[1] | Monica Mezzalama[1,2]

[1]AGROINNOVA – Centre of Competence for the Innovation in the Agro-environmental Sector, Università di Torino, Grugliasco, Italy

[2]DISAFA - Dept Agricultural, Forestry and Food Sciences, Università di Torino, Grugliasco, Italy

Correspondence
Monica Mezzalama, AGROINNOVA – Centre of Competence for the Innovation in the Agro-environmental Sector, Università di Torino, Grugliasco, TO, Italy.
Email: monica.mezzalama@unito.it

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Abstract

Fusarium oxysporum f. sp. *lactucae* (FOL), the causal agent of Fusarium wilt of lettuce, is a seedborne and soilborne pathogen that causes severe losses everywhere lettuce is cultivated. Genetic resistance is potentially the most effective strategy to control the pathogen, but susceptibility evaluation of breeding material with conventional methods carried out under controlled conditions require 3–5 weeks before symptoms severity can be accurately assessed on young plants. A race-specific quantitative real-time PCR (qPCR) protocol was developed and validated according to the international validation standard (EPPO, PM7/98) as an aid to speed up the process. The experiments were carried out on five lettuce cultivars grown in the greenhouse and four cultivars grown in microcosm with known levels of susceptibility to the pathogen. The qPCR test carried out on seedlings grown in the microcosm detected the pathogen already 7 days after the inoculation. However, the correlation between the n. cell/µl of the pathogen and the symptoms expression was reliable only at 14 days from the inoculation both, in the greenhouse and in the microcosm assay. A high quantity (from $2.9x10^2$ to $1.9x10^3$ cells/µl) of FOL race 1 corresponds to a high susceptibility of the cultivars and low quantity (0–56.9 cells/µl) is correlated to a resistant response of the cultivar.

KEYWORDS
Lactuca sativa L, qPCR, seedborne, varietal selection, wilt

1 | INTRODUCTION

Fusarium oxysporum f. sp. *lactucae* (FOL) is a seedborne and soilborne pathogen responsible of Fusarium wilt of lettuce (*Lactuca sativa* L.), a worldwide important leafy vegetable. It penetrates the plant through the roots and cause the development of stunted plants that presents yellow leaves and brown streaks in the root vascular system (Garibaldi et al., 2004). Fusarium wilt of lettuce was first observed in Japan in 1955 (Motohashi, 1960), and the causal agent was identified by Matuo in 1967. The pathogen was also detected in California (Hubbard & Gerik, 1993), Arizona (Matheron & Koike, 2003), Taiwan (Huang, 1998), Iran (Millani et al., 1999) and Brazil (Ventura & Costa, 2008). In Europe, FOL was first detected in Italy in 2002 and then in Portugal (Garibaldi et al., 2002; Marques Ramalhete et al., 2006). Race 1 was first described in Japan by Matuo and Motohashi (1967) and it is the prevalent race worldwide. Race 1 was found in Italy, first in Lombardy and then in Veneto, Piedmont and Emilia Romagna (Garibaldi et al., 2002).

In Italy, losses up to 70% were observed in susceptible lettuce varieties. Soil disinfestation and crop rotation can help avoiding the spread of the pathogen, but the ability of the fungus to survive beyond 2.5 years as dormant resting structures called chlamydospores

in the soil and in the crop residues must be considered (Paugh & Gordon, 2021; Scott et al., 2012). Genetic resistance can provide a complete control of the disease if not compromised by the introduction of new pathogenic races (Scott et al., 2014). There are different conventional methods to assess the resistance of lettuce cultivars, under controlled conditions, such as growing seedlings in infested soil in trays that allows to test many samples in a small area (Tsuchiya et al., 2004) or by the artificial inoculation of the seedling roots with a conidial suspension of the pathogen. Both methods are successful in the breeding and selection of resistant varieties, but they require 3–5 weeks before severity of symptoms can be accurately determined on the young plants and proper visual assessment of disease severity may be subjected to many variables, linked to the operator subjective rating or to the inconsistent expression of the symptoms.

