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Chip Digital PCR (cdPCR) to Identify and Quantify *Botrytis cinerea* Infection in Tomatoes

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Abstract: *Botrytis cinerea* is a fungal pathogen present in almost any environment, able to cause a severe postharvest disease on a wide range of crops, resulting in significant economic losses. Furthermore, *B. cinerea* is frequently found in plant tissues as a latent, asymptomatic infection that, when stimulated by favorable alterations in the environment or the physiology of the host, can swiftly develop into a significant symptomatic infection. In greenhouses, fields, and on propagation materials, the principal strategy adopted to control infection is the use of chemical fungicides or eco-friendly alternative methods. For the optimal success of conventional and biocontrol treatments, it is crucial to monitor the disease development and the fungal infection entity. The aim of this work was to develop a fast new method based on chip digital PCR (cdPCR) to estimate the extent of the *B. cinerea* infection in tomatoes. To better evaluate the amount of plant infection, a duplex assay able to co-amplify both fungal and host plant DNA was fine-tuned. The cdPCR assays were applied to quantify *B. cinerea* in tomato seedling samples, both naturally and artificially contaminated. The developed method offers sensitive detection, reliable identification, and precise pathogen quantification. The method can be used for *B. cinerea* diagnostics along the tomato production chain, starting from the seeds and transplanting seedlings to plants and crop residues in open fields and greenhouses. To the best of our knowledge, this is the first study directed at applying cdPCR to *B. cinerea* diagnosis in tomatoes.

Keywords: chip digital PCR; *B. cinerea*; tomato; diagnostic

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1. Introduction

The common and necrotrophic pathogenic fungus *Botrytis cinerea* [(Pers.: Fr.), Teleomorph: *Botryotinia fukeliana* (de Bary) Whetzel] can colonize more than 200 crop species worldwide, including tomato, pepper, bean, onion, potato, crucifers, cucurbits, sunflowers, strawberries, grapes, roses, and other flowers [1]. As a saprophyte, *B. cinerea* can survive in a field under a variety of conditions, colonizing flower residues, fruit juice drops, dead leaves, or other non-living plant tissue [2]. As a pathogen, known as gray mold, *B. cinerea* is a major cause of pre- and post-harvest losses in fruit and vegetable production, resulting in worldwide economic losses [3]. Indeed, this fungus has been classified as the second most important plant pathogen in the "world's top 10 fungal pathogens" in terms of economic and scientific relevance, preceded only by *Magnaporthe oryzae* [4]. It is especially harmful to dicotyledonous mature or senescent hosts; however, it typically accesses the tissues at early stages of crop growth and remains dormant for a considerable amount of time (days to months). During this period, infected plants remain asymptomatic until the fungus is reactivated by favorable environmental conditions or the host's physiology changes in association with decreased defenses. Under advantageous conditions, the saprophytic mycelium produces abundant conidia, forming a dense, velvety, and gray-brown spore

mass on colonized plant tissues. Consequently, a seemingly healthy plant can deteriorate and rot suddenly due to fungal activation. As a result, severe damage occurs after harvesting apparently healthy crops and transporting them to distant markets, enabling the disease to cause significant losses. Conidia can, in fact, accumulate in storage bins and containers and can be transported and air-dispersed in packinghouses. Conducive conditions, including injuries, high humidity, senescent plant tissue, and high sugar content, can determine pathogen attack during handling, storage, marketing, and after consumer purchase. *B. cinerea* is therefore one of the most important post-harvest diseases of fresh fruit and vegetables, as reviewed by Romanazzi et al. [2]. Moreover, this fungus is responsible for important losses in field and greenhouse-grown horticultural crops prior to harvest, starting from the seedling stage [1].

B. cinerea is difficult to control since it can attack the host in a variety of ways, uses a wide range of host plants as inoculum sources, and can survive as mycelia, conidia, or sclerotia in crop debris for extended periods of time. Infestation can occur along the whole lifecycle of the host, from the seedling stage until product ripening [4]. The main strategy adopted to reduce and contain the infection is the use of chemical fungicides, but also other eco-friendly alternatives such as antagonistic microorganisms (as yeasts and bacteria) and natural antimicrobial substances (as plant extracts and essential oils) [5]. For optimal success of conventional and biocontrol treatments, it is crucial to monitor disease development and the fungal infection entity. Understanding pathogen infection entities is fundamental to successfully predicting disease risk during the growth phases. For these reasons, the development of efficient tools to diagnose and control plant infections is highly requested. To study the shift from latent to aggressive infection, effective methods to control fungal growth, fungal inhibition after pesticide application, or screening for its presence are crucial [6]. Early detection of plant infections is critical to increasing crop productivity and meeting the world's growing population demand. Visual estimation has been shown to be inaccurate because of systematic bias in the measurements of different assessors. Traditional plant disease detection methods are time-consuming, laborious, and take 3–5 days to estimate disease incidence. Classical methods include spore counting by microscope, the spread culture method, and the isolation of microorganisms on selective media. The standard purification method is based on single-spore isolation. Using a sterile cotton swab, field samples are taken from spore-bearing lesions. Owing to the potential for many isolates with distinct characteristics to coexist on a single lesion, these samples are frequently called "micro-populations" and go through single-spore isolation before being further examined. Spores, which are frequently smaller than 10 µm in diameter, must be handled carefully under sterile conditions under a microscope. The accurate enumeration and correct identification of spores and conidia, as well as the retrieval and isolation procedures, are extremely laborious and require extensive skills and knowledge that are linked to the operator's expertise. An alternative method is based on the isolation of hyphal fragments under the stereomicroscope [7]. Molecular techniques can be used to facilitate pathogen recognition; in fact, methods based on DNA analyses are often sensitive, reliable, faster (requiring a few hours instead of days), and less laborious while resolving the difficulty of morphologically differentiating species. The rise of a variety of new molecular methods radically changed plant pathology study approaches [8]. The number of commercially available molecular protocols, based on PCR and Real-time PCR (qPCR), to identify fungal pathogens has exponentially increased.

