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Research Papers

A SYBR Green qPCR assay for specific detection of *Colletotrichum ocimi*, which causes black spot of basil

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Summary. *Colletotrichum ocimi* causes black spot of basil (*Ocimum basilicum*) and is a serious threat to basil cultivation as it compromises leaf production. The pathogen also infects seeds, which could become primary sources of inoculum for spread of black spot. A SYBR Green real-time PCR assay was developed to detect *Colletotrichum ocimi* in basil leaves and seeds, based on the partial *β-tubulin* (*tub2*) gene sequence. Two primer sets were designed and tested. The selected primer pairs produced amplicons of 130 bp. The real-time PCR assay was validated for analytical specificity, sensitivity, selectivity, repeatability and reproducibility. The assay was specific for *C. ocimi* with respect to ten *Colletotrichum* spp. and to another 12 pathogens of basil plants. Sensitivity was 1 pg μL^{-1} of genomic fungal DNA and amplification analyses were not influenced by basil genomic DNA. The assay detected and quantified *C. ocimi* in artificially inoculated basil leaves. This is the first specific primer set for *C. ocimi*, which allows rapid detection and quantification of the pathogen is a useful tool for diagnostics in plants. Detection in seeds would also be possible, but will require an optimized extraction method. The qPCR detection of *C. ocimi in planta* can contribute to adoption of effective preventive disease management strategies.

Keywords. Anthracnose, molecular diagnostics, *Ocimum basilicum*, seeds.

INTRODUCTION

Colletotrichum includes important species pathogenic to a range of plant hosts (Dean *et al.*, 2012). *Colletotrichum ocimi* causes black spot of basil (*Ocimum basilicum*), an economically important crop cultivated in many countries, and, particularly, in the Mediterranean area (Damm *et al.*, 2014). Black spot is the common name for this anthracnose disease, which can affect basil leaves and seeds (Gullino *et al.*, 1995), and is a serious threat to seed-production companies and basil producers (Cacciola *et al.*, 2020). Gullino *et al.* (1995) reported isolation of a *Colletotrichum* sp. from basil, which was initially identified as *C. gloeosporioides*. (Damm *et al.* (2014) later allocated these isolates to the *C. destructivum* species complex (SC), describing it as *C. ocimi*. Several other *Colletotrichum* species have been reported on basil: *C. capsici* in India (Alam *et al.*, 1981), *C. destructivum* in Italy (Cacciola *et al.*, 2020), *C. siamense* in Malaysia (Ismail *et al.*, 2021) and *Colletotrichum* sp. in Florida, United States of America (Alfieri *et al.*, 1984).

Before the 1990's, species of *Colletotrichum* were identified using a combination of morphological and cultural characters. The most investigated features were shape and size of conidia and appressoria, presence or absence of setae, sclerotia, acervuli, sexual morphs, and cultural characteristics (Cannon *et al.*, 2000). However, these characters alone were considered inadequate for species identification, due to their variability depending on environmental and culture conditions (Cai *et al.*, 2009). Molecular tools have been considered more reliable and were subsequently used along with morphological observations. Particularly, multi-locus phylogenetics is used for *Colletotrichum* species identification (Hyde *et al.*, 2009). Up to 13 different loci are available for delineation of species within a SC (Liu *et al.*, 2016; Talhinhas and Baroncelli, 2021). Bhunjun *et al.* (2021) considered the ITS region as useful for reaching a SC-level identifications, with *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) and *β-tubulin* (*tub2*) as the most informative loci for species-level identification. However, the polyphasic approach suggested for *Colletotrichum* species identification is laborious and time-consuming (Cai *et al.*, 2009; Du *et al.*, 2021).