Molecular techniques are rapid tools already used for the identification of different *F. oxysporum formae speciales* and races. Shimazu et al. (2005) developed sequence tagged site (STS) markers to identify races 1, 2 and 3 of FOL, using the sequence-characterized amplified region (SCAR) technique, while Pasquali et al. (2007) designed a series of specific primers for FOL race 1, using the inter-retrotransposon amplified polymorphism (IRAP) technique. Real-time PCR is a sensitive, reproducible and reliable tool used to quantify nucleic acids of a target (Schena et al., 2004). A specific quantitative real-time PCR (qPCR) protocol can be used by plant pathologists in early stages of cultivar development to detect and monitoring disease progression in infected plant tissues allowing the measurement of plant-pathogen interaction and enabling an earlier differentiation of the resistance in lettuce varieties reducing time of varietal selection in breeding programs (Fraaije et al., 2001). The aim of this study was to develop and validate a qPCR assay, using the specific primers for FOL race 1 to allow an early detection of the pathogen in seedlings, comparing the results with the visual assessment of the disease severity, and identifying plants that are not expressing symptoms.

2 | MATERIAL AND METHODS

2.1 | Lettuce cultivars and pathogen propagation

The commercially available lettuce cultivars Luna nera (Maraldi Sementi), Lunaverde, Cencibel (Rijk Zwaan) and the differential cultivars Romabella and Costa Rica 4, of known susceptibility to the race 1 of the pathogen (Gilardi et al., 2017), were used in this study. Their susceptibility was preliminary confirmed following the protocol described by Garibaldi et al. (2004). Lettuce seedlings were grown in plastic trays (4 L volume) under greenhouse conditions (25–30°C, natural daylight, HR 75%) for 15 days. A total of 15 seedlings per cultivar were inoculated with FL 7–18 strain of FOL race 1 (isolated from *L. sativa* L. in Italy, Agroinnova collection). The conidial suspension was obtained growing the strain in potato dextrose broth (PDB, Sigma-Aldrich, St. Louis, Missouri, USA) on a rotary shaker (120 rpm) for 10 days at room temperature. The suspension was measured for spore counts on a haemocytometer and adjusted to obtain a final concentration of 10^6 conidia/ml in a final volume of 1000 ml. The seedlings were inoculated by immersion of the roots for 10 min in the conidial suspension and then transplanted in plastic pots (12 L volume) filled with disinfected peaty moss substrate (TS3 and CICLAMEN 50:50). The pots were placed in the greenhouse with a temperature between 28 and 30°C. At 30 days post-inoculation, disease severity was assessed with a scale that ranged from 0 to 4, where 0 = healthy plants; 1 = initial symptoms of leaf yellowing, a slight reduction of the development and initial vascular browning; 2 = severe leaf yellowing, reduction of development and vascular browning; 3 = leaf chlorosis and inhibition of growth, evident deformation and severe vascular browning symptoms of wilting during the hottest hours of the day; 4 = plant strongly deformed with leaf chlorosis or completely necrotic leaves, totally wilted with severe vascular browning (Garibaldi et al., 2004). The data were expressed as disease index (DI) 0–100, calculated with the following formula:

$$DI = \left[\sum (i \times n_i) / (4 \times \text{total of plants}) \right] \times 100;$$

where $i = 0$–4 and n_i is the number of plants with rating i.

2.2 | Validation of the specific qPCR for FOL race 1

The qPCR reaction was set up using the race-specific primers Hani3' and Hani3lattrev (Pasquali et al., 2007). The assay was carried out on a StepOne Plus™ Real-Time PCR System thermal cycler (Applied Biosystems, Waltham, Massachusetts, USA), in a final volume of 25 µl containing: 1 µl of genomic DNA as template, 0.3 µl of each primer (10 µM) and 10 µl of SYBR Green PCR Master Mix (Applied Biosystems). The amplification protocol was carried out with an initial incubation at 95°C for 10 min and followed by 40 cycles at 95°C for 15 s, 60°C for 1 min and 72°C for 45 s and a melting curve with the following steps: 95°C for 15 s, 60°C for 1 min and an increase of the temperature to 95°C at 0.3°C/s to record the fluorescence. The protocol was validated by the evaluation of sensitivity, specificity, selectivity, repeatability and reproducibility according to the EPPO PM7/98 standard (EPPO, 2019). The limit of analytical sensitivity was obtained with a standard curve by the correlation of the Cycle threshold (Ct) and the concentrations of ten-fold serial dilutions of the DNA of one strain of FOL race 1 (FL 7–18). To verify the analytical specificity of the protocol to detect FOL race 1 an assay was set up with DNA samples extracted from 7 FOL race 1 isolates, 1 strain of FOL race 4, 9 isolates of phylogenetically closely related *Fusarium* spp. and other 3 lettuce pathogens: *Rhizoctonia solani*, *Verticillium dahliae* and *Alternaria alternata* (Agroinnova collection). To verify the influence of the plant DNA on FOL race 1 detection, the pathogen DNA was 5-fold diluted in *L. sativa* 'Romabella 58/16' and 'Luna nera 12/19' DNA. The repeatability of the method was tested through the degree of concordance among three independent tests carried out under unchanged test and instrument conditions, in the same laboratory and by the same operator, considering