Recently, highly sensitive molecular detection approaches based on digital PCR (dPCR) are renewing phytopathogen management [9]. dPCR is now a useful and innovative tool in plant pathogen diagnostics and crop protection.

dPCR is a third-generation PCR based on the subdivision of the analytical sample into multiple partitions that are individually amplified. In plant disease diagnostics, dPCR is a rapid and reliable tool to obtain absolute quantification for several classes of pathogens (such as fungi, bacteria, viruses, and phytoplasma). dPCR has several advantages in comparison with the widely adopted qPCR. Among the advantages, there is the higher

sensitivity, even in low-DNA samples, the quantification system, which is not dependent on certified material but on the absolute quantification strategy, and the low susceptibility to PCR inhibitors. Moreover, a high level of accuracy, reliability, and run-to-run reproducibility have been demonstrated [10,11]. Briefly, the ability to do absolute quantification without the use of a standard curve, the enhanced precision and accuracy in qualification and quantitation, and the decrease in false negatives are just a few of the benefits of employing the dPCR approach.

Building on already-available qPCR assays, it is possible to shift to a dPCR system; successful examples have already been shown in plant diagnostics. As reviewed by Morcia et al. [12], many examples of diagnostic assays developed ex novo or translated from similar qPCR assays have recently been published.

In the last 15 years, several molecular assays based on qPCR have been developed to identify *B. cinerea* in different types of hosts. A specific and sensitive Sybr green-based qPCR assay was developed for the detection and quantification of *B. cinerea* infection in grapes by Diguta et al. [13]. Si Ammour et al. [14] used the qPCR methodology based on TaqMan chemistry as a reliable tool to quantify *B. cinerea* in grape bunch trash. An EVAGreen-based qPCR protocol was applied to the early detection of the fungus in pelargoniums (House geraniums) to determine the level of cultivar resistance [15].

A droplet dPCR (ddPCR) tool was used to monitor *B. cinerea* natural inoculum in the grape at different phenological stages on asymptomatic samples [16]. A duplex ddPCR was developed for rapid and accurate quantification of *Alternaria* spp. and *B. cinerea* simultaneously in sweet cherries at different growth stages [17].

Several considerations led us to develop a dPCR diagnostic approach in tomatoes.

The choice of this pathosystem was based on the fact that among the primary fungal pathogens affecting tomatoes, *B. cinerea* stands out as the one that causes significant damage to production, particularly in intensive cultivation and greenhouse environments. In Mediterranean countries, in fact, *B. cinerea* is responsible for an average annual loss of approximately 20% in tomato production. Such losses can reach over 40% when the environmental conditions favor the development of the pathogen [18]. Additionally, tomatoes play a key role in the Italian agrifood scenario. Italy is in fact the seventh-largest producer of tomatoes worldwide (FAOSTAT), with a production of tomatoes for industrial transformation of around 6,000,000 tons annually. Moreover, in Italy, there are approximately 25,000 hectares dedicated to the production of table tomatoes, out of which more than 7 thousand are used for greenhouse cultivation. This last cultivation gives a mean annual production of 480,000 tons; table tomato is therefore the main greenhouse crop in Italy (http://dati.istat.it/Index.aspx?QueryId=33703, 29 September 2023) [19]. The fight against this pathogen is mainly agronomic, with the removal of all crop residues and infected organs, the ventilation of greenhouses, the adoption of a scattered planting system, and the use of fungicides. The adoption of such control methods is subject to the identification of the pathogen, and from this point starts the objective of the current study, i.e., to develop a chip digital PCR (cdPCR) assay for the detection and quantification of *B. cinerea* in tomato.

The rationale for the development of a further DNA-based method for *B. cinerea* quantification has been based on the following points:

a. In comparison with qPCR-based protocols already available, cdPCR does not require standard reference and is less sensitive to PCR inhibitors;
b. There are several commercially accessible dPCR systems available at the moment, and they all use different techniques to obtain absolute quantification. A comparatively new technique for dPCR is called Quant Studio® 3D digital PCR. It involves loading a PCR sample onto a microchip, where it is spread among 20,000 reaction wells, enabling the execution of 20,000 distinct PCR reactions. The Quant Studio® technology allows for absolute quantification without requiring reference to a standard control by using Poisson statistical analysis of fluorescent signals from positive and negative wells. Previous studies adopted a droplet digital PCR (ddPCR) platform for *B. cinerea* quantification. The availability of a new protocol based on Quant Studio® 3D digital

PCR can be beneficial for laboratories that use such a platform and for comparisons among methods;

c. Previous studies were focused on the *B. cinerea* quantification in fruits such as strawberries and cherries. The cdPCR assay developed in this study is directed at diagnostics for tomatoes. Both tomato samples, naturally contaminated or spiked with fungus, were analyzed.