The quantitative real-time PCR technique has been used as a rapid and sensitive diagnostic method for the identification of *Colletotrichum* spp. (Mirmajlessi *et al.*, 2015). Schena *et al.* (2017) developed a duplex qPCR TaqMan method to detect and quantify *C. godetiae* and *C. acutatum sensu stricto* in olive tissues. An assay for the simultaneous detection of *C. acutatum* and *C. gloeosporioides* from infected strawberry leaves by real-time PCR was developed by Rahman *et al.* (2019). Du *et al.* (2021) elaborated a real-time PCR method to detect, quantify and monitor *C. siamense* infecting rubber trees, and Kamber *et al.* (2021) developed a specific quantitative real-time TaqMan PCR assay that allowed rapid and reliable detection and quantification of *C. lupini* in infected seeds and plant material. These specific molecular techniques provide effective and rapid tools for *Colletotrichum* species identification and quantification in symptomatic and asymptomatic plant tissues. These methods could be implemented by phytosanitary services and companies to assess the health status of seeds and propagation material (Kumar and Gupta, 2020). However, no methods have yet been developed to identify *C. ocimi* or other species of the *C. destructivum* SC.

Considering the important losses that black spot disease can produce on basil seedlings and plants, the present study aimed to develop a SYBR Green qPCR assay to facilitate the detection and quantification of *C. ocimi* in basil leaves and seeds.

MATERIALS AND METHODS

Fungal isolates and DNA extraction

Isolates of *C. ocimi* (Table 1) were taken from stock cultures maintained at -80°C in the culture collection of the AGROINNOVA Centre of Competence (University of Torino), and grown on potato dextrose agar (PDA, VWR Chemicals) amended with 25 μg μL^{-1} of streptomycin sulphate (PDA-S, Sigma-Aldrich). Genomic DNA was extracted from 0.1 g of mycelium, using the E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-Tek), following the manufacturer's instructions, and was stored at -20°C. The DNA concentration of each sample was measured using NanoDrop 2000 (Thermo Fisher), and was then adjusted to 1–50 ng μL^{-1}.

Species-specific primer design

Damm *et al.* (2014) reported that the partial *tub2* gene sequence of *C. ocimi* was less than 97% similar to sequences of this locus of other *Colletotrichum* spp., so this region was selected for primer design. Sequences of the *tub2* region retrieved from the GenBank database were: *Colletotrichum ocimi* (CBS 298.94, KM105502; CVG189, MN535124; CVG190, MN535125; CVG193, MN535126; CVG200, MN535128; CVG 202, MN535129; CVG 203, MN535127; CVG 204, MN535130 and CVG 205, MN535131), and *Colletotrichum* spp. within the *C. destructivum* SC (*C. americae-borealis* CBS 136232, KM105504; *C. bryoniicola* CBS 109849, KM105461; *C. destructivum* CBS 136228, KM105487; *C. higginsianum* CPC 19379, KM105464; *C. lentis* CBS 127604, JQ005850; *C. lini* CBS 172.51, JQ005849, and *C. utrechtense* CBS 130243, KM105481). Selection of species within the *C. destructivum* SC was based on phylogenetic distance within the complex, with closely related species selected. All sequences were aligned with the software Mega v. 7 (Kumar *et al.*, 2016), and a region with high polymorphism with respect to the non-target species was selected for primer design. Two primer pairs were designed and

Table 1. List of isolates used in this study to assess the specificity of the primer pairs designed for the detection of *Colletotrichum ocimi* (TubOc_23fw - TubOc_190rev and TubOc68fw - TubOc_197rev).