the results of biological and technical replication. The reproducibility of the method was tested by different operators on different days.

2.3 | Comparison of disease severity and quantity of FOL race 1 detected with qPCR

Two different methods were used to assess the progress of the infection on lettuce seedlings. Each assay was performed in triplicate and the data obtained expressed as mean value of the three replications carried out.

2.3.1 | Microcosm test

The first method was carried out in microcosm with four different lettuce cultivars: Romabella (resistant), Costa Rica 4 (resistant), Cencibel (susceptible) and Luna nera (susceptible). A total of 432 seeds per cultivar (24 replications of 18 seeds) were sown in plastic boxes (12.5 cm × 6.5 cm) filled with 150 ml of disinfected peaty substrate and irrigated with 40 ml of sterile water. The boxes were closed with their lid and incubated at 27°C, under a 12 h near-ultraviolet light (NUV):12 h dark cycle. The boxes should be placed in a climatic chamber that ensure a good light distribution on each one of them to allow an optimal growth of the plants and a better evaluation of the disease development. After two days 12 boxes of each cultivar were inoculated with a conidial suspension of FL 7–18 strain of FOL race 1 with a final concentration of 10^6 conidia/ml, injecting 1 ml of the inoculum at the base of each sprout with a micropipette and sterile tips. The other 12 boxes of each cultivar were treated with 1 ml of sterile water and used as healthy controls. The boxes were incubated at the same conditions described above for 7 days, before to proceed with the count of the germinated seedlings and the first sampling for real-time PCR testing. Symptoms were recorded as number of dead plants over the total number of germinated seeds because the small size of the seedlings did not allow the assignment of an index of severity of the disease.

A total of 80 plants per cultivar (4 replications of 20 plants randomly collected in the boxes) were harvested from the inoculated samples and from healthy controls of each cultivar. The seedlings were removed with tweezers, rinsed under tap water to remove soil debris, washed with a 1% sodium hypochlorite water solution for 1 minute, rinsed with sterile deionized water and let it dry on sterile filter paper. The seedlings were then sectioned with a scalpel to sample 5 mm of hypocotyl (Figure S1). Six portions from each biological replication, including healthy control, were plated on Potato Dextrose Agar (PDA) added with streptomycin sulphate S (25 mg/L) to isolate the pathogen. The same protocol was applied 14 days after the inoculation.

2.3.2 | Greenhouse test

The second method was carried out following the protocol used to confirm the susceptibility to FOL race 1 of the five lettuce cultivars used in this study, as described previously (Garibaldi et al., 2004). Fifteen days after sowing a total of 45 plants per cultivar (3 replicates of 15 plants) were inoculated and transplanted in plastic trays (12 L volume) filled with peaty substrate. The pots were placed in the greenhouse with a random scheme, with a temperature between 28 and 30°C. The symptoms were recorded after the appearance of the first signs of leaf yellowing and stunted plants, 14 days after the artificial inoculation, and the severity was assessed with a scale that ranged from 0 to 4 and expressed as disease index (DI) 0–100 as mentioned above (Garibaldi et al., 2004). After 14 days from the inoculation, concurrently with the assessment of disease severity, 3 plants from each replication, including the healthy control, were sampled, rinsed under tap water to remove soil debris, washed with a 1% sodium hypochlorite water solution for 1 min, rinsed with sterile deionized water and dried on sterile filter paper.