2. Materials and Methods

2.1. Fungal Samples

B. cinerea strains, supplied by "Università Cattolica del Sacro Cuore" (Piacenza, Italy), were kept on potato dextrose agar (PDA, Liofilchem, Teramo, Italy) and stored at 4 °C until use. The cultures were validated as *B. cinerea* prior to use in the cdPCR assays by morphological analysis. Two strains of *B. cinerea* were in fact isolated from naturally contaminated eggplant (*Solanum melongena* L.) and tomatoes cultivated in northern Italy using the "Tissue Isolation Method". Plant tissues (leaves and stems) exhibiting suspicious symptoms were washed for 10 min with running water, disinfected briefly (1 min) with a low concentration of hypochlorite (NaClO 0.5%), washed three times with sterile water, dried with adsorbent paper, and cut into small pieces (3–5 mm) and incubated on agar-H_2O medium at 25 °C for 5–7 days. Small pieces of grown molds were transferred to a PDA medium at 25 °C for 7–10 days in light conditions to stimulate conidia production. An optical microscope was used to observe and examine colony and conidial morphology. *B. cinerea* on PDA medium produced grey and white mycelia in the early stages which later became grey and flocculant. Conidia were oval and subspherical, while sexual spores were colorless and spherical. The nuclei, which are black and irregular in shape, were produced on the colonies after about one month of culture.

2.2. Plant Samples

To test the assays' applicability on infected samples, four *Solanum lycopersicum* cultivars commercially available (Sailor, Mariner, Wilson, and Rossoro) were used. Twenty seeds of each variety were put in Petri dishes on a PDA medium, with or without *B. cinerea* inoculum. To obtain inoculated samples, the seeds were placed at equal distances around the perimeter of the 90 mm Petri dish and inoculated in the middle with 8 mm PDA plugs from actively growing cultures. The inoculation plug was collected from the growing margins of *B. cinerea* 10 days old. After one week at 25 °C, the mycelium reached the seeds, which in the meantime had germinated. The plants, pooled in two groups consisting of ten plants each, were collected after germination (seedling stage, 1 week at 25 °C), reduced to fine powder by mortar and pestle in the presence of liquid nitrogen, and the DNA was extracted using a DNeasy Plant Mini Kit (Qiagen® Italia, Milan, Italy) in accordance with the manufacturer's protocol. Utilizing a Qubit fluorometer and the Qubit dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA), the amount of extracted DNA was evaluated.

2.3. DNA Extraction and Quantification

Following the method outlined by Al-Samarrai and Schmid [20], fungal DNAs were isolated in duplicate from lyophilized mycelium. 30 mg of freeze-dried mycelium was mixed in 500 µL of lysis buffer containing 40 mmol/L Tris-acetate, 20 mmol/L sodium acetate, 1 mmol/L EDTA, and 1% *w*/*v* SDS pH 7.8. The mixture was pipetted multiple times until the suspension became foamy. After adding 2 µL of RNAse A (10 mg/mL), the mixture was incubated for 5 min at 37 °C in order to remove RNA. Polysaccharides and proteins were precipitated with the addition of 165 µL of 5 mol/L NaCl. After centrifugation at 13,000 rpm for 20 min at 4 °C, the obtained supernatant was mixed with 400 µL of chloroform and 400 µL of phenol and centrifugated at 13,000 rpm for 10 min at 4 °C. Two volumes of 95% ethanol were used to precipitate DNA, and 70% ice-cold ethanol was used

to wash the obtained precipitate three times. Following washing, the DNA was dried, resuspended in 50 µL of Tris-EDTA buffer, and stored at −20 °C.

Total plant DNA was obtained from tomato leaves (in triplicate, 100 mg per extraction) employing the DNeasy® Plant Mini Kit (Qiagen® Italia, Milano, Italy).

The amount of extracted fungal and plant DNA was evaluated using a Qubit™ fluorometer and the Qubit™ dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Primers and Probes

For cdPCR-based diagnostics of *B. cinerea*, the assay BC3-dig, previously developed by Suarez et al. [6] and validated by Si Ammour et al. [14] was transferred from a qPCR environment to a cdPCR one. This assay was used in combination with the assay Tom-dig, tested by Morcia et al. [21] for tomato (*Solanum lycopersicum*) identification, to estimate the infection entity. Table 1 reports primers and probes sequences of the two assays. BC3-dig primers and probe sequence specificity were evaluated using the Blastn suite [22].

Table 1. Primers and probe sequences used in multiplex cdPCR to target *B. cinerea* and tomato host plants.