Sample number	Species/Sample	ID Code	Host
1	*Colletotrichum ocimi*	CVG189	*Ocimum basilicum*
2	*Colletotrichum ocimi*	CVG190	*Ocimum basilicum*
3	*Colletotrichum ocimi*	CVG193	*Ocimum basilicum*
4	*Colletotrichum ocimi*	CVG200	*Ocimum basilicum*
5	*Colletotrichum ocimi*	CVG202	*Ocimum basilicum*
6	*Colletotrichum ocimi*	CVG204	*Ocimum basilicum*
7	*Colletotrichum ocimi*	CVG205	*Ocimum basilicum*
8	*Colletotrichum fioriniae*	CVG175	*Salvia leucantha*
9	*Colletotrichum bryoniicola*	CVG257	*Salvia nemorosa*
10	*Colletotrichum fructicola*	CVG170	*Salvia greggii*
11	*Colletotrichum utrechtense*	CBS 130243	*Trifolium pratense*
12	*Colletotrichum americae-borealis*	CBS 136232	*Medicago sativa*
13	*Colletotrichum lentis*	CBS 127604	*Lens culinaris*
14	*Colletotrichum higginsianum*	CPC 19379	*Brassica chinensis*
15	*Colletotrichum lini*	CBS 172.51	*Linum usitatissimum*
16	*Colletotrichum destructivum*	CBS 136228	*Crupina vulgaris*
17	*Stagonosporopsis vannaccii*	PHB1	*Ocimum basilicum*
18	*Stagonosporopsis vannaccii*	PHB8	*Ocimum basilicum*
19	*Alternaria arborescens*	PHB29	*Ocimum basilicum*
20	*Alternaria alternata*	BASALT 5/10	*Ocimum basilicum*
21	*Alternaria tenuissima*	BASALT 2/10	*Ocimum basilicum*
22	*Plectosphaerella cucumerina*	CVG886	*Ocimum basilicum*
23	*Rhizoctonia solani*	22reis	*Ocimum basilicum*
24	*F. oxysporum* f. sp. *basilici*	FOB001	*Ocimum basilicum*
25	*Myrothecium verrucaria*	BAS 5-18	*Ocimum basilicum*
26	*Myrothecium follicola*	BAS 4-18	*Ocimum basilicum*
27	*Myrothecium roridum*	BAS cv Eleonora	*Ocimum basilicum*
28	*Sclerotinia* sp.	36bas	*Ocimum basilicum*
29	*Peronospora belbahrii*	-	*Ocimum basilicum*
30	*Colletotrichum nigrum*	CVG171	*Salvia greggii*

validated *in silico* using the reference strains listed above, using NCBI Primer-Blast (Ye *et al*., 2012): TubOc_23fw - TubOc_190rev amplifying 168 bp and TubOc_68fw - TubOc_197rev amplifying 130 bp (Table 2).

End-point PCR amplification, analytical specificity and sensitivity

Preliminary tests were carried out to define the optimal final concentrations of $MgCl_2$ (0.5, 1 and 1.5 mM),

Table 2. Sequences of the primers designed for the specific detection of *Colletotrichum ocimi.*

Primer name	Primer sequence 5'->3'	Amplicon (bp)
TubOc_23fw	GCCTTTTGGTGCGTAGTCA	168 bp
TubOc_190rev	GGTGAATACGTGGTCAGGGC	
TubOc_68fw	CGACCTGGAAAGGAATAACTCGT	130 bp
TubOc_197rev	GGTTAGCGGTGAATACGTGGT	

primers (0.5 and 1 μM), DMSO (0.5, 0.7 and 1.2 μL) and template (20, 50 and 100 ng μL^{-1}). The end-point PCR reactions were carried out using a Taq DNA polymerase kit (Qiagen), in a total volumes of 25 μL each. The optimized mixture composition was as follow: 2.5 μL of Qiagen Buffer 10×, 0.5μL $MgCl_2$ (25mM), 0.5 μL dNTPs (10mM), 0.5 μL of each primer (10μM), 0.2 μL of Qiagen Taq DNA polymerase (5U) and 1 μL of DNA as template (20-50 ng μL^{-1}) The thermal cycler conditions were: 94°C for 3 min, followed by 30 cycles each of 94°C for 45 s, 64°C for 45 s, and 72 °C for 1 min, and a final extension cycle at 72 °C for 5 min. The PCR products were examined by electrophoresis on 1% agarose gels (VWR Life Science AMRESCO^R biochemicals), stained with GelRed^{TM} in Tris-acetate buffer. The primers were tested to evaluate their specificity using strains of species within the *C. destructivum* SC (Damm *et al*., 2014) and of species reported as pathogens on basil plants and seeds (Garibaldi *et al*., 1997; Gilardi *et al*, 2018). All strains used are listed in Table 1. Analytical sensitivity tests were carried out by conducting PCR reactions using *C. ocimi* DNA of strain CVG190 (Guarnaccia *et al*., 2019), which was 10-fold serially diluted (10 ng μL^{-1} to 100 fg μL^{-1}). The Limit of Detection (LOD) of these tests was assessed.