2.3.3 | Pathogen quantification via qPCR

A total of 18 hypocotyls per replication, sampled at 7 and 14 days from seedlings grown in the microcosm assay, and 3 hypocotyls per replication, sampled at 14 days from the greenhouse test, of each cultivar tested, were grounded with liquid nitrogen with mortar and pestle to obtain a powder. One hundred mg of the powder was transferred to a 2 ml microcentrifuge tube and used for the extraction of the DNA with the Omega E.Z.N.A.® Plant DNA kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturers' instructions, after a 30-min cycle, at 25 Hz, in Tissuelyser (Qiagen®, Hilden, Germany), that improves the yield of DNA extraction. The extracted DNA was quantified with the spectrophotometer Nanodrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stored at −20°C. The DNA was tested with the race-specific qPCR protocol validated. All qPCR tests were performed including three technical replications of each DNA sample, including a positive (FL 7–18 DNA) and negative controls. The average of the three technical replications 'Quantity mean', obtained with the qPCR assay, were used to calculate the number of cells of the pathogen present in the DNA extracted from artificially infected lettuce samples. The Ct values obtained with the molecular assay were compared with the standard curve to calculate the amount (pg) of the pathogen DNA in 1 µl, of each sample. In order to calculate the number of cells/µl, the elution volume of the DNA extracted (40 µl) was considered. The approximate number of fungal cells in each sample was calculated dividing the DNA quantity by the weight in pg of the genome of the pathogen (0.0377 pg) (Pasquali et al., 2006).

2.4 | Data analyses

Baseline range, threshold cycle (CT) values and qPCR standard curves were automatically generated using StepOne software.

The data were subjected to the analysis of variance (ANOVA), following an arcsine-based transformation of DI and percentage of dead plants values for data normalization. The Tukey's test was used

to explore differences between multiple group means ($p \leq .05$). Data were finally back-transformed to the original. Statistical analysis was performed with the Statistical Package for Social Science (SPSS, IBM, Chicago, IL, USA) version 27.0.

3 | RESULTS

3.1 | Lettuce cultivars

The 5 lettuce cultivars used in this study showed different degrees of susceptibility to FOL race 1 FL7-18 and confirmed their suitability for validation of the molecular detection protocol (Table 1).

3.2 | Validation of the specific real-time PCR for FOL race 1

Analytical sensitivity of the real-time PCR reaction, determined with a standard curve obtained by correlating the cycle threshold (Ct) and ten-fold serial DNA dilutions extracted from fresh mycelium of 7–18 strain of FOL race 1, showed the amplification of the first five points (from 1 ng/µl to 100 fg/µl – from 26.5 to 2.7 copies), determining the amount of 100 fg/µl as the sensitivity limit of the reaction. The regression line was obtained with the determination coefficient (R^2) value of .9978, and the average reaction efficiency, calculated from the slope of the line, is 92% (Figure 1a,b). The analytical specificity of the reaction, tested using 7 strains of FOL race 1, 1 strains of FOL race 4, 9 strains of phylogenetically closely related *Fusarium* spp. and other 3 isolates of *Rhizoctonia solani*, *Verticillium dahliae* and *Alternaria alternata*, was confirmed by the amplification of the 7 strains of FOL race 1 only (Table 2).

No influence was observed on the selectivity of the real-time PCR assay when FOL race 1 DNA, serially diluted in DNA of *L. sativa* 'Romabella', and of 'Luna nera' DNA, was used (Figure 2). The amplifications showed similar PCR efficiencies and a reliable correlation between the Ct values and the amount of measured FOL race 1 DNA. The repeatability and reproducibility of the real-time PCR assay for FOL race 1 were confirmed by producing 100% reliable amplifications among replications, both biological and technical, when tested under unchanged analysis conditions or with different operators.