Assay ID	Primers and Probes ID	Primers and Probes Sequences	Biological Target	Target Gene	Amplicon Size	References
Tom-dig	Tom-F Tom-R Tom-pr	gcaatatcaagagccccgtc ggagcgcttagcacacat VIC-tgcaacatccttctttcttctcgtg-MGB	*Solanum lycopersicum*	Prosystemin GenBank: M84800.1.1	91 bp	[21,23]
BC3-dig	BC3-F BC3-R BC3-Pr	gctgtaatttcaatgtgcagaatcc ggagcaacaattaatcgcatttc FAM-tcaccttgcaatgagtgg-MGB	*Botrytis cinerea*	Ribosomal IGS spacer GenBank: AM233400.1	94 bp	[6]

2.5. Chip Digital PCR for B. cinerea Diagnostic in Tomatoes

The BC3-dig and Tom-dig assays (Table 1) were used in a duplex reaction for cdPCR analysis. For cdPCR tests, the QuantStudio™ 3D Digital PCR System (Applied Biosystems by Life Technologies, Monza, Italy) was employed. 8 µL of QuantStudio™ 3D Digital PCR 2× Master Mix, 0.72 µL of each primer at 10 µM (final concentration of 450 nmol), 0.32 µL of FAM and VIC-MGB probes at 10 µM (final concentration of 200 nmol), 2 µL of DNA, and 2.48 µL of nuclease free-water were used in the duplex reaction with a final volume of 16 µL. In the negative template control (NTC), 2 µL of nuclease-free water was employed as the template. The QuantStudio™ 3D Digital chip loader was used to load a total of 15 µL of reaction mixture onto the QuantStudio™ 3D Digital PCR chips in accordance with the manufacturer's instructions. The following cycling protocol was used to carry out the amplifications in the ProFlex™ 2Xflat PCR System Thermocycler (Applied Biosystems by Life Technologies, Monza, Italy): 96 °C for 10 min, 47 cycles of 98 °C for 30 s (denaturation) and 58 °C for 2 min (annealing and extension), and a final step of 10 °C (hold temperature). The QuantStudio™ 3D Digital PCR Instrument was used to capture end-point fluorescence data, and the files produced were analyzed using the cloud-based QuantStudio™ 3D AnalysisSuite dPCR software, version 3.1.6. Each sample was examined twice under repeatability conditions. The estimation of repeatability was carried out according to Hougs et al. [24].

3. Results

The set of primers/probes designed for *B. cinerea* diagnostics in tomato crops were evaluated on three different sets of samples:

1. Test samples, obtained by spiking tomato DNA with *B. cinerea* DNA dilutions;
2. Tomato seedlings obtained from commercial seed stocks;
3. Tomato seedlings artificially contaminated with *B. cinerea.*

3.1. Test Samples, Obtained Spiking Tomato DNA with B. cinerea DNA Dilutions

The specificity of the primers/probes was previously in lab-evaluated by Si Ammour et al. [14] against the most common grape pathogens. Their specificity towards the major fungal and bacterial diseases of tomato was further in silico evaluated. No significant sequence homology was detected for etiological agents of the following tomato pathologies: anthracnose fruit rot, early blight, Septoria, late blight, buckeye rot, leaf mold, bacterial wilt, bacterial spot, tomato pith necrosis, fusarium wilt, southern blight, damping off.

The sensitivity of the assays was evaluated on samples prepared by spiking a fixed amount of tomato cv. Mariner DNA (20 ng) with *B. cinerea* DNA serial dilutions. Figure 1 shows the results obtained after cdPCR analysis of tomato DNA spiked with serial dilutions of *B. cinerea*. A coefficient R^2 equal to 0.999 (Figure 1, Table 2) was obtained between the fungus DNA dilution factors and fungus copies/µL estimated after cdPCR analysis. The assay can identify the lowest amount of *B. cinerea* of sample C (Figure 1 and Table 2), quantifying it in about 24 copies of the fungal target sequence/µL of the DNA sample.