SYBR green real-time PCR (qPCR) assay development

The primers which provided the best results in conventional PCR analyses were selected and used for qPCR with SYBR Green. The real-time PCR assays were carried out using a StepOne Plus™ Real-Time PCR System thermal cycler (Applied Biosystems). Preliminary tests were conducted to define the optimal final concentrations of the primers (3, 1 or 0.3 μM) and the optimal annealing temperature (60 or 64°C). Reactions were carried out with the optimised mixture composition in a total volume of 10 μL, using 5 μL of 10× Power SYBR Green Mastermix, 0.3 μL of each primer (100 μM) and 1 μL of DNA as template (20–50 ng μL^{-1}). The optimal amplification conditions were: 95°C for 3 min, followed by 40 cycles each at 95°C for 15 s and at 64°C for 35 s. The melting curves were acquired after each run by ramping the temperature from 60°C to 95°C. Each reaction was performed in triplicate, and the results were displayed using the StepOne software.

SYBR green real-time PCR (qPCR) assay validation

The protocol was verified by evaluation of analytical specificity, sensitivity, selectivity, repeatability and reproducibility. For specificity, qPCR was performed in triplicate on all the strains listed in Table 1, using strains of *C. ocimi* as positive controls. Analytical sensitivity of the qPCR assay was evaluated by a standard curve obtained using *C. ocimi* DNA of strain CVG190 10-fold serially diluted, ranging from 10 ng μL^{-1} to 100 fg μL^{-1}. The LOD of the method was assessed, along with the correlation coefficient (R^2) between the cycle threshold (Ct) and the initial concentration of genomic DNA and the mean relative efficiency. Another test was conducted on *C. ocimi* genomic DNA mixed with host plant DNA, to simulate interference from host plant DNA and to establish selectivity. Plant DNA was extracted after grinding in liquid nitrogen, using an E.Z.N.A. Plant DNA kit according to the manufacturer's instructions. *Colletotrichum ocimi* DNA of strain CVG190 was ten-fold serially diluted (from 10 ng μL^{-1} to 100 fg μL^{-1}) with basil genomic DNA (10 ng μL^{-1}) extracted from healthy leaves. Each reaction was performed in triplicate and the results were displayed through the StepOne software. The standard curve was used as internal control to quantify *C. ocimi* DNA in different samples. Three independent assays were conducted to determine the repeatability of the method. The reproducibility was assessed by two different operators on different days.

Detection of Colletotrichum ocimi *in artificially inoculated basil leaves*

Nine artificially inoculated leaf samples of *O. basilicum* were collected from basil plants of the 'Genovese' type cultivar 'Italiano classico' (Royal Seeds). Conidial suspensions (10^6 conidia mL^{-1}) were sprayed onto pathogen-free 2-month-old plants, which were considered pathogen-free because no visual symptoms were found on leaves examined under a stereo-microscope (Leica EZ4). Control plants were sprayed with sterile water. The plants were then covered with transparent plastic film to maintain high relative humidity, and were transferred to a growth chamber maintained at 25°C with a 12 h light 12 h dark regime. The plastic film was removed at 3 d post-inoculation (Guarnaccia *et al*., 2019). DNA was extracted from 0.1 g fresh weight of symptomatic or non-inoculated leaves. DNA was measured using NanoDrop 2000 (Thermo Fisher Scientific) and diluted to reach a concentration of 10 ng μL^{-1}. The obtained DNA was analysed with the SYBR green Real-time PCR (qPCR) assay described above.

Detection of Colletotrichum ocimi *in artificially inoculated and non-inoculated commercial basil seeds*