TABLE 1 Cultivar used as controls and their susceptibility to FOL race 1 (ANOVA and Tukey $p < 0.05$)

Cultivar	Disease index 0–100 at 30 days after inoculation		Susceptibility
Luna nera	68.3	d	Susceptible
Cencibel	36.7	c	Susceptible
Romabella	0.0	a	Resistant
Costa Rica 4	0.0	a	Resistant
Lunaverde	10.0	b	Partially resistant

3.3 | Comparison of disease severity and quantity of FOL race 1 detected with qPCR

The presence of the pathogen and the progress of the infection over time was confirmed by isolation on PDA + S, after 7 and 14 days from the inoculation, using portions of the hypocotyl of seedlings from each replication. After 7 days of incubation at room temperature (25–27°C), the plates were observed and the presence of the inoculated pathogen was verified and confirmed, at each time point by observation of fungus morphology (Table 3).

The results of the analyses carried out on the DNA extracted from lettuce tissues are listed in Table 3. Positive amplifications were obtained with real-time PCR assays both with DNA extracted from lettuce grown in microcosm at 7 and 14 days and in the greenhouse at 14 days. Data of disease index and percentage of dead plants were correlated with the amount of pathogen quantified with the real-time PCR. The protocol did not detect the pathogen in the resistant cultivar Romabella that was asymptomatic (DI = 0), while detected a low amount (21.2 cells/µl) of FOL race 1 in the partial

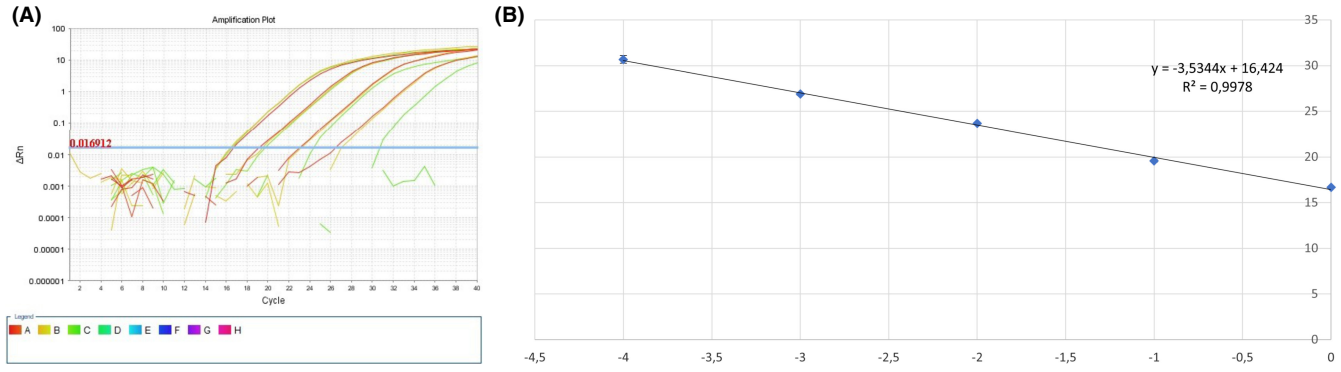

FIGURE 1 (a) Amplification with real-time PCR assay of serial dilutions of DNA of FOL race 1 strain FL 7–18. The threshold cycle values (Ct) of the fungal DNA ten-fold serial dilutions are represented in red and green. (b) Regression line obtained. The equation of the line and the coefficient of determination are shown in the graph

TABLE 2 Strains used in this study to check the specificity of the real-time PCR assay for FOL race 1 (Ct, melting °C). Standard deviation was included for each Ct and melting temperature. Neg = negative, melting = melting temperature