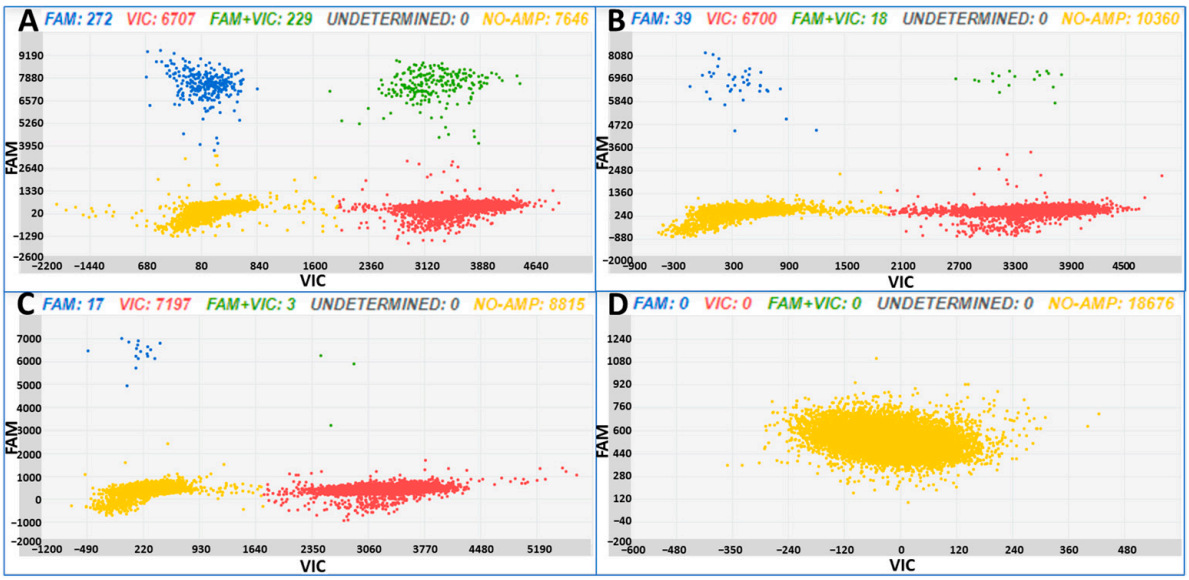

Figure 1. Two-dimensional scatter plot after cdPCR with Tom-dig assay (which gives a VIC-positive signal, visualized as a red dot) and the BC3-dig assay (which gives a FAM-positive signal, visualized as a blue dot). The signals from the VIC reporter dye are on the X-axis and the FAM reporter dye is on the Y-axis. Fixed amounts of tomato DNA were spiked with decreasing quantities of *B. cinerea* DNA as follows: (**A**) 20 ng tomato DNA spiked with *B. cinerea* stock DNA; (**B**) 20 ng tomato DNA spiked with *B. cinerea* tenfold diluted stock DNA; (**C**) 20 ng tomato DNA spiked with *B. cinerea* a hundred-fold diluted stock DNA; (**D**) no template control-NTC. Blue dots stand for FAM-positive PCR partitions, indicating an amplification of the *B. cinerea* DNA target. Red dots are PCR partitions, which are VIC-positive to the tomato endogenous gene. Green dots stand for partitions in which co-amplification of both targets occurred. Yellow dots stand for PCR partitions without any target.

Table 2. Copies/reaction µL obtained in cdPCR with Tom-dig assay and BC3-dig assay on samples prepared by spiking a fixed amount of tomato cv. Mariner DNA (20 ng) with *B. cinerea* DNA serial dilutions, Standard Deviation (SD), and Confidence Intervals (CI).

Sample	*B. cinerea* ng	Tomato ng	Copies/µL *B. cinerea* ± SD	CI Copies/µL *B. cinerea*	Copies/µL Tomato ± SD	CI Copies/µL Tomato
A	0.001	20	46.23 ± 3.02	42.39–50.42	836.24 ± 18.14	816.41–856.54
B	0.0001	20	4.73 ± 0.97	3.68–6.08	662.27 ± 13.13	646.42–678.52
C	0.00001	20	2.89 ± 0.78	2.08–4.03	792.90 ± 14.8	774.47–811.77
D	0	0	0	0	0	0

Replications of the same DNA samples were used to evaluate the repeatability of the assays. The standard deviations found were ≤25% for both assays, according to the acceptance criterion reported by Hougs et al. [24].

3.2. Tomato Seedling Samples Obtained from Commercial Seed Stocks

cdPCR was conducted on DNA extracted from the seedling stage (1 week) of each tomato cultivar (Sailor, Mariner, Wilson, and Rossoro) grown in Petri dishes on a PDA medium at 25 °C. All the samples resulted free from fungus infection, except Rossoro which showed a natural *B. cinerea* contamination with 3.5 copies/µL (Figure 2 and Table 3).

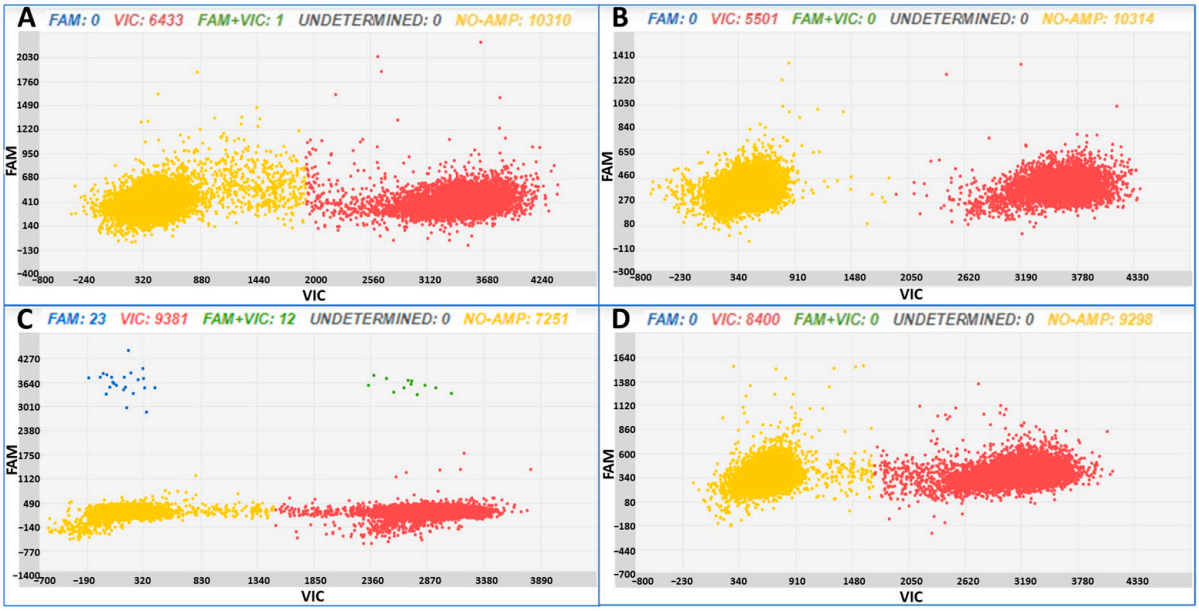

Figure 2. Chip digital PCR (cdPCR) analysis using Tom-dig assay (which generates a VIC-positive signal, visualized as a red dot) and BC3-dig assay (which generates a FAM-positive signal, visualized as a blue dot). Scatter plots show the VIC signals on the X-axis and the FAM signal on the Y-axis. All data points from every well on the chip are shown in the figure. The samples analyzed are as follows: (**A**) 20 ng of Mariner DNA, (**B**) 20 ng of Sailor DNA, (**C**) 20 ng of Rossoro DNA, and (**D**) 20 ng of Wilson DNA. Amplifications were obtained in all samples for tomato (red dots) and for *B. cinerea* in sample C only (blue dots). Green dots (present in Figure (**C**) only) are partitions in which both amplifications (for tomato and *B. cinerea* DNAs) occurred, whereas yellow dots are negative PCR partitions without any target amplification.