Conidial suspensions in water could not be used to inoculate seeds, due to gum production by basil seeds after their immersion. Glycerol has been reported as plasticiser of this gum (Amini *et al*., 2015). Preliminary tests were therefore conducted by soaking seeds in glycerol solutions to find the best concentration to avoid gum production (data not shown). To inoculate basil seeds, a conidial suspension of *C. ocimi* was prepared in 70% glycerol (VWR Chemicals), with a final concentration of 10^6 conidia mL^{-1}. Seed samples were exposed to thermal shock for 30 s in liquid nitrogen and then soaked for 1 h in the suspension. Control seed samples were soaked in 70% glycerol. During soaking, samples were gently shaken at 95 rpm. Each seed sample was then recovered on plastic plates with sterile absorbent paper and incubated at 25°C for 72 h in the dark. Two inoculation trials were conducted, each using six batches of seeds (1 g of seeds for each batch) of the 'Genovese' type cultivar 'Edwina' (Enza Zaden), with three replicates per batch for a total of 18 samples. After the first trial, DNA of each inoculated sample was extracted directly after the inoculation. After the second trial, each inoculated sample was first washed with 5 mL of sterile distilled water to remove glycerol residuals, and DNA extraction was then carried out. Ten commercial batches of seven basil cultivars of the 'Genovese' type were each sampled in triplicate (30 samples) of commercial basil seeds. (Table 3). The seeds had no visible symptoms under a stereo-microscope (Leica EZ4). The cultivars were selected among those known to be susceptible to black spot (Guarnaccia *et al*., 2019; Cacciola *et al*., 2020). After grinding in liquid nitrogen, DNA of 0.1 g of each seed sample was extracted using an E.Z.N.A. Plant DNA kit, according to the manufacturer's instructions. DNA concentrations were measured using NanoDrop 2000. The obtained DNA was analysed with the SYBR green Real-time PCR (qPCR) assay described above.

Table 3. List of commercial basil seed cultivars of the 'Genovese' type reported as susceptible to black spot, and used in the present study to detect and quantify the presence of *Colletotrichum ocimi*.

Batch number	Cultivar
1	Aromatico
2	Genovese Gecom FT
3	Genovese ISI 602 F1
4	Italiano classico
5	Italiano classico
6	Italiano classico
7	Italiano classico
8	Italiko
9	Profumo
10	Superbo

RESULTS

Species-specific primer design, analytical specificity and sensitivity

The two designed primer sets were tested to assess their analytical specificity and sensitivity in end-point PCR. The primer pair TubOc_23fw - TubOc_190rev amplified DNA of *C. ocimi* strains, but also gave non-specific amplification with DNA of *C. bryoniicola* (CVG257), *C. americae-borealis* (CBS 136232), *C. lini* (CBS 172.51), *C. destructivum* (CBS 136228) and *Sclerotinia* sp. The primer pair TubOc_68fw - TubOc_197rev gave best results, by amplifying only DNA of *C. ocimi* strains (Figure 1, A and B). In analytical sensitivity tests, the LOD of the end-point PCR was 1 ng μL^{-1} for the primer set TubOc_23fw - TubOc_190rev, and 0.1 ng μL^{-1} for the primer set TubOc68fw - TubOc_197rev (Figure 2). The analytical specificity and sensitivity assays showed that the primer pair TubOc68fw - TubOc_197rev was appropriate for subsequent assays (Supplementary Figure 1).

SYBR green real-time PCR (qPCR) assay validation

The SYBR green real-time PCR with the selected primer pair was able to amplify the 130 bp fragment of the *tub2* partial gene of the *C. ocimi* strains, but not of the other species listed in Table 1. The specificity of the primers was also confirmed by presence of a single dissociation peak in the melting curve at 81.84 ± 0.22°C (Figure 3). The DNA of *C. ocimi*, serially diluted from 10 ng μL^{-1} to 100 fg μL^{-1} in sterile distilled water, was used to build a standard curve to evaluate the analytical sensitivity of the detection method (Figure 4, Supplementary Table 1). The LOD of the test was at 1 pg μL^{-1} (Ct = 36.45 ± 0.44). The correlation coefficient (R^2) between the cycle threshold (Ct) and the initial concentration of genomic DNA was >0.99. The mean value of the regression slope was –3.22, and the mean relative efficiency was 104%, which showed good qPCR efficiency (Adams, 2006). The presence of plant DNA together with fungal DNA had no influence on the selectivity of the primers in the SYBR green assay (Supplementary Table 2). A similar PCR efficiency and a reliable correlation between the Ct values and the amount of DNA of *C. ocimi* was