				Real-time PCR	
Species	Origin	Year of isolation	Isolate code	Ct	Melting (°C)
F. oxysporum f.sp. *lactucae* race 1	Italy	2007	Mya 3040	16.00 ± 0.34	74.04 ± 0.00
F. oxysporum f.sp. *lactucae* race 1	Italy	2018	FL 7/18	16.37 ± 1.27	73.94 ± 0.09
F. oxysporum f.sp. *lactucae* race 1	Italy	2016	FL 8/16	16.30 ± 0.13	73.99 ± 0.08
F. oxysporum f.sp. *lactucae* race 1	USA	2006	SB1-1 MAFF 244120	17.46 ± 0.41	73.88 ± 0.07
F. oxysporum f.sp. *lactucae* race 1	Japan	2005	JCP 043	16.37 ± 0.15	73.78 ± 0.00
F. oxysporum f.sp. *lactucae* race 1	USA	2002	AZ - 2002-01	18.11 ± 0.26	74.19 ± 0.00
F. oxysporum f.sp. *lactucae* race 1	France	2016	FOL 8/16	18.60 ± 0.82	74.33 ± 0.21
F. oxysporum f.sp. *lactucae* race 4	Italy	2019	FL 8/19	Neg	61.35 ± 0.00
F. proliferatum	Italy	2020	P 9.6	Neg	61.34 ± 0.00
F. fujikuroi	Italy	2006	I1.3	Neg	61.35 ± 0.21
F. verticillioides	Italy	2020	P12.3	Neg	61.30 ± 0.08
F. avenaceum	Italy	2020	AMBR 3–1	Neg	63.29 ± 3.36
F. oxysporum f. sp. *conglutinans*	Italy	2019	CVG1	Neg	61.39 ± 0.38
F. oxysporum f. sp. *cucumerinum*	Italy	2019	CVG3	Neg	61.25 ± 0.09
F. oxysporum	Italy	2019	CVG116	Neg	61.35 ± 0.00
F. oxysporum f. sp. *spinaciae*	Italy	2019	CVG346	Neg	61.40 ± 0.09
F. oxysporum f. sp. *radicis-capsici*	Italy	2020	P17.D	Neg	61.34 ± 0.00
Alternaria alternata	Italy	2017	19–53	Neg	61.35 ± 0.00
Rhizoctonia solani	Italy	2018	RH	Neg	61.35 ± 0.00
Verticillium dahlie	Italy	2013	19–48	Neg	61.39 ± 0.09

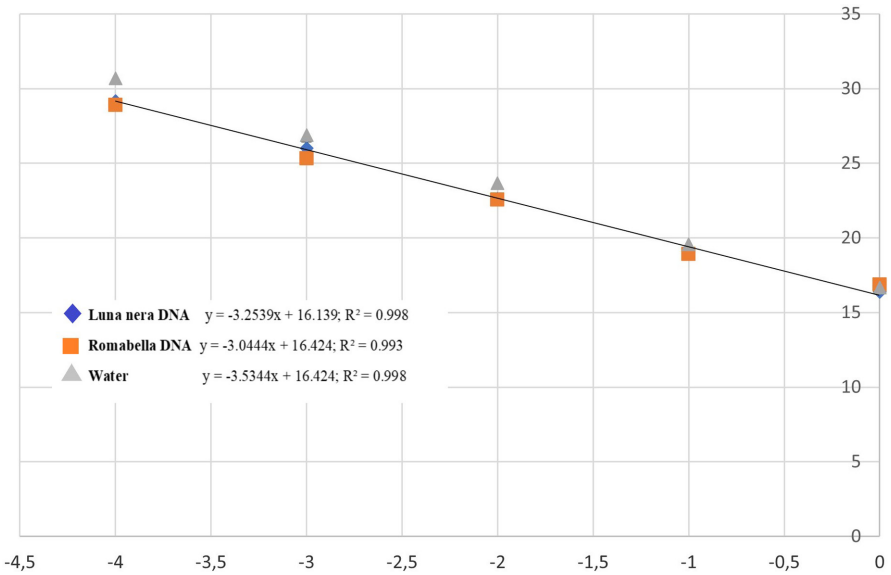

FIGURE 2 Regression lines obtained by correlating the cycle threshold (Ct) and ten-fold serial dilutions of FOL race 1 DNA (1 ng/μl) diluted in sterile, ultrapure water or in DNA (1 ng) extracted from hypocotyl of lettuce cv Luna nera and cv Romabella

resistant cultivar Lunaverde, that showed a low disease index (4.4) in the greenhouse. High quantity of FOL race 1 (3×10^2 cells/μl and 1.9×10^3 cells/μl) and high disease index (40 and 62) were instead recorded in susceptible cultivars, such as Cencibel and Luna nera. The results obtained from the analysis carried out in the microcosm assay showed the detection of the pathogen in all the cultivars tested, at 7 days from the inoculation. The protocol detected the pathogen in the susceptible cultivars, Luna nera and Cencibel (5.3 and 3.1×10^2 cells/μl, respectively), that showed the 11.9% and 9.9% of dead seedlings, but also in the resistant cultivars Romabella and Costa Rica 4 (10.6 and 7.9 cells/μl). At 14 days from the inoculation, the assay detected the pathogen only in the susceptible cultivars Luna nera (56.9 cells/μl) and Cencibel (2.9×10^2 cells/μl), where the highest percentages of dead plants corresponded to high amount of