Table 3. Copies/µL obtained in cdPCR with Tom-dig assay and BC3-dig assay on seedlings tomato cultivars Mariner (A), Sailor (B), Rossoro (C) and Wilson (D), Standard Deviation (SD) and Confidence Intervals (CI).

Sample	*Varieties*	Tomato ng	Copies/µL *B. cinerea* ± SD	CI Copie/µL *B. cinerea*	Copies/µL Tomato ± SD	CI Copie/µL Tomato
A	Mariner	20	0.27 ± 0.26	0.08–0.73	644.08 ± 14.6	628.35–660.21
B	Sailor	20	0	–	568.13 ± 12.9	553.17–583.50
C	Rossoro	20	3.49 ± 0.87	2.60–4.70	1102.50 ± 12.2	1097.70–1125.70
D	Wilson	20	0	–	856 ± 17.1	837.70–875.11

3.3. Tomato Seedlings Artificially Contaminated with B. cinerea

Twenty seeds/cultivars (Sailor, Mariner and Wilson) free from fungus infection were placed on PDA in Petri dishes and infected with *B. cinerea*. After one week at 25 °C, infected seedlings were collected, and DNA was extracted. In Figure 3 and Table 4, amplification in cdPCR was obtained from the DNA extracted from Mariner tomato seeds artificially

infected by *B. cinerea*. Because of the strong infection of the seedlings, it was necessary to reduce the amount of DNA to be analyzed in dPCR to avoid chip saturation. Tenfold dilutions of DNA samples were therefore analyzed.

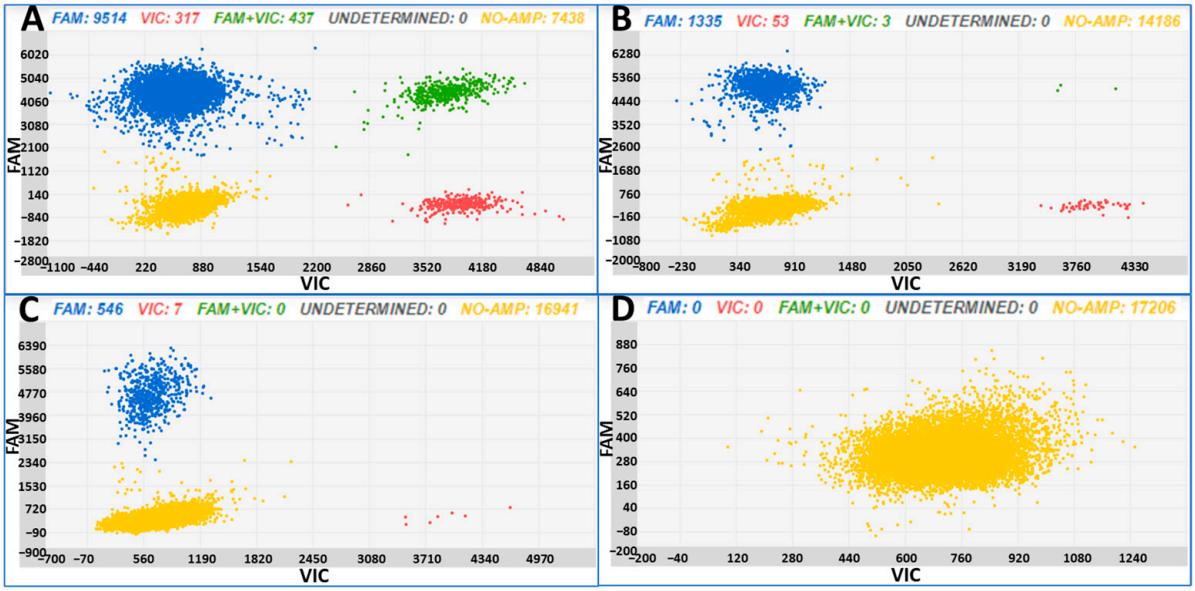

Figure 3. The samples analyzed are as follows: (**A**) 2 ng of total DNA extracted from infected Mariner plants, (**B**) 0.2 ng of total DNA extracted from infected Mariner plants, (**C**) 0.02 ng of total DNA extracted from infected Mariner plants, (**D**) No template control (NTC). Amplifications were obtained in all samples for tomato (red dots) and for *B. cinerea* (blue dots) except in sample D (blank control without DNA). Green dots are partitions in which both amplifications (for tomato and *B. cinerea* DNAs) occurred, whereas yellow dots are negative PCR partitions without any target amplification.

Table 4. Copies/µL obtained in cdPCR with Tom-dig assay and the BC3-dig assay, Standard Deviation (SD), and Confidence Intervals (CI). Total DNA was extracted from Mariner artificially infected plants.