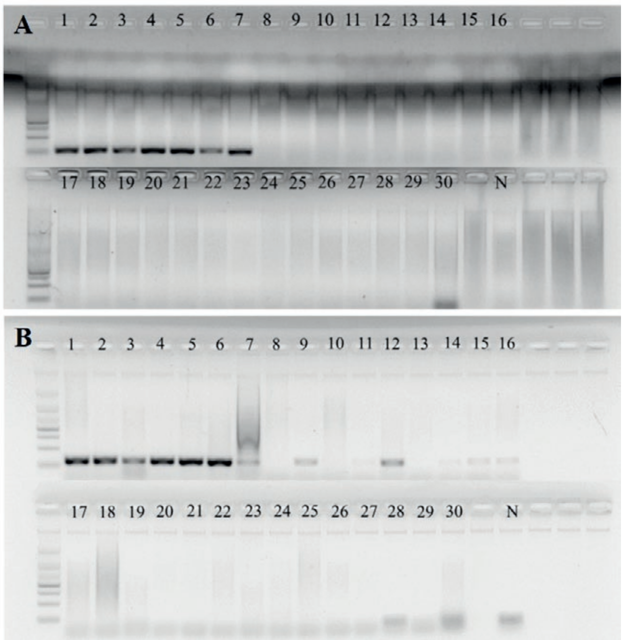

Figure 1. A) End-point polymerase chain reaction (PCR) specificity of the primer set TubOc_68fw TubOc_197rev. The numbers of tested *Colletotrichum ocimi* strains correspond to those reported in Table 1. N = negative control. **B)** End-point polymerase chain reaction (PCR) specificity of the primer set TubOc_23fw TubOc_190rev. The numbers of the tested strains correspond to those reported in Table 1. N = negative control.

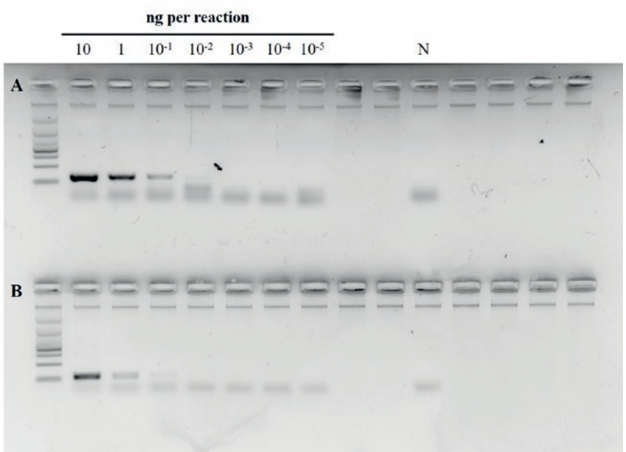

Figure 2. End-point polymerase chain reaction (PCR) analytical sensitivity of the two primer sets with a 10-fold serial dilution at the indicated concentration of genomic DNA of *Colletotrichum ocimi* strain CVG190. A = primer set TubOc_68fw TubOc_197rev; B = primer set TubOc_23fw TubOc_190rev. N = negative control.