TABLE 3 Results of disease index (0–100) recorded at 14 days in the greenhouse assay; isolation of FOL race 1 from the hypocotyl at 7 and 14 days from the inoculation in the microcosm assay; percentage of dead plants due the infection of FOL race 1 at 7 and 14 days from the inoculation; average quantity of cell/µl of FOL race 1 detected with real-time PCR in the assays carried out in the greenhouse and in the microcosm (ANOVA and Tukey $p < 0.05\%$)

Sample			Greenhouse			Microcosm							
Cultivar	Treatment	Susceptibility	Disease index (0–100) at 14 days		Average quantity of FOL race 1 (cells/µl) at 14 days	Isolation at 7 days	Percentage of dead plants at 7 days		Average quantity of FOL race 1 (cells/µl) at 7 days	Isolation at 14 days	Percentage of dead plants at 14 days		Average quantity of FOL race 1 (cells/µl) at 14 days
Romabella	Inoculated	Resistant	0.0	a	nd	a	Positive	1.4 ± 0.0	a	10.6	Negative	11.6 ± 0.1	a	nd
	Not inoculated		0.0	a	nd	a	Negative	nd		nd	Negative	nd		nd
Cencibel	Inoculated	Susceptible	40.0 ± 0.2	c	$3.0*10^2$	d	Positive	9.9 ± 0.1	b	$3.1*10^2$	Positive	41.0 ± 0.2	b	$2.9*10^2$
	Not inoculated		0.0	a	nd	a	Negative	nd		nd	Negative	nd		nd
Costa Rica 4	Inoculated	Resistant	0.0	a	7.9	b	Positive	3.8 ± 0.3	ab	7.9	Negative	6.2 ± 0.1	a	nd
	Not inoculated		0.0	a	nd	a	Negative	nd		nd	Negative	nd		nd
Luna nera	Inoculated	Susceptible	62.0 ± 0.1	d	$1.9*10^3$	e	Positive	11.9 ± 0.1	b	5.3	Positive	28.3 ± 0.2	b	56.9
	Not inoculated		0.0	a	nd	a	Negative	nd		nd	Negative	nd		nd
Lunaverde	Inoculated	Partially resistant	4.4 ± 0.1	b	21.2	c	NOT tested							
	Not inoculated		0.0	a	nd	a								

Note: Data are expressed as mean value of the three replication performed for each assay. Standard deviation was included for each disease index and percentage of dead plants value recorded. *nd = not detected.

FOL race 1 detected. In not inoculated controls, no dead plants due to pathogen infection were recorded, and no cells/µl were detected with qPCR assay.

4 | DISCUSSION

The qPCR assay with the specific primers for FOL race 1 (Pasquali et al., 2007) used in this study confirms its reliability as diagnostic tool for the quantification of the pathogen in early stages of infection. The analytical sensitivity was determined setting up a curve of standards, and it corresponds to a concentration of 100 fg/µl. The weight of the genome of *F. oxysporum* is approximately 0.0377 pg (Pasquali et al., 2006), and considering that the limit of detection estimated was 100 fg, the sensitivity of the real-time reaction corresponds to 2.7 copies. The analytical specificity of the reaction was confirmed by the amplification of FOL race 1 isolates only, and no influence was observed on the selectivity of the qPCR assay when FOL race 1 DNA, serially diluted in DNA of *L. sativa*. Repeatability and reproducibility were confirmed by replication of qPCR tests under unchanged and modified test conditions.