Sample	Total DNA (ng)	Copies/µL *B. cinerea* ± SD	CI Copie/µL *B. cinerea*	Copies/µL Tomato ± SD	CI Copie/µL Tomato
A	2	1097.70 ± 17.98	1075.7–1120.2	56.65 ± 3.71	53.63–61.92
B	0.2	119.07 ± 4.79	112.86–125.63	4.95 ± 1.07	3.81–6.40
C	0.02	42.08 ± 2.68	38.69–45.76	0.53 ± 0.39	0.25–1.11
D	0	0	–	0	–

The mean copies of fungus and tomato target sequences detected by cdPCR assay in serial DNA dilutions were given in Table 5. The linearity was evaluated (Figure 4) by plotting the copies of the fungus and tomatoes against scalar dilutions of the total DNA extracted from artificially infected tomato plants.

Table 5. Mean copies/µL of fungus and tomato target sequences detected by cdPCR assay in serial dilutions of total DNA extracted from the three varieties Mariner, Sailor, and Wilson artificially infected with *B. cinerea*. Confidence intervals (CI) and Standard Deviation (SD) are reported.

Dilution Factor	Copies/µL *B. cinerea* ± SD	CI Copies/µL *B. cinerea*	Copies/µL Tomato ± SD	CI Copies/µL Tomato
10	2854 ± 384.1	2592–3423	53 ± 8.12	43–61
	327 ± 40.81	281–380	4 ± 0.98	3–5
	59 ± 10.01	47–71	0.8 ± 0.24	0.51–1

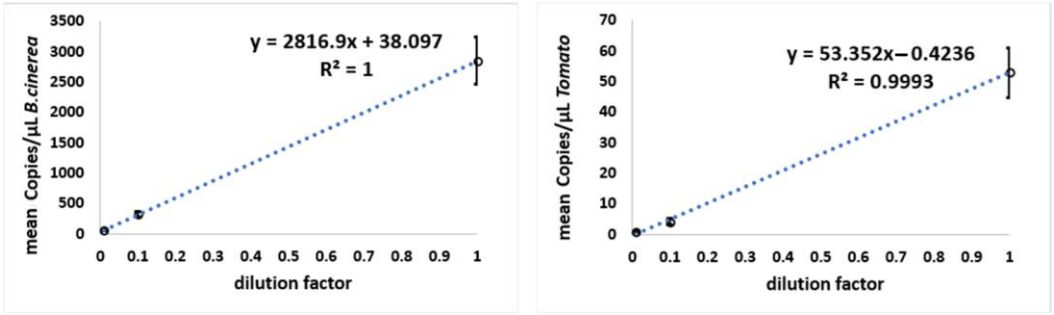

Figure 4. Regression curves obtained by plotting the copies of *B. cinerea* (**left**) and of tomato endogenous gene (**right**) against dilutions of total DNA extracted from tomato-infected plants. Error bars are reported.

4. Discussion

During their lifespan, plants are frequently exposed to attack by unwanted pathogenic microorganisms. *B. cinerea* is probably one of the tomato diseases more difficult to control. Botrytis can spread very rapidly and cause polycyclic diseases: the conidia colonized the tissues of healthy plants through wounds caused by pruning, insects, or weather conditions, and, in conductive environmental conditions, the risk of disease outbreaks increases. The prevention of grey rot requires therefore great vigilance in inspection to detect possible symptoms. However, correct identification of this infection is not trivial, because, on the basis of the symptoms, it might be mistaken for other diseases [25].

B. cinerea is a versatile opportunist pathogen that adopts different strategies during the process of pathogenesis. Plant pathogen infection can initiate through wounds, or sites previously produced by other pathogens. In addition, *B. cinerea* can enter in substomatal cavity via stomatal openings and is able to penetrate intact host surfaces [26]. The main damage caused by *B. cinerea* infection occurs on the stems at pruning wounds in tomatoes grown in greenhouses, the fungus can rot the entire stem. Soft rotting of mature tomato fruits occurs primarily after harvest; in unripe tomatoes, the 'ghost spot' symptom is associated with successful host defense, but the stained fruits are not marketable [1]. At the beginning of colonization, *B. cinerea* enters a quiescent and asymptomatic period as a biotroph in which nutrients are obtained from the living host cells. After that, it starts a very aggressive phase as necrotrophy, which is believed to be triggered by biochemical changes in host tissues, such as an increase in volatile organic compounds, sugar, and nitrogen. The fungus secretes virulence factors such as oxalic acid that stimulate pectin degradation, cell wall-degrading enzymes (such as cellulase, protease, pectinase, laccase, and cutinase), plant hormone analogs and elicitors to disrupt the host's metabolism and cellular structure, to suppress host immunity system, and to promote susceptibility by altering the hormone balance in the host [27]. Cell wall lysis, in addition to facilitating the entry, provides nutrients, as carbon sources, for fungal sustenance and growth. *B. cinerea* produces enormous masses of gray conidia that allow it to grow rapidly on the surface of the plant and simultaneously on nearby ones. *B. cinerea* is necrotrophic and after host killing, it can continue to grow saprophytically on plant remains as mycelia and sclerotia. Sclerotia represent an important fungal survival mechanism, are extremely resistant to environmental changes and thanks to their melanized rind and β-glucan matrix which protects them from desiccation, UV radiation and microbial attack, and intracellular nutrient reserves (protein, lipids, glycogen, and polyphosphate) they can survive in the soil for up to 1 year [1,28].