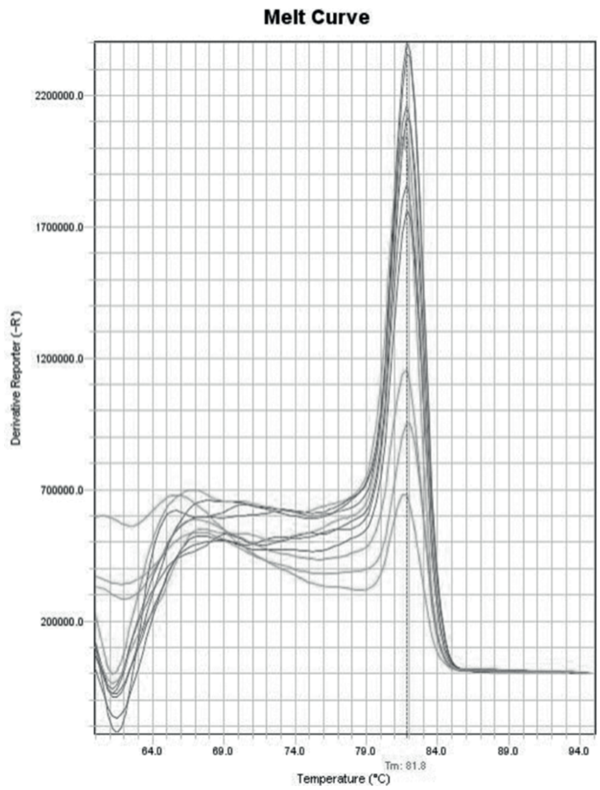

Figure 3. Single dissociation peaks of melting curves at 81.84 ± 0.22°C, which confirmed the specificity of the primer set TubOc_68fw TubOc_197rev. The peaks were obtained from the amplification of the dilutions (from 10 ng μL^{-1} to 100 fg μL^{-1}) in water of the *Colletotrichum ocimi* genomic DNA of strain CVG190.

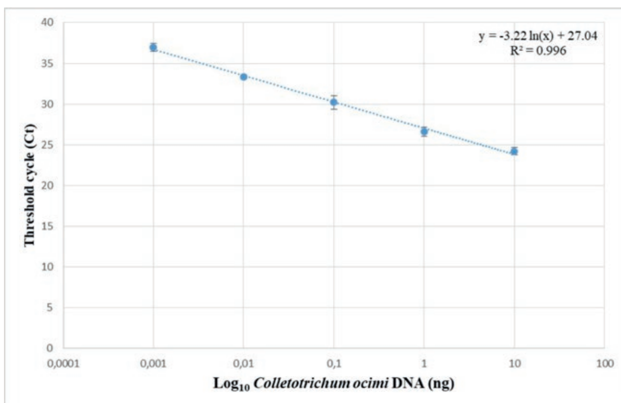

Figure 4. Standard curve obtained with *Colletotrichum ocimi* genomic DNA (strain CVG190). The Ct values were plotted against the *C. ocimi* DNA concentrations (from 10 ng μL^{-1} to 1 pg μL^{-1}). Standard deviation bars, the linear regression equation and the R^2 value are displayed on the graph. The dilutions were run in triplicate.

found from the amplification of *C. ocimi* DNA of strain CVG190 diluted in basil genomic DNA, compared to amplification of the samples diluted in water. The assay was performed on different days by two different operators and its reproducibility was confirmed.

Detection of Colletotrichum ocimi *in artificially inoculated basil leaves*

The SYBR green assay was used to detect and quantify *C. ocimi* in symptomatic basil leaves. The assay detected presence of *C. ocimi* in eight of the nine samples tested. In seven samples, the pathogen was detected at 0.247 to 0.001 ng μL^{-1}. One sample was below the LOD of the test (Ct = 38.26 ± 0.72). DNA samples obtained from non-inoculated basil leaves were negative for the presence of *C. ocimi*.

Detection of Colletotrichum ocimi *in artificially inoculated and commercial basil seeds*

The SYBR green assay was used to detect and quantify *C. ocimi* in artificially inoculated and in commercial basil seeds. For the artificially inoculated seeds, the first assay was conducted with DNA of basil seeds directly extracted after inoculation. The Ct values determined from four samples (one replicate each) were below the LOD of the assays (Ct = 37.13; 38.20; 39.03 or 38.15). For all other replicates, the assay was negative for the presence of *C. ocimi*. A second assay was therefore conducted on DNA of seeds extracted after inoculated seed washings. This second assay was detected presence of *C. ocimi* in four out of 18 tested samples. In two samples, the pathogen was found in a range of 0.004 and 0.001 ng μL^{-1}. The other two samples were below the LOD (Ct = 37.73 ± 0.09 and Ct = 38.93 ± 0.26). DNA samples obtained from non-inoculated basil seeds were confirmed as negative for the presence of *C. ocimi*. The assay was negative for the presence of *C. ocimi* in the commercial basil seeds sampled.