The results of the molecular assay highlighted the sensitive and specific identification of FOL race 1 in symptomatic and asymptomatic lettuce seedlings, and a high correlation between the levels of disease severity recorded in the greenhouse and the quantity of FOL race 1 detected with qPCR, showing the absence of the pathogen in the asymptomatic and resistant cultivar Romabella, a low quantity of FOL race 1 (21.2 cells/µl) in the partial resistant 'Lunaverde' that showed a low disease index (4.4), while high quantity of the fungus was detected in susceptible cultivars, Cencibel and Luna nera. The results obtained from the analysis carried out in the microcosm assay showed the detection of the DNA of the pathogen after 7 days from the inoculation, but no correlation between the percentage of dead plants and the quantity of the pathogen recorded was found, considering the known susceptibility of the cultivars tested. These results could be due to the small size of the seedlings (about 5–8 mm) that did not allow a clear separation of the hypocotyl portion of the stem at this stage. Repeating the experiments increasing the number of seedlings eliminated this inconvenience at 14 days from the inoculation, where it is possible to notice significantly higher concentrations of the pathogen in the susceptible cultivars compared to the resistant ones, producing reliable results between the number of dead plants recorded and the quantity of pathogen detected with the qPCR protocol, as it occurred in the greenhouse assay. In previous studies where the serial dilution plate method of different portions of taproots was used, the colonization of resistant cultivars by the pathogen was observed at six weeks after inoculation (Scott et al., 2014). In this case it is possible that the age of the seedlings influenced the results. Results similar to ours were obtained in the detection of other pathogens responsible of important economic losses in vegetables crop, such as *Synchytrium endobioticum* on potato (Van Gent-Pelzer et al., 2010), Cucumber vein yellowing virus

(CVYV) in cucumber (Picó et al., 2005), *Fusarium oxysporum* f. sp. *ciceris* in chickpea (Jiménez-Fernández et al., 2011), *Alternaria alternata* and *A. solani* in potato leaves (Leiminger et al., 2015), where the highest concentration of DNA of the targets were detected in the most susceptible cultivars that showed high levels of disease severity, while the lowest quantity of DNA were found in samples of cultivars classified as the most resistant to the pathogens.

The DI recorded in the greenhouse assay and the percentage of dead seedlings recorded in the microcosm test highly correlated with the quantity of pathogen detected by the qPCR confirming the applicability and the usefulness of the method as a fast, effective, reliable and sensitive diagnostic technique to assess the disease severity on lettuce and to evaluate the resistance of new lettuce cultivars, already after 14 days from the artificial inoculation. The microcosm assay highlighted also the advantage of significantly reducing the space needed to test the susceptibility of the lettuce cultivars, allowing to analyse a greater number of samples in each experiment. Further studies could be done to optimize the environmental variables that influence the seedlings growth, under artificial conditions of the microcosm assay. On the basis of the results obtained it is possible to establish the amount of 50 cells/µl the threshold to consider a genotype as susceptible.

Marker Assisted Selection (MAS) is a tool that supports breeders in the selection of lines with the desired characteristics, but in a breeding program for plant disease resistance, field or greenhouse assessments under disease pressure are complementary steps to be taken to confirm both genetic resistance and evaluate the plant phenotype (Mahdavi Meighan et al., 2020). This stage can take several weeks, even months with some pathogens. The possibility to use a molecular tool to shorten the field or greenhouse evaluation and discarding false negative susceptible lettuce can represent a step forward in obtaining the expected results. qPCR represents an efficient and less time-consuming way to screen the resistance in lettuce cultivars as well as to evaluate the efficacy of fungicide treatments in early stages (Munkvold & O'Mara, 2002), and the levels of contamination of the seeds and of the soil (Pollard & Okubara, 2019) or latent infections of *F. oxysporum* strains to manage the post-harvest losses in onion and for testing batches of onion sets (Latvala et al., 2019). Although an analysis of the costs needs to be done, the method can be modified and validated for other races of the same pathogen, with possible time saving in the screening process, allowing also the detection of the pathogen in plants not yet expressing typical symptoms of Fusarium wilt.

AUTHOR CONTRIBUTIONS

MS involved in experimental procedures, data analysis and writing. GG involved in greenhouse test, review and editing; MLG involved in funding acquisition, review and editing; MM involved in experiment design, supervision, writing, editing, review and corresponding author.

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CONFLICT OF INTEREST

We declare that there is no conflict of interest for publishing this article, and that it fulfils the scope of Journal of Phytopathology.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/jph.13128.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Giovanna Gilardi https://orcid.org/0000-0002-6420-7444
Monica Mezzalama https://orcid.org/0000-0003-2558-8878

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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