In tomatoes, *B. cinerea* causes damping off (death of seedlings), foliar blighting, flower and green or ripe fruit infections and stem girdling. Infections in postharvest cause soft decayed, which together cause 20–40% damage to tomato crops. During epidemic periods, this damage can increase to 50–70% [29].

A pillar in tomato protection is the early diagnosis of the disease which permits to activate the more suitable protective measures in greenhouse and field plantations. Diagnostic labs and inspection organizations are therefore increasingly looking for quick routine techniques that offer sensitive detection, reliable identification, and precise quantification of potentially plant-harmful organisms.

Focusing on *B. cinerea* diagnostics, several technologies have been developed over time, with different specificity, sensitivity, speed to result, quantification efficiency, portability, and cost, as reviewed by Bilkiss et al. [30]. Among PCR-based methods, capable of returning a quantification of the target pathogen, Chilvers et al. [31] developed a real-time PCR assay for the quantification of *B. aclada*, *B. allii*, and *B. byssoidea* in onion seed. A TaqMan qPCR assay was developed by Carisse et al. [32] to quantify the conidia of *B. squamosa* in onion. Diguta et al. [13] developed a qPCR assay based on an IGS sequence for the diagnosis of *B. cinerea* on grapes, whereas Zhang et al. [33] and Fan et al. [34] developed assays to detect *B. cinerea*, *B. fabae*, and *B. fabiopsis* in broad bean. Malarczyk et al. [35] developed a triplex qPCR assay for the detection of the berry pathogens *Botrytis* spp., *Colletotrichum* spp., and *Verticillium* spp.

In this work, we proposed a molecular tool based on cdPCR for *B. cinerea* detection and quantification in tomatoes. The analysis is organized as a duplex assay to simultaneously quantify the fungus and the host plant. Two main reasons are behind the choice of simultaneous plant-pathogen detection:

> "*it is crucial that the diagnostic assays are thoroughly validated regarding specificity and sensitivity, not only with pure cultures or pure DNA samples, but also with plant samples spiked with the target pathogen*" Venbrux et al. [36]

Usually, in *B. cinerea* host species, the disease severity is correlated with the amount of fungal DNA [37]. The ratio between host plant DNA and *B. cinerea* DNA can provide a value of the fungal infection compared to a fixed unit of measurement of the plant. The information deriving from such a ratio can be exploited as a rapid and cost-effective tool for assessing botrytis risk in the field and for better management of fungicide-based control measures.

Linearity evaluations confirmed that the duplex assay can quantify both pathogen and plant over a wide dynamic range.

Its novelty, in comparison with other dPCR assays for *B. cinerea* quantification, is that it has been specifically developed for tomato sample analysis. The assay, in the subsequent step, has been evaluated in two sets of samples, i.e., on commercial seeds and leaf tissue obtained after artificial infection. The rationale behind this choice is that such samples are significant for the tomato cultivation chain: on one hand, the seed health is at the basis of the plant health, on the other hand, the assay must be able to detect the pathogen in plant infected tissues. On the basis of the results obtained, the assay is able to give diagnostic answers in both situations. Further tests on a large number of infected plants could provide information on the correlation between visual symptoms and the extent of the infection, also taking into account that the molecular target used in the assay is a sequence characterized by variable numbers of copies even within the same species. Consequently, may be that a robust connection between disease symptoms and Botrytis DNA quantity might be difficult to get. The high sensitivity of the probe set targeting rDNA can be due to the high copy number of the target. The high copy number makes rDNA a desirable target for sensitive detection, but the copy number might vary among isolates of *B. cinerea* and therefore variability due to copy number variation can be present between samples. On the other hand, a probe set based on a single-copy gene may suffer from false negative errors when the plant tissue colonization is low. Given that it is imperative to diagnose even low levels of infection, a probe set based on multi-copy sequences is of greater interest for disease control. The fact that the analytical target is multicopy suggests that a dPCR-based approach is more reliable than a qPCR-based one. The latter technique, in fact, relies on standard curves for quantification, but the variability in the number of copies of the target can be problematic for the construction of a universal calibration curve.

5. Conclusions

In conclusion, the developed assay can be proposed for the *B. cinerea* diagnostics along the tomato production chain, starting from the seed control and plant seedling production arriving at the control of cultivations in open fields and of crop residues. A further, interesting perspective of application could be in the analysis of air samples. As reviewed by West and Kimber [38] the development and use of air sampling devices in plant pathology is a sector that is experiencing renewed growth since the first description of the Hirst spore trap, used as the workhorse of aerobiological sampling for over 60 years. Now, several different air samplers have been proposed. Among the others, many impactors have been adapted for samples to be analyzed by DNA-based diagnostics (West and Kimber). Several examples of joint applications of spore traps and real-time PCR have already been proposed. Among others, Quesada et al. [39], developed a simple, low-cost spore trap that, coupled with a real-time PCR analysis, allows surveying *Fusarium circinatum* spore abundance in outdoor environments. Klosterman et al. [40] coupled spore traps and quantitative PCR assays for detection of the downy mildew spores. Given the mobility of the Botrytis conidia may be important to monitor the air for an early identification of the pathogen and its quantification in the environment. Building on the sensitivity and robustness of the cdPCR assay proposed, a further important field of its application could be therefore the analysis of air samples to monitor the level of Botrytis conidia and consequently whether to activate actions to combat the disease.

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