DISCUSSION

Molecular techniques are reliable and effective for the specific detection and quantification of plant pathogens (Mirmajlessi *et al.*, 2015). The present study describes the development of end-point PCR and SYBR Green real-time qPCR assays for the specific detection of *C. ocimi* in basil leaves and seeds. Both techniques were used to assess their analytical specificity and sensitivity. Selectivity, repeatability and reproducibility of the SYBR Green real-time qPCR assay were also determined. The amplification reactions were performed by increasing the annealing temperature to 64°C. With the obtained increased stringency, one primer set (TubOc_23fw -TubOc_190rev) still gave non-specific amplification. However, the amplification reaction with the other primer set (TubOc68fw - TubOc_197rev) allowed the specific detection of *C. ocimi*, avoiding the problem of cross-amplification. The LOD of the test was 1 pg μL^{-1} of *C. ocimi* DNA. This detection threshold was within the range of different assays that target other *Colletotrichum* spp. such as *C. lupini* (10 pg μL^{-1}), *C. godetiae* and *C. acutatum s.s.* (10 pg μL^{-1}) and *C. theobromicola* (1.4 pg μL^{-1}) (Schena *et al.*, 2017; Kamber *et al.*, 2021; Kaur *et al.*, 2021). Both analytical sensitivity and selectivity tests showed good qPCR efficiency within the established range of acceptability for developing a new detection method, from 90 to 110% (Adams, 2006).

This new assay can be applied to DNA directly extracted from fresh plant material and was not affected by co-extracted plant DNA. The assay allowed detection and quantification of *C. ocimi* in symptomatic artificially inoculated basil leaves. The pathogen was also detected from 0.1 g fresh weight of symptomatic leaves, in a range of 0.247 and 0.001 ng μL^{-1}. Future studies are planned to test the method also on naturally symptomatic leaves.

For seeds, the assay conducted with DNA directly extracted from seeds inoculated with conidial suspension of *C. ocimi* in glycerol permitted the detection of the pathogen on one replicate of four samples. The obtained values were positive, but below the LOD of the method, and since they were observed only in one replicate, the repeatability parameter of the assay could be affected (Cardwell *et al.*, 2018). A second assay was therefore conducted washing inoculated seeds before DNA extraction to reduce glycerol residuals. This second trial gave detection the pathogen on four inoculated samples out of 18, and quantification was possible on two of these samples. The second extraction method was used for quantification of *C. ocimi* on a subset of commercial seeds selected for testing, where the pathogen was not detected since no amplification curves and no peaks in the melting curves were observed during the qPCR analyses.

Globalization of markets and the centralization of companies in some regions have contributed to long distance exchanges of plant materials and seeds. Due to this international trade, different pathogens can be transmitted *via* uncertified material, causing disease outbreaks in new areas (Gullino *et al.*, 2014; Munkvold and Gullino, 2020). Specific pathogen detection methods are therefore required. The assay developed in the present study is the first molecular diagnostic tool developed to detect *C. ocimi*, and is a useful tool for the rapid identification and quantification of the pathogen on symptomatic basil leaves. For seeds, the method had a low detection success rate. Further research is therefore required to improve DNA extraction. This could be the most important step, due to the production of gum and presence of possible inhibitors within the basil seed coats or endosperm.

Traditional approaches to identify *Colletotrichum* spp. based on morphological and molecular data require isolation, DNA extraction and multi-locus sequencing, are time-consuming, and are applicable only to a restricted range of samples due to the high cost of the analyses (Bhunjun *et al*., 2021). Although a precise cost analysis should be carried out, SYBR Green real-time PCR with melting curve analysis is reported to be cost-effective, easy to use, and have optimal efficiency for small amplicons (Capote *et al*., 2012). For this reason, this technique was selected to develop the protocol described in the present study. The technique produced reliable and accurate diagnoses, and can reduce the necessary analysis time to a few days instead of weeks.

Colletotrichum ocimi can be transmitted *via* basil seeds, as reported in previous studies (Guarnaccia *et al*., 2019; Cacciola *et al*., 2020). Thus, detection of the pathogen on inoculated seeds, demonstrated in the present study, even at a low rate, confirms that this diagnostic tool could be useful for further investigations of commercial seeds, with an improved DNA extraction method. Furthermore, the end-point PCR assay could be used as a screening method to detect the presence or absence of *C. ocimi*, and to estimate its relative quantity through agarose-gel visualization.

No specific primers have been previously designed to detect *C. ocimi*. The developed and validated assay described here was shown to be specific for its target organism. Further studies are planned, aiming to improve detection of the pathogen on seeds or seedlings and to develop effective and preventive disease management strategies for basil production.

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